ABSTRACT

SILVERMAN, EMILY J. *Stevia rebaudiana:* Seed Longevity Studies and Estimates of Heritability of Agronomic Traits Using Parent-Offspring Regression (Under the direction of Todd C. Wehner).

Stevia rebaudiana, a member of the Asteraceae family, produces sweet compounds in its leaves called steviol glycosides. The genus contains over 230 species, and *S. rebaudiana* produces steviol glycosides in its leaves that are 300 times sweeter than sucrose. Steviol glycosides, also called rebaudiosides, are non-toxic and non-caloric, making them a good solution for replacing artificial sweeteners as well as part of the sucrose market. Plant breeding efforts are directed at increasing the concentration of steviol glycosides and maximizing yield (Mg/ha).

Stevia is a new crop in the United States and is well-suited to replace tobacco since it uses the same production methods and equipment. Growers looking for alternative crops will be interested as researchers develop adapted cultivars and suitable methods.

Stevia seed lots were harvested from field cages in 2016 and used in germination experiments. Families were evaluated for the effect of cold and room temperature storage conditions on seed longevity over a 53-month period. Twelve seed lots were selected for the seed longevity study and germinated in 3 replications over 24 time periods (quarterly) to identity optimum storage temperature and determine how viability over time. Seed lots with low initial viability were not able to withstand long-term seed storage and deteriorated at a faster rate than seed lots with higher initial viability. For maximum viability, seeds should be used within one year if stored at room temperature (21°C), and within 1.5 or 2 years if stored cold (4°C).

Field trials were conducted in 2015 and 2016 to assess diversity and heritability in stevia populations developed by intercrossing off-patent cultivars and landraces. Analysis of variance

showed wide variation among genotypes in two locations for phenotypic, agronomic, and steviol glycoside traits. Narrow-sense heritability was highest for rebaudioside (Reb-)A (0.56), Reb-C (0.50), Reb-D (0.40), and stem height (0.30). Pearson correlations comparing plant densities (6-plant and 30-plant plots) were high for early subjective ratings of stem height (0.72). A positive correlation was observed for early subjective branch width ratings in 6-plant and 30-plant plots (0.62). Early season subjective branch width ratings of 6-plant plots were correlated with early season stem height ratings in 30-plant plots (0.66). Early season subjective stem height of 30-plant plots was correlated with branch width ratings of 6-plant plots (0.63). These results indicate plant density played a role in the plants' phenotype for measured traits.

Stevia rebaudiana: Seed Longevity Studies and Estimates of Heritability of Agronomic Traits Using Parent-Offspring Regression

by
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BIOGRAPHY

Emily Jean Silverman was born in Raleigh, North Carolina on Oct. 30, 1987, to Janet Shurtleff and David Silverman. Emily grew up on a small farm now maintained as the Baily and Sarah Williamson Preserve in Shotwell, NC. As a child, she spent many days walking in the woods, gardening with her mother, and watering plants at the NCDA greenhouse where her mother worked. Emily got a summer job working for the corn breeding program led by Dr. Major Goodman, Department of Crop Science, North Carolina State University. She instantly knew she wanted to pursue a career in field research and continued working for the program for eight summers. Emily obtained a B.S. in Horticultural Science at NCSU in May 2011. Emily took part in independent projects focused on petunia genetics and *Botrytis* resistance with Dr. Dennis Werner and Dr. John Williamson, which led her to pursue a degree in plant pathology. In August 2011, Emily was admitted as a graduate research assistant to the Plant Pathology Department at NCSU under the direction of Dr. Frank J. Louws, where she conducted research on tomato grafting to combat bacterial wilt disease and contributed to a mapping population project to develop markers for bacterial wilt resistance. After she finished her M.S. in 2015, she obtained a research specialist position with Dr. Todd Wehner on the stevia and cucurbit breeding project. She started her Ph.D. in 2016 and is looking forward to completing her doctoral degree.

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CHAPTER 1

Stevia Breeding and Production, a Review

Introduction

Sugar consumption has risen drastically in the past several decades and has been associated with increased medical problems such as diabetes, obesity, and dental caries (Grenby, 1991). The high demand for sugar substitutes and alternative plant-derived sweeteners has increased because of these public health issues. *Stevia rebaudiana* provides a solution for the food industry because of its superior sweetness profile coupled with benefits to human health such as anti-inflammatory, anti-cancer, antitumor, and immunomodulatory properties (Kinghorn, 2002). In 2004, the World Health Organization approved stevia as a food additive (JECFA, 2008). Stevia has been grown commercially in Japan since the 1970s and is popular there (Kinghorn and Soejarto, 1985; Ashwell, 2015). *S. rebaudiana* leaf extracts are used widely in over 60 countries to sweeten soft drinks, yogurt, ice cream, and other foods (Food Insights, 2021). In 2008, the United States Food and Drug Administration approved purified stevia leaf extracts as a food additive. However, crude extracts and whole dried leaves are not approved until toxicology research is completed (FDA, 2019). *S. rebaudiana* is native to the Amambay Mountain region of Paraguay. It grows naturally between altitudes of 200-500m in the Rio Monday valley at latitudes of 22º to 24º south and 53º to 56º west (Pande and Gupta, 2013; Hossain et al., 2017). The species is well adapted to many environments and can tolerate saline stress conditions in lowland marshes and high-altitude grasslands. Stevia grows as a shrub in mountainous regions, dry valleys, open forests, and riverbanks (Robinson, 1930).

Stevia is a small, herbaceous, perennial shrub that the Guarani Indians of eastern Paraguay first used as a sweetener and medicinal tea (Shock, 1982a). The shrub has many names,

including kaà-heê-ê, sweet herb, honey yerba, sweetleaf, and stevia. A Swiss scientist, Moises Santiago Bertoni, first described stevia in 1887 and 1899. He subsequently characterized the species in 1905 (Bertoni 1905; Lewis et al., 1992). The sweetness of stevia is attributed to ent-kaurane diterpene compounds, called steviol glycosides (SG) that accumulate in leaves (Kinghorn and Soejarto, 1985). The SG profile of each plant is unique owing to its varying ratio of multiple SGs, which are dependent on genotype and influenced by environmental factors, nutrient management, and cultivation practices.

Taxonomy and Classification

Stevia rebaudiana Bertoni is a member of the Asteraceae family (Lester, 1999). It belongs to the Eupatorium tribe and is considered one of the more adapted genera in that tribe (Schilling et al., 1999). *S. rebaudiana* is one of over 230 species within the genus; other notable species include *S. micrantha, S. ovata*, *S. plummerae*, *S. salicifolia*, and *S. serrata*; they are indigenous to the southern United States, Mexico, Central and South America (Grashoff, 1972; Soejarto et al., 1983*)*. *S. rebaudiana* is unique because it produces diterpene glycosides that give the leaves a sweet taste (Soejarto et al., 1983).

The taxonomic classification for stevia is described below:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superphylum	Spermatophyta
Phylum	Magnoliophyta
Class	Magnoliopsida

Subclass	Asteridae
Group	Monochlamydae
Order	Asterales
Family	Asteraceae
Subfamily	Asteroideae
Tribe	Eupatorieae
Genus	*Stevia*
Species	*rebaudiana*

Cytogenetics

Schilling et al. (1999) found evidence of evolution from a high base chromosome number to a low base chromosome number during the analysis of chloroplast DNA. Two other research teams confirmed chromosome numbers reported by Monteiro, stated that Stevia is a diploid with 11 chromosome pairs (2n=22) (Monteiro, 1980). He found diploids (2n=22), triploids (2n=33), and tetraploids (2n=44) in several accessions of stevia. Others have reported similar chromosome counts in the genus; the triploids and tetraploids showed a high degree of male sterility, likely due to chromosome abnormalities (Frederico et al., 1996; de Oliveira et al., 2004; Yadav et al., 2011). A total of 73 *Stevia* species and 244 accessions collected from Mexico had x=11 or x=12, with a mix of diploids, triploids, tetraploids, pentaploids, and hexaploids (Watanabe et al., 2001). A cytogenetics study of stevia conducted by Cimpeanu et al. (2005) reported 11 chromosome pairs with lengths of 1.70 - 3.76 µm.

Botany

Nesom (2006) described the genus *Stevia* as containing annuals, perennials, subshrubs, and shrubs measuring 50 to 120 cm. Stems are erect, leaves are primarily opposite or mostly alternate, sessile, and lanceolate-elongate shaped, with entire or serrated margins (Nesom, 2006). The shrub has two main growth habits, erect single-stem or highly branched. Branches can be opposite, alternate, or whorled depending on accession. Leaves are lanceolate, serrated, small, and arranged in an opposite pattern along the stem of new wood (Chan et al., 2000). *S. rebaudiana* has been described as a small shrub that perennializes in sub-tropical and tropical environments. The plant has an extensive, shallow, fine root system with brittle stems and small lanceolate-elliptical-shaped leaves (Ferrazzano et al., 2015; Gutiérrez et al., 2016).

The inflorescence is formed in irregular cymes in indeterminate heads. Florets are usually in groups of five to six with white-purple corollas and branched filiform styles. Flowers are perfect, containing five small, recessed anthers surrounding a bi-lobed stigma that is exerted above the anthers and corolla (Goettemoeller and Ching, 1999). Flowers bloom over multiple days within the same cluster and over the whole shrub (Taiariol, 2004). The pollen sheds before bud break; pollen grains are large, with few per flower, making pollen dispersal difficult. Pollen viability is 0 to 65% in the accessions studied. Anthers are small and recessed, and all five surround the base of the style. The stigma is receptive after the flower has opened, and the stigma forks extend above the corolla; asynchronous flowering encourages outcrossing in stevia (de Oliveira et al., 2004). Stevia exhibits sporophytic self-incompatibility, which prevents self-pollination and the production of inbred lines (Chalapathi et al., 1997a).

Stevia initiates flowers under short-day conditions (Singh and Rao, 2005). Flowers can be initiated as early as the 4-node stage (Yadav et al., 2011). Flowering depends on genotype

response to environmental conditions such as day length and temperature (Brandle et al., 1998a). Environmental conditions such as photoperiod, temperature, water, and nutrient availability can impact plant growth, SG content, and flowering period (Ramesh et al., 2006). Flowers bloom over eight weeks, and seeds persist on the plant for 32 days while they mature on the mother plant. Stevia is insect-pollinated, and flowers require pollen from a different plant source for proper fertilization. It is recommended to provide hives of honeybees in seed production fields for adequate pollination (Oddone, 1999), although they are not needed in glycoside production fields. Seed viability can be affected by collecting seeds before physiologically mature (Colombus, 1997).

High germination rates are possible when seed harvest timing aligns properly with seed maturity; seed quality is also dependent on genotype and environment (Carneiro and Guedes, 1992a). For instance, heavy rain during flowering can reduce seed set, and shade can delay flowering time (Slamet and Tahardi, 1988). Stevia seeds are small, about 3mm in length, with little endosperm and a persistent pappus containing 20 bristles that enables wind dispersal (Goettemoeller and Ching, 1999). Black seeds are fertile, while tan seeds are infertile. Open-pollinated stevia crops produced 0 to 60% black seeds compared to the controlled cross-pollinated treatment that resulted in 86% black seeds. The black seeds had 60 to 86% germination (Raina et al., 2013). A study on seed longevity demonstrated stevia seeds could be stored for up to three years in cool, dark conditions without losing viability; that disagrees with a later study which observed 50% loss in viability after three years of storage at 0ºC (Kawatani et al., 1977; Chen and Shu, 1995). Seed yield was reported up to 8.1 kg ha $^{-1}$ (Carneiro, 1990). Lester (1999) reported a one ha stevia seed nursery would be sufficient to transplant 200 ha of stevia plants for leaf production. Brandle et al. (1998) proposed the optimum seed production

location is between 20º and 30º North latitude in the northern hemisphere with transplanting scheduled in late winter-early spring and seed harvest in late summer-early fall.

Seeds germinate in 4 to 14 days in flats in a greenhouse float bed; it takes 4 to 6 days for 62 to 90% germination at 25ºC (Shock, 1982a; Carneiro and Guedes, 1992a). Breeding progress can be improved by using populations having faster germination. The optimal germination temperature is between 20 and 25ºC, but it can occur at higher temperatures. Enhanced germination can be achieved when using a heating pad set to 40ºC for less than 24 hours; longer times will lower the total germination rate (Tanaka, 1985). In stevia, seeds germinate slowly, and seedlings develop slowly. Seed are direct sown into greenhouse flats or float bed trays and require 45 to 60 days to reach transplant size (Colombus, 1997; Brandle et al., 1998a). Seed germination of most accessions is generally low and highly variable because seed viability is inconsistent; 50% germination is standard (Goettemoeller and Ching, 1999; Lester, 1999; Shock, 1982a; Carneiro et al., 1997). Tetrazolium chloride treatment demonstrated that black seeds had higher viability compared to tan seeds, with 76.7% and 8.3% germination, respectively (Goettemoeller and Ching, 1999). Seeds germinated under light had a higher germination rate than seeds germinated in darkness, indicating light may be required for germination. In that same study, researchers examined pollination methods using bumblebee thoraxes to cross-pollinate, hand cross-pollination, wind cross-pollination, and self-pollination by hand, with open pollination as a control. They determined that self-incompatibility was not an issue with the genotypes used for their study and that hand self and cross-pollination methods produced the highest germination rates in the study, with 93% and 92% seed germination, respectively. The control treatment had the lowest germination rate, and they concluded that the best seed production is using the active transfer of pollen (Goettemoeller and Ching, 1999).

Growth Stages

A description of plant phenological and morphological growth stages helps standardize research and production practices (Feekes, 1941). Decimal code scales have been used for decades to record the growth stages of cereal crops**.** Easily recognizable morphological stages are assigned a number from 1 to 11, starting from seedling emergence to grain ripening **(**Zadoks et al., 1974**)**. Vegetative and reproductive growth stages are distinctive and are subdivided further depending on the most prominent feature **(**Zadoks et al., 1974**).** Tomato researchers use another standardized protocol, the tomato plant descriptor, which lists over 100 traits to characterize tomato germplasm when collected or created for future research (IPGRI, 1996). Characterization descriptors enable easy discrimination of different phenotypes that are highly heritable, visually observed, and expressed in all environments. Environmental descriptors are helpful whenever a new accession is collected and characterized so site-specific parameters can be used to help interpret results (IPGRI, 1996; Zadoks et al., 1974). Schneiter et al. (1981) developed an accurate, standardized system for characterizing growth stages in single- and branched-inflorescence sunflower (*Helianthus annuus* L) by dividing vegetative and reproductive stages into two and nine subdivisions each, respectively; vegetative growth is based on the seedling emergence and number of true leaves which accumulates as the plant grows, while reproductive growth is based on the appearance of inflorescences, time of anthesis, and time of seed maturity.

Carneiro developed a decimal code to aid researchers in evaluating *S. rebaudiana* plant growth from seed imbibition through flower bud initiation. Two distinctive categories: vegetative and reproductive, each with five subdivisions: V_0, $V_{1.i}$, $V_{2.i}$, $V_{3.i.k}$, $V_{4.i.k}$, and $R_{1.i}$, $R_{2.i}$, $R_{3.i}$, $R_{4.i}$, $R_{5.i}$, respectively (Carneiro, 2007). The seed germination stage, V_0, begins when the seed absorbs water from the medium, and only the radical and/or the cotyledons are present. The normal

seedling stage, $V_{1.i}$, is noted when the hypocotyl is visible. The *i*-tenth represents the number of normal seedlings that have developed. The seedling growth stage, $V_{2.i}$, is noted by counting the number of nodes with true leaves at least 5mm long. The plant growth and branching stage, $V_{3.i.k}$, is observed by counting the number of i-nodes on the plant's main stem, and the k-node is the first axillary branch, counting from the ground to the top of the canopy. Regrowth is the final vegetative stage, $V_{4.i.k}$, where the i-node is counted on all emerging stems, and the k-node represents the uppermost axillary branch with at least two internodes (Carneiro, 2007). Flower bud formation denotes the first reproductive stages, $R_{1.i}$, followed by crop flowering, $R_{2.i}$, when pollination occurs. The seed development stage, $R_{3.i}$, overlaps with $R_{2.i}$ because flowering and seed development is asynchronous; at the end of seed development, the petals turn brown, and the pappi change position and spread out. Fruit dispersal, $R_{4.1}$, occurs once seeds are ripe, dark in color, and wind dispersed. The final stage of plant growth is senescence, $R_{5.i}$, when the plant dies (Carneiro, 2007).

Le Bihan et al. (2020) documented phenotypical stages of stevia using the Biologische Bundesanstalt Bundessortenamt and Chemical Industry (BBCH) scale to standardize scientific characterization and create a tool for researchers to evaluate populations for genetic improvement in a uniform manner. Researchers describe ten growth stages, including germination, bud development, foliar development, side shoot appearance, stem elongation, harvestable plant part development, the emergence of the inflorescence, the start of anthesis, seed development, seed ripening, and finally, plant senescence. Growing degree days were used to describe each growth stage's chronological order and timing under temperate climates (Le Bihan et al., 2020). Researchers can use this standardized growth stage scale to plan data collection for

specific traits at approximate times to compare genotypes in different testing or production regions objectively.

Crop Production

Stevia is native to South America and has been commercially available in Paraguay and Brazil for decades. One of the earliest reports of stevia production for use as a sweetener was made by Dieterich (1909), who noted its widespread cultivation in Paraguay as early as 1909. Stevia has been cultivated in Japan as an alternative to sugar since the 1970s (Kinghorn and Soejarto, 1985). Stevia production has expanded far beyond Paraguay, and cultivation has been reported in India, Indonesia, Japan, Korea, Taiwan, Brazil, China, Canada, Kenya, Malaysia, Argentina, Colombia, Vietnam, the United States, and Thailand (Kinghorn, 2002; Chatsudthipong and Muanprasat, 2009; Singh et al., 2019; Ashwell, 2015).

Stevia grows well in fertile, sandy loam to loamy soil and requires consistent soil moisture for adequate plant growth (Shock, 1982b; Hossain et al., 2017). The optimum climate is semi-humid subtropical with an average temperature of 23ºC (Brandle and Rosa, 1992). Stevia is sensitive to water stress and requires frequent watering; it can grow in acid-to-neutral soils ranging from pH 4.0 to 7.5 (Shock, 1982b; Hossain et al., 2017).

Crop Production Timeline

In Paraguay, stevia seeds are directly sown into beds (pH 5.5 to 6.5, temperature 15 to 25ºC) of sand with added organic matter and phosphorus hydrated to soil water capacity levels. Seeds are sown at high density, 10 to 15gr/m^2; then, seedlings are thinned to 600/m^2. Approximately 170 m^2 seedbed will be used to transplant 1 ha for production. A cover on the

seedbed with a 50% shade cloth helps reduce seed loss from wind and prevents seed desiccation. Seeds begin to germinate 5 to 7 days post-sowing and are ready to transplant after 60 days once they reach 15cm height with 5 to 7 nodes on the main stem. Seedlings will reach 10 to 15cm after 60 to 90 days, with strong stems and an average of 20 leaves per plant. Five people must transplant 1000 m^2 of seedlings from the seedbed to the field. Transplanted fields are watered daily for the first two weeks of crop establishment and then watered multiple times per week after establishment (Stevia-Store, 2021).

Stevia is harvested just before flowering (at the end of the vegetative stage) when the steviol glycosides are at their highest concentration. The plants are cut just above soil level, and one laborer can collect leaves from 1000 m^2 of field per day. Machines are also used to harvest the crop. Stevia can be harvested multiple times per season, with the first harvest at 60 days post-transplant and the second harvest at 60 to 90 days after the first. The average yield in Paraguay is 4000 kg/ha/yr in five- to six-year-old production fields. Seed production is reserved for 5% of the area; these plants are not harvested but instead let flower and go to seed. After harvest, watering is reduced to two to three times per week while plants recover. Each week, a drying surface of 100 m^2 is needed for each ha harvested. Synthetic fabrics cover the solar drying area, and leaves are collected once leaves become brittle. Alternatively, mechanical dryers held at 70ºC can dry stevia with a target of 10 to 12% leaf moisture. Plastic, synthetic fabric, and jute bags store dry leaves in a cool, dark, dry facility for up to six months until final processing. (SteviaLand Paraguay, 2011).

In Ethiopia, 15cm raised beds with 40 to 60 cm between-row spacing and 20 to 25cm between-plant spacing is recommended for a plant density of 70,000 to 100,000 plants/ha (Mengesha et al., 2014). Clonal propagation is suggested to maintain crop uniformity and a

dependable SG concentration. The 15cm tall clones are transplanted two months after hardening off. Plants are transplanted before the rainy season when irrigation is a limiting factor; otherwise, they are planted year-round in Ethiopia. Fertilizer for stevia is 50 tons/ha manure and 60:30:45 kg N:P:K. Slow-release nitrogen is applied in three splits: pre-plant, before the first harvest, and after the second harvest. Stevia may only need to be watered once per week in the rainy season and increased to two to four times per week in the dry season. Hand weeding and hoeing are performed on a 15-day interval starting two weeks post-transplant. Growing points are pinched at two weeks and four weeks post-transplant to encourage branching (Mengesha et al., 2014).

Stevia is harvested three times a year, starting atfour months post-transplant and then every 40 to 60 days after that. They also suggest leaving 10cm of growth after harvest for crop regrowth. Mengesha et al. (2014) suggested using drying racks in a glasshouse or transplant polyhouse with fans running to air-dry the plants after harvest at 40 to 50ºC for 24 hours or until 12% moisture is reached. Once dry, plants are threshed to separate stems and leaves, and the powdered leaves are passed through a 2 to 3-mm sieve to remove impurities. Dry leaf weight ranges from 7.26-9.04g per plant in field trials evaluating stevia at four locations in Ethiopia (Mengesha et al., 2014).

In Southern Ontario, seedlings are produced in plug trays and transplanted in the field after seven to eight weeks in May with row spacing of 52 to 61 cm and plant density of 100,000 plants/ha (OMAFRA, 2021). Prepared fields received a pre-plant application 100 kg/ha of 6:24:24 N:P:K fertilizer and a split post-plant application of 140 kg/ha urea. Stevia needs frequent irrigation and mechanical cultivation to manage weeds. Plants were harvested just before flowering for optimum yield and SG accumulation (OMAFRA, 2021). Leaves are dried

using mechanical driers set at 40 to 50ºC forced air temperature for 24 to 48 hours (OMAFRA, 2021). Once the dried plant material becomes brittle to the touch (10 to 12% moisture), it is passed through a leaf-stem separator. A total fresh weight of 21,500 kg/ha and dry weight of 6,000 kg/ha has been reported in Southern Ontario. They reported that dried leaf weight of upwards of 3,000 kg/ha could be produced in their region with a yield potential of 2,850 kg/ha (OMAFRA, 2021). Leaves are stored in plastic, sealed bags in clean, dark, and dry facilities with low humidity (OMAFRA, 2021).

Clonal Propagation

Stem cuttings root easily under high moisture conditions and effectively preserve germplasm that does not breed true from seed (Shock, 1982a). Fresh shoots from the field can be treated with pesticides and cut into two-node segments, removing the lower leaves to encourage root formation; success depends on the age of the plant material used for propagation (Shock, 1982a). Cuttings from young shoot tips with two to four nodes root best under high humidity mist conditions. Rooting success is determined by the time of year and plant tissue location where cuttings are harvested. Cuttings root better during winter than in early spring or summer months (Tirtoboma, 1988).

Rooting hormones such as indole butyric acid (IBA) and indole-3-acetic acid (IAA) can improve survival rates in the clonal propagation system. Plants grown from cuttings are more uniform in growth and SG content than those produced from seeds (Ramesh et al., 2006). Clonal propagation for commercial production is labor-intensive, requiring a large mother plant nursery to be maintained, disease and pest-free, for multiple years. If used with perennial production

practices, it could be cost-effective for five to six years under favorable climate and cultivation practices.

Seed Propagation

Stevia seed germination is poor (Shock, 1982a; Carneiro et al., 2007). The production of stevia plants from seed in flats or trays is required to produce vigorous plants for mechanical field transplanting. Stevia seeds are small and have a poor germination rate, preventing growers from the direct sowing of seeds in the field. Stevia seeds have a high frequency of empty or clear achenes and are sown in high density to ensure adequate plant stands for production (Oddone, 1997; Carneiro, 1990). Studies of potting media showed that stevia seedlings having higher shoot dry weights were produced in soil mixtures containing a sand-clay-loam as the primary component (Carneiro et al., 1997). Seedlings transplanted early in the season produce higher biomass (Lee et al., 1978). May was suggested as a planting time for northern hemisphere production (Brandle et al., 1998a). Seed propagation does not produce a reliable SG profile because the species is self-incompatible, making for a high variability from plant to plant. Seed propagation is mainly used in the annual production system because seeds are relatively inexpensive, and little labor is needed to manage a seedling greenhouse.

Micropropagation

Micropropagation is often used to preserve selections in asexually propagated species such as sweet potato (*Ipomoea batatas*), blueberry (*Vaccinium* spp.), and stevia. Micropropagation is an effective method to mass-produce clones that are uniform, disease-free, and high-quality. Leaves, axillary buds, shoot primordia, and internodal explants can be used to

grow a small amount of tissue into an explant in controlled environment conditions. Marín et al. (2016) conducted a disinfection study to introduce nodal segments of 'Morita II' into tissue culture using sodium hypochlorite at 4% and 5% for 5, 10, 15, or 20 minutes. They concluded the best disinfection treatment was 5% sodium hypochlorite for 20 minutes because 76.75% of nodal segments were sanitized, and 58.91% of nodal explants sprouted after treatment *in vitro*.

In tissue culture, a basal medium can maintain germplasm for extended periods, and plant growth regulators can be applied to the medium to maximize growth. Debnath (2008) developed a protocol to regenerate stevia from nodal segments *in vitro* culture using 2.0 mg/L N6-benzyl amino purine and 1.13 mg/L indole-3- acetic acid to supplement the basal medium. The author reported that each nodal explant could produce 39 shoots after 30 days. Multiplication can be achieved with repeated subculturing before transferring elongated shoots to rooting medium supplemented with 2.0 mg/L indole-3-butyric acid (Debnath, 2008); he also developed a screening protocol using agar diffusion methods to test stevia leaf extract for antimicrobial activity against medically important bacteria and fungi. Chloroform and methanol extraction methods were reported to be concentration-dependent in their ability to inhibit growth (Debnath, 2008). Further, using cytokinin such as thidiazuron, benzylaminopurine, and kinetin to supplement nutrient medium increased shoot production and enhanced SG content in plants raised *in vitro* (Singh and Dwivedi, 2014).

Ferreira and Handro (1988) developed a cell suspension protocol for propagating stevia from 2n cells; they demonstrated that cultures can be maintained for three years and still regenerate normal stevia explants from actively growing calli. In addition, stevia can be mass-produced with temporary immersion bioreactors, with 64,600 shoots produced from 460 g of

propagules (Akita et al., 1994). This method would be helpful to commercially produce a large number of explants for field transplant of a single cultivar.

Several researchers have concluded that plant growth hormones are required in micropropagation to promote shoot formation, elongation, and root development (Sivaram and Mukundan, 2003; Mitra and Pal, 2007; Khan et al., 2016; Ahmed et al., 2007; Kaur, 2012). Sivaram and Mukundan (2003) evaluated shoot apex, nodal, and leaf culture *in vitro* using Murashige and Skoog (MS) medium supplemented with auxin compounds; they found that 6-benzyl adenine (BAP) and IAA at 8.87 µM and 9.80 µM, respectively, were effective to produce large numbers of explants with high concentrations of SG. Nodal explants were produced using MS medium with the addition of indoleacetic acid, kinetin, and adenine sulfate; 90% of axillary buds sprouted and regenerated within four weeks of *in vitro* culture. A survival rate of 60% was observed once explants were transplanted to field conditions, and SG content was similar for explants and nodal mother plants (Mitra and Pal, 2007). In Bangladesh, IBA, 1-Naphthaleneacetic acid (NAA), and IAA growth promoters were used in supplemented media for *in vitro* studies evaluating methods for mass micropropagation; IAA treatment had the highest rooting percentage of 97.66% (Ahmed et al., 2007). Khan et al. (2016) demonstrated that MS medium amended with urea effectively increased the number of shoots produced in an *in vitro* study conducted with nodal explants, reducing the culture time to six months. Kaur (2012) reported that a medium with 2,4-dichlorophenoxy acetic acid and bezyladenine was effective for the mass micropropagation of *S. rebaudiana* in India. Sairkar et al. (2009) outlined an approach to improve all three stages of micropropagation: amend MS medium with 0.1 mg/L N^6-benzyl amino purine for enhanced shoot formation, 3.5 mg/L N^6-benzyl amino purine to increase shoot multiplication and add 100 mg/L activated charcoal to half strength basal medium to improve

explant rooting. Alternatively, half-strength MS medium amended with 0.4 mg/L NAA produced 96% rooting of stevia nodal explants (Thiyagarajan and Venkatachalam, 2012). Ali et al. (2010) found callus formation to be enhanced when leaf explants were grown in MS medium amended with three mg/L 2, 4-D, while nodal and internodal explants were more successfully cultured as callus when the medium was fortified with three mg/L $^{-1}$ NAA and one mg/L^{-1} BAP. Shoot tips were shown to be more vigorous at shoot induction than nodal propagules and leaf explants when cultured on a half-strength Nitsch medium amended with 1 mg/L IAA (Anbazhagan et al., 2010). Das et al. (2011) observed that shoot tips were the quickest to induce bud formation compared to nodal and axillary bud explants in another study comparing different tissue types and concentrations of plant growth-promoting hormones. MS medium amended with two mg/L kinetin produced more than 11 shoots/single shoot tip explant in 35 days of *in vitro* culture. They also found that plant growth hormones had a negative impact on root development.

Synthetic seed technology can be used to maintain stevia germplasm as demonstrated in a study examining encapsulated (5% high-density sodium alginate hardened with 50mM $CaCl_2$) and non-encapsulated (control) nodal segments containing a single axillary bud stored in MS medium supplemented with thidiazuron and stored at 5, 15 and 25C for 4, 8, and 12 months (Lata et al., 2014). A 77% survival rate was reported in encapsulated nodal segments stored at 25C for eight months. Explants regenerated from encapsulated nodal segments were able to acclimate in the growth room after transplant, with an 87% survival rate. Further, they found SG content to be consistent between the mother plant and explants (Lata et al., 2014).

Alvarado-Orea et al. (2020) reported that *in vitro* green root cultures of stevia produced higher SG content under long-day conditions compared to dark culture, and SG was 2.4 times higher when treated with 250uM hydrogen peroxide compared to the control. Root cultures

treated with 250 μM or 500 μM hydrogen peroxide and 250 μM or 500 μM methyl jasmonate results in higher flavonoid content under long day length. It was concluded that applying elicitors stresses stevia, causing the plant to increase the production of secondary metabolites.

Plant Density

There is not yet a clear consensus on optimum plant density, and several studies contradict reports. Lee et al. (1980) evaluated plant density, with plant spacing of 50 to 70 cm between rows and 10 to 30 cm within rows; high density did not affect plant height, the number of branches, or the number of nodes, but did reduce dry leaf yield per plant. Another study reported and that 60 x 20 cm plant spacing resulted in a higher growth rate and yield per plant and that 60 x 10 cm spacing produced higher dry leaf yield (Murayama et al., 1980). Another study reported that a density of 80,000 to 100,000 plants/ha was recommended to be harvested four times per year (Donalisio et al., 1982). Shock (1982b) reported no advantage to plant more than 16 plants/yd^2; high mortality was observed at the density of 32 plants/yd^2. A narrow row spacing of 25 x 25 cm was suboptimal for biomass production because the root spread of the plant extends 30 cm within the first year of growth (Angkapradipta et al., 1986). The crop density of 200,000 plants/ha produced low yield because of intense light competition and was not recommended for biomass production (Basuki, 1990). High dry leaf yields were observed at plant density up to 96,618 plants/ha, 45 x 22.5 cm plant spacing, and SG yield in a field study conducted in clay loam soil in India (Chalapathi, 1996; Chalapathi et al., 1997b). A 50 x 20 cm row spacing produced adequate yields (Filho et al., 1997a). In Canada, a high plant density of 160,000 plants/ha was recommended to produce high yields (Colombus, 1997). In an annual

production system, plant density up to 110,000 plants/ha demonstrated an increase in leaf yield (Brandle et al., 1998a).

A South American research group evaluated transplant density and plant spacing, which concluded that 80,000 plants/ha had the highest yield and double-spacing plants at a rate of 35 per 65 cm produced the lowest results at 50,000 to 80,000 plants/ha (Carneiro and Martins, 1992b). Planting density has been reported from 40,000 to 400,000 plants/ha from research conducted in Japan. They observed lower yields at plant densities of 83,000 to 111,000 plants/ha (Katayama et al., 1976). A Moroccan research team reported that high plant density had a positively impacted on fresh leaf weight, dry leaf weight, stevioside and SG content when comparing densities 70 x 30 cm, 70 x 20 cm, and 70 x 10 cm. They also reported that a single harvest at 95 days post-transplant produced a higher yield than a second harvest 72 days after the first harvest. SG content also decreased as the season continued despite plant density (Benhmimou et al., 2017). A field trial conducted in Turkey showed that 30 x 60 cm spacing (55,556 plants/ha) produced a higher dry leaf yield of 110.9 kg/ha compared to 45 x 60 cm and 60 x 60 cm spacing with 70 kg/ha and 45.7 kg/ha, respectively (Samadpourrigani et al., 2019). According to a two-year field study conducted in Ethiopia, 20 x 40 cm plant spacing (125,000 plants/ha) produced the highest whole plant yield, fresh leaf weight, and dry leaf weight compared to fourteen other treatment combinations examining intra-row spacing of 20 cm, 25 cm, 30 cm, 35 cm, and 40 cm and inter-row spacing of 40 cm, 50 cm, and 60 cm. They proposed using 25 x 40 cm spacing (100,000 plants/ha) for maximum yield and ease of crop management (Btru et al., 2017).

Nutrients

Nutrient management plays a crucial role in plant growth and development. Murayama et al. (1980) reported a higher growth rate and dry leaf weight with an application of 2.1 kg/ha commercial mineral fertilizer N:P:K. Filho et al. (1997a) reported 1 ton/ha dry leaf yield was produced with the application of 64.6 kg N/ha, 7.6 kg P/ha and 56.1 kg K/ha fertilizer. Rashid et al. (2013) reported that 60 kg/ha nitrogen produced maximum dry leaf yield and whole plant yield compared to lower nitrogen levels in a field trial conducted in Punjab, India.

Nitrogen deficiency in stevia causes reduced growth, small leaves, and reduced branching, and generally, the plant looks chlorotic (Filho et al., 1997b). Phosphorous deficiency symptoms of stevia caused reduced growth, and plants were stunted. Stevia leaves curve downward under potassium-limiting conditions and forms smaller and fewer branches; lower leaves turned chlorotic and then necrotic. Calcium deficiency causes multiple symptoms ranging from small dark lesions, chlorosis, and necrosis of leaves, and the upper portion of stems and shoots can wilt and collapse. Magnesium deficiency was evident when older leaves showed interveinal chlorosis in an inverted V shape on affected leaves. Boron deficiency symptoms were observed in young leaves turning chlorotic, necrotic, and defoliating; branching was reduced, and stems exhibited reddish-colored cracks (Filho et al., 1997b). A foliar spray of Borax 6% was suggested to mitigate boron deficiency (Mengesha et al., 2014). Boron toxicity was observed in leaves with small brown spots and leaf tip burn on older foliage that turned necrotic. Zinc toxicity symptoms included irregularly shaped necrotic spots on lower leaves, progressing to whole plant wilt and death (Filho et al., 1997b).

In northern India, Pal et al. (2015) evaluated three nitrogen levels, two phosphorous levels, and three potassium levels in three field locations to understand spatial and nutritional

variability better. They reported that SG content was dependent on genotype and environmental conditions. The location had an impact on stevioside content and leaf yield, of which the authors concluded the best location for biomass production and SG content was at the Council of Scientific and Industrial Research, Institute of Himalayan Bioresource Technology, under a nutrient regime consisting of 90:40:40 kg/ha N:P:K. Nutrient availability is strongly controlled by soil properties and the climate they are growing in, and thus nutrient management needs to be addressed for specific locations. They also noted that branch number and leaf area were essential traits that related to yield and should be used by breeders to make selections (Pal et al., 2015).

Growth, biomass, and mineral nutrition were evaluated in a fertilizer study using organo-minerals in northwestern Morocco (Benhmimou et al., 2018). Treatments included a water control, 300:100:240 kg/ha N:P:K, 40 Mg/ha organic fertilizer, and 75:60:20 kg/ha N:P:K with an additional 10 Mg/ha organic matter. They reported an increase in biomass with a higher fertilizer rate and higher nutrient content in the leaves. They found a once-over harvest at 85 days post-transplant to be more productive than harvesting a second time 56 days after the first harvest and concluded harvest timing is critical to maximizing SG content and biomass. Finally, they also suggested that fertilizer trials need to be conducted on a regional basis to optimize production for specific locations. The best treatment for their region was 300:100:240 kg/ha N:P:K (Benhmimou et al., 2018).

Díaz-Gutiérrez et al. (2021) examined the effect of poultry manure combined with inorganic fertilizer to mineral fertilizer on stevia nutrient uptake and yield grown at two altitudes, 2569 and 1487 m above sea level (MASL), under greenhouse conditions for two years. Poultry manure mixed with inorganic fertilizer improved soil cation exchange capacity, nutrient uptake, and biomass at both altitudes compared to the mineral fertilizer control treatment. At higher

elevations, the poultry manure treatment enhanced nitrogen, calcium, potassium, magnesium, sulfur, and manganese uptake by 40%, 34%, 63%, 75%, 44%, and 371%, respectively. Plants grown at the 2569 MASL had higher nutrient content in the leaves and increased biomass production than the 1,487 MASL location, regardless of fertilizer treatment. The authors stressed that the nutrient regime depends on environmental factors (precipitation, soil type, cation exchange capacity, water holding capacity) and adjustments in the nutrient regime are specific to production location (Díaz-Gutiérrez et al., 2021).

Verma et al. (2020) studied the effect of three rates of nitrogen (300, 200, 100 kg/ha), three rates of phosphorous (180, 120, 60 kg/ha), and two potassium rates (160, 80 kg/ha) on biomass production in India. Plant height, branch count, and leaf count were observed on a per-plant basis, and biomass parameters included fresh plot weight, fresh leaf weight, and dry leaf weight. Harvest was conducted four times per year during the two-year study. At the 300 kg/ha rate, nitrogen produced the highest dry leaf yield, with 45.68 Mg/ha, followed by 200 kg/ha nitrogen, with 43.76 Mg/ha, and the 100 kg/ha rate produced the lowest yield of 38 Mg/ha. Potassium level did not have a significant influence on yield. The authors reported N:P:K rate of 300:180:160 kg/ha was the optimal fertilizer regime for their location (Verma et al., 2020).

A research team hypothesized the addition of potassium humate would increase rates of photosynthesis of stevia grown under three photoperiods using natural light in a greenhouse as control, adding 1.5 or 2.5 hours of supplemental light at the end of the day to extend day length for treatment two and three, respectively (Fazeli Kakhki et al., 2019). Under their natural light conditions, adding 1.5 hours of supplemental light and applying potassium humate as a foliar spray four times in seven-day intervals increased plant growth, biomass, and dry leaf weight. These results confirm previous reports that increased day length increases plant growth and

suggest further research is needed to determine if supplemental foliar fertilizers are necessary for greenhouse production in other regions (Fazeli Kakhki et al., 2019). Another research group investigated the effect of foliar application of abscisic acid and fluridone and decapitation on branching, leaf yield, and SG content on stevia in a greenhouse experiment (Tavakoli et al., 2020). Cutting the tops off plants reduced apical dominance and increased bud break of lower auxiliary buds. Abscisic acid after decapitation increased bud break more than decapitation alone; increased plant size and increased SG accumulation were observed. The best treatment to increase biomass and SG content was a foliar application of 50 µM abscisic acid and 50 µM fluridone (Tavakoli et al., 2020). This experiment demonstrates that stevia growth and SG content are flexible and that these chemicals could be further evaluated in applied production studies to identify commercial use.

Harvest and Drying Methods

A 900 g/m^2 dry matter yield was achieved in a once-over harvest at the end of the season in Davis, California (Shock, 1982b). Plants are harvested by cutting them at 10 cm above the soil surface and then removing leaves from stems once dry (Hossain et al., 2017). Yield in Mississippi was highest, from one harvest per 180 days rather than two harvests per 90 days or three harvests per 60 days (Moraes et al., 2013). Two to three harvests require more labor and resources than a single harvest event, and thus breeding programs need to focus on collecting data with fewer labor inputs. Tavarini et al. (2013) studied the effects of cultural practices, nitrogen fertilizer, harvest time, and crop age on stevioside and rebaudioside (Reb-)A content and propagation method, micropropagation, stem cutting, and direct seeding. A significant effect

of genotype on yield and glycoside content and a significant effect of cultural practice and propagation method on glycoside concentration were reported.

The SG levels change as plants develop, and SG accumulation is also dependent on photoperiod, temperature, and other environmental conditions (Singh and Rao, 2005). Soluble sugars and proteins increase with enhanced water uptake early during plant development and more rapidly during the final stages of vegetative growth. Viana et al. (1980) reported that free sugars decreased as leaf growth decreased. They indicated that protein synthesis depended on photosynthesis carbon supply and was only achieved at 80% full leaf area. Harvesting late in the growing season, under long-day conditions, leading to increased biomass production and higher SG yield (Tavarini et al., 2013; Metivier and Viana, 1979; Ermakov et al., 1994).

Drying is an essential process of stevia production. Post-harvest handling of dry leaves needs to be considered to maintain high quality, prolong storage life, maintain low moisture, and inhibit microorganism activity and decay (Chakraborty and Dey, 2016). Al Amrani et al. (2018) conducted a study examining the effect of the drying method on the content of stevioside in dry leaf matter. Out of eight drying temperature treatments, solar radiation and oven drying at 50ºC produced the highest stevioside and Reb-A range of 7.86% and 7.84%, respectively. Drying leaves at 60ºC and microwaving for 2min produced the highest percentage of stevioside and Reb-A with 0.355% and 0.350%, respectively (Al Amrani et al., 2018). Stevia leaves need to be processed quickly after harvest because they contain 80% moisture and wilt rapidly (Al Amrani et al., 2018). Another research group evaluated temperatures ranging from 30 to 80ºC; it was discovered that phenolics and flavonoids were highest after drying leaves below 50ºC, antioxidant content was highest when dried at 40ºC, and SG content was highest when dried up to 50ºC; there was no benefit to applying 60 to 80ºC hot air (Lemus-Mondaca et al., 2016).

Abiotic and Biotic Stress

Environmental stress can include drought, waterlogging, salinity, nutrient deficiency, toxicity, and cold and hot temperatures, impacting plant growth and production (Tzortzakis, 2010). Tolerance is the ability of a plant to produce adequate yield without reduction despite being subjected to stress (Debnath et al., 2019). Cold tolerance is a desirable trait for a perennial production system, especially in areas with a cold winter. Stevia is sensitive to low temperatures during the seedling growth stage (Soufi et al., 2016). A pre-treatment of endogenous salicylic acid, hydrogen peroxide, 6-benzylaminopurine, and calcium chloride was shown to induce chilling tolerance upon exposure to 10/6ºC day/night temperatures for 120 hours in a controlled environment study conducted on stevia. Endogenous application of signaling compounds positively affected dry weight, leaf area, electron transport rate, and quantum photosynthetic yield. The content of chlorophyll and carotenoids were not affected by low-temperature treatment and the application of signaling compounds (Soufi et al., 2015; Soufi et al., 2016). Two Romanian genotypes were identified that would overwinter reliably, and two other genotypes were identified in central Italy that overwintered successfully for up to 8 years (Viînatoru et al., 2019; Andolfi et al., 2006). These studies helped demonstrate the ability of stevia to overwinter in different climates. However, a reliable protocol to evaluate germplasm in controlled environments would allow researchers to observe cold tolerance without directly confounding from other environmental factors. Kozik et al. (2020) developed a protocol using 14 genotypes, subjecting a subplot of each genotype to cold treatments of 2, 0, -2, and -4ºC at five durations from 2-10 days. Treatments with the highest genetic variance included 2ºC for two days, 0ºC for eight days, and -2ºC for six days. Three genotypes were identified with high tolerance; the best genotype was 7947-3 which was able to tolerate six days of -2ºC treatment

(Kozik et al., 2020). The methods from this research can be used in developing tolerant cultivars.

Salt stress is a limiting factor for plant growth and production in arid and semi-arid regions (Machado and Serralheiro, 2017). Soufi et al. (2019) suggested using saline irrigation to evaluate and improve germplasm for crop production in saline soils. They found stevia to be moderately tolerant to saltwater irrigation using rates of 3.40 and 4.64 dS $m^{-1,}$ while 7.72 dS m^{-1} reduced root: shoot ratio, leaf number, and leaf area. Research conducted by Mahajan et al. (2020) evaluated using exogenous application of KNO_3 to enhance salt tolerance in stevia. In that study, plants were subjected to four NaCl levels (0, 40, 80, and 120mM) and five KNO3 concentrations (0, 2.5, 5.0, 7.5, and 10g/L). They demonstrated that biomass was reduced as salinity levels increased because nutrient uptake was reduced. Application of KNO_3 increased yield up to 26% under saline conditions. SG content was highest with 5.0g/L KNO_3. Stevia demonstrated moderate salt tolerance to 80mM NaCl treatment in this experiment.

Two salt-tolerant cultivars, ‘Zhongshan No. 3’ and ‘Shoutian No.2’, were crossed with two high Reb-A cultivars, ‘Zhongshan No.4’ and ‘Shoutian No.3’ to develop hybrids for salinity tolerance evaluation (Yang et al., 2012). Researchers compared sand and hydroponic culture using clones of hybrids generated from seven cross combinations. They observed that seed set and germination were lowest in self-pollinated treatments compared to cross-pollinated treatments; ‘Shoutian No. 2’ x ‘Zhongshan No.3’ had the highest seed set rate of 74.9%, and germination varied from 63.8 to 89.0% across all hybrids. No significant differences were observed among hybrids under salt treatment for seven days with 100mmol/L NaCl in sand culture; after 28 days, hybrids generated from ‘Shoutian No. 2’ x ‘Shoutian No.3’ and self-pollinated progeny of ‘Zhongshan No.3’ had significantly higher survival rates. The hydroponic

culture was maintained for 14 days using treatments 100, 150, 200, and 250 mmol/L NaCl; high salt tolerance was observed in hybrids derived from ‘Shoutian No. 2’ x ‘Zhongshan No.3’, ‘Zhongshan No.3’ x ‘Shoutian No. 2’, and ‘Zhongshan No.3’ x ‘Shoutian No. 3’ (Yang et al., 2012). Results indicated that improvement in salt tolerance could be achieved in stevia, and hydroponic culture can be used to conduct seedling evaluations to develop salt-tolerant cultivars.

A salinity study was conducted using micropropagation of calli produced *in vitro* from two cultivars of stevia, ‘Sugar High-A3’ and ‘Spanti’, grown on basal medium supplemented with 0, 500, 1000, 2000, and 3000 mg/L NaCl (Al-Taweel et al., 2019). Callus survival, regeneration rate, explant height, shoot number, leaf count, root count, and root length were all negatively affected by the increasing concentration of NaCl. ‘Sugar High-A3’ was more tolerant to salinity stress than ‘Spanti’. They also found that cDNA-SCoT markers were helpful in validating phenotypic observations of salinity tolerance; three of five primers were present in ‘Sugar High-A3’, which was not observed in ‘Spanti’, indicating cDNA-SCoT markers can assist breeders in selection for salt tolerance in stevia (Al-Taweel et al., 2019).

Debnath et al. (2019) evaluated the effect of waterlogged conditions, drought conditions, and salinity conditions on two cultivars of stevia, ‘Fengtian’ and ‘Shoutian-2’, in a greenhouse container study. Waterlogging had the worst impact on biomass production for both cultivars. ‘Fengtian’ could tolerate saline conditions and even had an increased Reb-A content, while ‘Shoutian-2’ had a high loss of biomass under the same conditions and no increase in Reb-A content. ‘Shoutian-2’ was able to tolerate drought stress and had increased stevioside content under limited water conditions (Debnath et al., 2019). Breeders can use those methods to identify germplasm with tolerance to salinity, drought, and waterlogged conditions. In a hydroponic salinity study, a differential accumulation of SG was observed in salt-tolerant

‘Fengtian’ and salt-sensitive ‘Shoutian -2’; also, Reb-A content increased by 53% while stevioside content decreased by 38% in ‘Fengtian’ when exposed to salt stress (Debnath et al., 2018). Researchers suggested salt stress treatment could regulate the amount of SG a cultivar produces.

In southern Italy, three irrigation treatments (100% water restitution, 66% water restitution, and 33% water restitution) were evaluated in a field study to determine water consumption’s effect on yield and quality of stevia (Lavini et al., 2008). The trial was harvested twice per year; the second harvest in both years had higher evaporative demand late in the growing season when the air temperature was higher. Researchers observed that 100% water restitution resulted in 40% more dry leaf yield than 33% water restitution treatment. Lavini et al. (2008) reported no significant differences in SG content for years or irrigation treatments, while harvest index and water efficiency values decreased with increased water restitution. Water consumption was found to be proportional to the irrigation supplied. Increased water supply did not significantly increase yield, indicating water volume can be decreased if water is limited or expensive. The authors stressed the importance of developing cultivation guidelines as well as breeding cultivars adapted to specific environments (Lavini et al., 2008).

A field study was conducted in rice fallow in India for two years where stevia plants were subjected to three drip-irrigation treatments (100%, 80%, and 60% water restitution), and three fertility levels (100%, 75%, and 50% of N-P-K at rate 110-45-45 kg/ha) with a control of surface water and fertilizer application (Behera et al., 2013). The 100% water restitution had a high fresh leaf, dry leaf, and biomass yields with 8.95 Mg/ha, 2.74 Mg/ha, and 3.44 Mg/ha, respectively; the 100% fertility treatment produced 8.21 Mg/ha fresh leaves, 2.53 Mg/ha dry leaves, and 33.50 Mg/ha biomass. The application of 100% water restitution and 100% fertility treatment resulted

in the highest yields with a fresh leaf weight of 9.12 Mg/ha, dry leaf weight of 2.90 Mg/ha, and biomass weight of 3.50 Mg/ha (Behera et al., 2013).

Two *Streptomyces* isolates from the roots of *Cucumis sativus* L., and *Salicornia europaea* L. were applied to stevia subjected to salt stress to observe plant growth-promoting rhizobacteria impact (Tolba et al., 2019). Both *Streptomyces* isolates increased plant growth parameters and may prove useful in developing a biological product to enhance growth and production under abiotic stress conditions. The use of biochar containing beneficial microorganisms and biochar containing inorganic nitrogen was compared with commercial synthetic N-P-K fertilizer as a control in a field study conducted in Egypt (Mahmoud et al., 2020). One of the biochar treatments contained *Spirulina platensis*, *Anabaena azollae, Azobcter chrococcoum, Bacillus subtilis,* and *Pseudomonas fluorescens* produced results most similar to the control treatment. No significant difference was observed in the SG content of biochar treatments compared to the control. The study demonstrated that biofertilizers effectively enhanced plant growth, increasing nutrient content, and improved the soil microbiome (Mahmoud et al., 2020).

Stevia Plant Diseases and Pests

Many plant pathogens attack *S. rebaudiana* including *Septoria steviae*, *Alternaria alternata*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum, Rhizoctonia solani*, Tomato spotted wilt virus, Aster yellows phytoplasma, *Fusarium armeniacum*, *Cerabasidium*, *Botrytis cinerea*, and *Pythium spp.* (Maiti et al., 2007; Ishiba et al., 1982; Koehler and Shew, 2014; Koehler et al., 2017b; Collins et al., 2016; Koehler et al., 2016, Koehler and Shew, 2019; Thomas, 2000). *S. steviae* causes Septoria leaf spot disease and was first reported in

Japan (Ishiba et al., 1982), later in Canada (Loving and Reeleder, 1996), and now it can be found across production regions in North Carolina (Koehler, 2018a).

Septoria steviae is a particularly devasting disease that does well in hot, humid conditions commonly found in the southeast US. This disease is characterized by olive-gray leaf lesions surrounded by chlorotic halos that coalesce, become necrotic, and eventually defoliate the entire plant, causing crop losses when untreated (Koehler and Shew, 2018b). Crop debris remaining in the field can harbor *S. steviae*, allowing it to overwinter; in perennial crop systems, this can impact the following growing season by providing fresh, young, new shoots for the pathogen to infect (Koehler and Shew, 2018b). Resistance to *S. steviae* in stevia was identified by Reeleder (2001) in Canada, who developed a screening protocol and identified resistant clone 598-1 during field and greenhouse trials. Koehler and Shew (2018b) evaluated the efficacy of seven fungicides on multiple *S. steviae* isolates and found that all of them were sensitive to single and combined product treatments of Azoxystrobin, chlorothalonil, flupyram, fluxapyroxad, pyraclostrobin, and tebuconazole in a two year, two field site study. Koehler and Shew (2017a) also found the overwintering survival of stevia to be improved by the application of QoI fungicides for perennial cropping systems. Another important root-infecting pathogen in perennial stevia systems that causes large-scale destruction is *Macrophomina phaseolina* (Koehler and Shew, 2018c, 2019). Charcoal rot was first reported in North Carolina in 2018 and is characterized by small, black root lesions visualized on infected roots of crowns removed from the field (Koehler et al., 2018c). Several fungal diseases of stevia have been reported in Egypt, including *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *F. semitectum*, *F. solani*, *Rhizoctonia solani*, *Alternaria steviae*, and *Botrytis cinerea* (Hilal and Baiuomy, 2000).

Few studies focus on the impact of insect infestation on biomass production. Aphids, mealy bugs, red spider mites, and whiteflies have been reported on stevia (Thomas, 2000). Also, pollinator studies are needed in various production regions to understand the species that are pollinating the crop besides honeybees; this could help scientists determine why seed set may be low in specific areas.

Steviol Glycosides

The SGs are secondary metabolites; they are tetracyclic diterpene compounds derived from the same kauenoid as the gibberellic acid precursor (Brandle and Telmer, 2007). Stevia favors the production of SG via an altered biochemical pathway over the production of gibberellic acid (Richman et al., 2002). It begins when kaurene is converted into steviol (Yadav et al., 2011). Then, steviol is synthesized for glycosylation reactions to occur and glucose groups to attach, forming different SG compounds (Brandle and Telmer, 2007). Most of the SGs are created from undergoing glycosylation four times, starting with a synthesis of steviol and ending with Reb-A formation (Richman et al., 2005). Synthesis begins in the chloroplast, then compounds are transported to the endoplasmic reticulum, Golgi apparatus, and then transported to the vacuole (Yadav et al., 2011). Kaurene synthase and *ent*-copalyl diphosphate are intermediate steps in the synthesis pathway, and stevia has a duplicated gene for kaurene-synthase (Richman et al., 2002). Both genes are highly expressed in mature leaves, making stevia a unique species for its biochemistry as it accumulates these SG intermediate products in its leaves. The enzyme responsible for glycosylation and formation of SG is uridine diphosphate glycosyltransferase (UGT). The many UGTs in stevia play a critical role in the pattern of glycosylation of steviol into different SGs (Madhav et al., 2013).

Extraction and purification of SG was first conducted by two French scientists in the 1930s; stevioside and Reb-A were the first reported compounds (Bridel and Lavielle, 1931). SGs are reportedly 250-300 times sweeter than sucrose, heat stable, pH-stable, and non-fermentable (Brandle, 2004; Geuns, 2003). The chemical structure of SG is composed of an aglycone, steviol, and various glucose molecules can attach to steviol to form different SGs (Chatsudthipong and Muanprasat, 2009). Stevioside is the best documented SG studied to date; however, there are over 30 SGs that can be found in stevia, including Reb-A to G, steviobioside, steviolmonoside, rubusoside, and dulcoside A (Gardana et al., 2010; Chaturvedula et al., 2011; Wölwer-Rieck, 2012**)**. Each SG has been reported to have different levels of sweetness compared to sucrose: Stevioside is 150 to 300 times sweeter, Reb-A is 200 to 400 times sweeter, Reb-B is 300 to 350 times sweeter, Reb-C is 50 to 120 times sweeter, Reb-D is 200 to 300 times sweeter, Reb-E is 250 to 300 times sweeter, rubusoside is 110 times sweeter, steviobioside is 100 to 125 times sweeter, and dulcoside A is 50 to 120 times sweeter than sucrose (Ashwell, 2015). Another researcher reported stevioside is 250-300 times sweeter than sucrose, Reb-A is 350-450 times sweeter than sucrose, Reb-B is 300-350 times sweeter than sucrose, Reb-C is considered 50-120 times sweeter than sucrose, Reb-D is 200-300 times sweeter than sucrose, Reb-E 250-300 times sweeter than sucrose, dulcoside A is 50 to 120 times sweeter than sucrose, and steviobioside is 100 to 125 times sweeter than sucrose. Rubusoside was the most bitter compound tasted while Reb-D was the least bitter, making this compound a higher priority for food and beverages (Hellfritsch et al., 2012).

Cultivars produce varying amounts of SG ranging from 4 to 20% in dry weight. Stevioside ranged from 5 to 10% of dry weight and made up 60 to 70% of total steviol glycosides. Reb-A ranged from 2 to 4%, and Reb-C ranged from 1 to 2% of dry weight (Yadav

et al., 2011). SG accumulates in the plant as it ages; older leaves have a higher concentration of SG than younger leaves, and because SG accumulates in the chloroplast, SGs are highest in leaves compared to stems and roots (Singh and Rao, 2005). Reb-A is usually present in 30 to 40% of the sweetness profile, and stevioside makes up 60 to 70% of the profile. The ratio of Reb-A: stevioside is a good indicator of sweetness. Reb-A is more desirable than stevioside because it does not have a lingering licorice aftertaste (Yadav et al., 2011). SG yield is altered by tissue type, tissue age, propagation method, day length, and cultivation (Singh and Rao, 2005; Tamura et al., 1984; Metivier and Viana, 1979; Shock, 1982a).

Extraction Methods

There are several methods to extract SG such as water-based extraction, extraction using organic solvents like chloroform-methane, glycerol and propylene glycol, and solid-phase extraction (Kinghorn and Soejarto, 1985; Pasquel et al., 2000; Phillips, 1987; Bovanová et al., 1998; Vaněk et al., 2001; Pól et al., 2007). Other extraction methods include pressurized liquid extraction (PLE), pressurized hot water extraction (PHWE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and supercritical fluid extraction (SFE) (Tan et al., 1988; Kienle, 1990; Kovylyaeva et al., 2007; Liu et al., 2010). Jaitak et al. (2009) compared three different extraction methods: conventional solvent extraction, MAE, and UAE. The MAE method resulted in the highest concentration of stevioside and Reb-A with 8.64% and 2.34%, respectively. Conventional extraction produced 6.54% stevioside and 1.20% Reb-A, while UAE yielded 4.20% stevioside and 1.98% Reb-A (Jaitak et al., 2009). Speed has been a major consideration for extraction techniques; MAE requires 1 minute to extract SG, UAE extraction takes about 30 minutes, and conventional extraction using the Soxlet method takes 16 hours

(Jaitak et al., 2009; Ferrazzano et al., 2015). Periche et al. (2015) also evaluated conventional, UAE, and MAE extraction methods and reported that UAE and MAE produced higher yields of SG than conventional heating methods. They also concluded that the ideal drying method for stevia leaves depends on their final use and recommended 180°C hot air for optimal drying conditions to use leaves for a sweetener or antioxidants (Periche et al., 2015). SG quantification can be measured using an ultra-high-performance liquid chromatography (U-HPLC) and tandem mass spectrometry (Shafii et al., 2012). The authors evaluated field and greenhouse-grown 'Eirete', 'Criolla', and a population developed at Michigan State University. Reb-A content ranged from 0.25 to 16.4% in plant leaves, indicating high genetic and environmental variability (Shafii et al., 2012). Another method used to measure SG is HILIC-MS/MS, which was reported to be faster and more accurate than U-HPLC (Well et al., 2013).

Essential Oils

Hossain et al. (2010) discovered 62 compounds in stevia leaf essential oil, and concluded it was predominantly composed of monoterpenes and sesquiterpenes. Of importance are α-cadinol, caryophyllene oxide, (-)-spathulenol, and ß-guaiene. The antimicrobial activity of stevia essential oil was attributed to the terpene compounds listed above (Hossain et al., 2010). Markovic et al. (2008) found sesquiterpenes: ß-caryophyllene, spathulenon and caryophyllene oxide were responsible for antimicrobial activity, making up 50 of 88 compounds in the essential oil extract. It was concluded that these compounds play a major role in antimicrobial activity. Also identified were nerol, ß-cyclocital, safranal, aromadenrene, α-amorphene, and T-muurolol, which were the first report of these compounds in stevia (Markovic et al., 2008).

Breeding Objectives

Some breeding programs are focused on improving total SG content, Reb-A: stevioside ratio, and leaf yield (Yadav et al., 2011). Higher Reb-A content is desirable to enhance a sweet flavor profile, and lower stevioside content reduces the lingering bitter aftertaste that some consumers find undesirable. Attention has been paid to improving the leaf: stem ratio because leaves accumulate the most SG compared to stems and roots, and a higher leaf yield is important to maximize agriculture field use. As demonstrated by previous research, gains can be made in these traits (Lee et al., 1982; Morita, 1987; Brandle and Rosa, 1992). The high level of heterogeneity in stevia is due to genetically-enforced natural outcrossing and can be used by breeders to select for high performance of agronomically important traits (Huang et al., 1995).

Other traits of lesser importance are rapid growth rate and regrowth rate, increased photosynthetic activity, high adaptability, resistance to abiotic and biotic stresses, photoperiod insensitivity, and self-compatibility (Tavarini et al., 2018). The selection of plants having longer and broader leaves, higher leaf area, and leaf thickness can help improve yield (Tavarini et al., 2018). The ability to regrow rapidly after harvest is a desirable trait for more than one harvest per season. Darker green leaves have more chlorophyll content and, subsequently a higher photosynthetic rate which might improve leaf production and SG accumulation (Tavarini et al., 2018). Stevia has demonstrated resilience in its ability to grow in diverse environments but is sensitive to frost and cold temperatures found in temperate and cold-weather regions. Temperate regions would benefit from cold-tolerant stevia; genotypes that overwinter reliably produce abundant shoots that regrow quickly should be selected from perennial trials to adapt stevia cultivars for cool climates (Tavarini et al., 2018). Disease resistance to *Septoria steviae* and *Sclerotinia sclerotiorum* is needed since these are the most devasting pathogens; *Septoria steviae*

can cause complete loss of crop when left untreated (Tavarini et al., 2018; Koehler et al., 2018b). Photoperiod insensitivity would be a valuable trait to identify and introgress into a cultivar to avoid flowering under short-day lengths. Self-pollination in stevia is generally unsuccessful due to self-incompatibility; however, it is important to identify genotypes with the ability to self-pollinate and produce abundant high-quality seeds. Identifying genes controlling self-compatibility may help breeders develop markers to select appropriate genotypes for the development of inbreds.

Knowledge of correlation among traits is helpful for breeders for cultivar development. Brandle and Rosa (1992) found no correlation between leaf:stem ratio and leaf yield with stevioside concentration, indicating that improvements can be made to select for decreased stevioside content and for increased yield and leaf:stem ratio. Shyu (1994) reported that leaf thickness was correlated with Reb-A concentration, and leaf size was correlated with dry leaf yield. Selection for larger and thicker leaves might be associated with high dry leaf yield. Selecting SG content late in the season is effective because SGs accumulate as plants age; evaluating seedling SG content is not effective to make selections for increased SG content in mature plants (Weng et al., 1996). The correlation between dulcoside A and stevioside is positive, as is the correlation of Reb-A and Reb-C. Stevioside and Reb-A concentration are negatively correlated and dulcoside A and Reb-C (Nakamura and Tamura, 1985). Guang Xi and Yan conducted a correlation analysis of agronomic traits in 36 genotypes from Canada, Israel, China, and Japan. They concluded that higher yield was achieved when plants were harvested at the end of the growing season because they reached maximum plant height. A high leaf:stem ratio was positively correlated with leaf thickness, dry matter content, and total SG content. Also, Reb-A and Reb-C were positively correlated; when Reb-A and Reb-C were high, stevioside

concentration was lower (Guang Xi and Yan, 2018). A collection of 15 clonal selections was evaluated in field trials in 2004 and 2005 to characterize genetic divergence and examine trait association in stevia (Anami et al., 2010). They reported high genetic diversity among genotypes; plant height and Reb-A: stevioside ratio were positively correlated, and fresh weight, dry weight, and branch count per plant were positively correlated. These results indicate a high genetic diversity of agronomic traits within a relatively small gene pool and traits can be improved in groups if they are positively correlated (Anami et al., 2010). Five genotypes from India were evaluated for morphological and phytochemical traits; stevioside and Reb-A content were correlated with leaf length and width, indicating those traits can be used to improve stevia (Singh et al., 2017).

Breeding Methods

The selection of appropriate clonal parents, proper pollination technique, leaf harvest timing, seed handling methods, and SG quantification method determine the breeding program's success. Stevia is self-incompatible and insect-pollinated; breeding methods have been developed to take advantage of that system (Chalapathi, 1997a; Oddone, 1997). Inbreeding methods are ineffective due to self-incompatibility, turning the focus of breeders toward population improvement and the development of full-sib cultivars.

Population improvement has been achieved using mass selection and recurrent selection in many cross-pollinated crops, including stevia. Single plant selections are maintained clonally, and seeds of selections are harvested separately to evaluate the progeny and select superior families using half-sibling methods. Seeds are harvested from each plot for population improvement using replicated field trials. This selection system is continued for multiple cycles,

increasing the frequency of desired alleles from which to select single plants having superior performance. In North Carolina, plants are allowed to intermate in the field, and seeds of each half-sibling family are collected from single plant selections; the best plants can also be maintained as clonal selections. In the greenhouse, clear mesh cages enclose two plants to make cross-pollinations. Biparental hybrids can be bred using clonal selections. Bluebottle flies can be used as pollinators in the greenhouse, while bees and other insects pollinate stevia in the field.

Genetic variability is generated by crossing two plants to develop a full-sib family or crossing more than two genotypes to develop a synthetic cultivar; once genetic variability has been created, it can be evaluated and then preserved by making a clonal cultigen from a superior single plant selection. Maintaining clonal germplasm is an important aspect of breeding a difficult crop to propagate from seed, like stevia. Single-plant selections are common with asexually bred crops. Clonal selections can be maintained and used as parents for full-sib cultivar development to generate uniform, high-quality seeds reliably each year. Stevia is not maintained in the USDA-ARS germplasm system, so germplasm is not widely available to researchers interested in obtaining research and cultivar development.

Traditional Breeding

There is high phenotypic and genotypic variation within and among stevia populations. Gaurav et al. (2008) found that broad-sense heritability was 99% for leaf yield, 92.8% for stevioside content, and 97% for plant height in a study of 10 stevia cultivars grown in three replications at one location. Brandle et al. (1998b) studied Reb-A and Reb-C inheritance in two crosses derived from unrelated parents. Reb-A was found to be controlled by a single dominant gene in the first cross, and a single additive gene controlled steviol glycoside content in the

second cross. Reb-A and Reb-C were linked in coupling in the first cross and repulsion in the second cross. Brandle identified a gene controlling high Reb-C, *Rce*, from the selection 95-002A-336, the type line. There were no reciprocal differences observed in the two crosses. Reb-A and Reb-C can be manipulated through selection simultaneously when the appropriate parent is selected for those traits. Brandle and Rosa (1992) estimated the narrow-sense inheritance of leaf yield, leaf: stem ratio, and stevioside content, which were 62.1, 78.8, and 76.7%, respectively. This high level of heritability in agronomic traits enables breeders to make high genetic gains.

Stevia is an outcrossing species with high genetic and phenotypic variability providing breeders the opportunity to identify novel genotypes with desirable SG profiles and agronomic traits (Handro et al., 1993). The open-pollinated behavior of the crop accounts for a portion of the observed phenotypic variation (Tateo et al., 1998). Wild-type stevia is reported to have 2 to 3% SG content of dry leaf matter (Yadav et al., 2011). Morita (1987) identified a cultivar with 9.1:1 Reb-A: stevioside content. Variation in SG within the same clone was observed by a research group in China, who reported stevioside content of 1.48 to 6.98%, and Reb-A content of 4.5 to 12.1%, and total SG of 10.26 to 19.57% (Huang et al., 1995).

Ceunen and Geuns (2013) found that plant growth under a 16-hour photoperiod, simulating long-day conditions, had higher stevioside than those grown under 8-hour photoperiod (short-day conditions); the young shoot tissue had four times more stevioside content than mature lower canopy leaves. Stevia plants have variable sensitivity to daylength of 8 to 14 hours (Valio and Rocha, 1997; Zaidan et al., 1980). Stevia can flower 54 to 104 days following transplant and can be classified into three photoperiod categories: short-day, day-neutral, and long-day (Zaidan et al., 1980).

Molecular Breeding

Marker-assisted selection can be used to aid phenotypic selection; it is considered more reliable than phenotypic selection because markers can be tested on plants in any growth stage, and markers consistently detect genes in plants regardless of environmental conditions (Tavarini et al., 2018). A linkage map was created from 183 RAPD markers covering 1389 cM, with 35.5% of the markers polymorphic and 62.5% of the markers segregating in a 1:1 ratio (Yao et al., 1999). These results demonstrate there is an abundance of polymorphisms in the stevia genome. Chester et al. (2013) used 20 random operon RAPD markers to evaluate the genetic diversity of 11 stevia accessions. Eighty-seven amplicon products were produced using ten of the 20 markers, and 67 were polymorphic, indicating high genetic variation among the 11 accessions. Also, Thiyagarajan and Venkatachalam (2015) used RAPD markers to examine genetic variation in three accessions and discovered nine primers that produced 111 amplicons, 90 of which were polymorphic, indicating there was high diversity among the three genotypes in their collection. Simple sequence repeat markers were developed and tested on five greenhouse-grown genotypes and 33 field-grown genotypes, and results indicated that genotypes could be distinguished using five markers (Hastory et al., 2015). Kaur et al. (2015) identified 168 single sequence repeats (SSR) from 5548 expressed sequence tags (ESTs). They synthesized 18 SSR primers derived from 18 singleton ESTs making up 61.11% polymorphism among 16 stevia accessions from different geographic regions. Those markers can be used to generate a genetic map in a segregating stevia population to aid in genomic analysis. Inter simple sequence repeat (ISSR) markers have been developed and used to assess morphological and phytochemical proprieties of 17 accessions collected from Malaysia and Paraguay; 32 ISSR markers produced 332 bands, 264 were polymorphic and successfully used to distinguish genotype relatedness.

These research findings provide yet another example of high genetic diversity in stevia and gives breeders another resource to aid in the selection of superior genotypes (Othman et al., 2018). Eighteen EST-SSR markers were developed from The Compositae Genome Project and used to genotype 145 stevia individuals, including 31 cultivars and 31 landraces from diverse geographical locations, to determine genetic diversity in the germplasm. The genotypes clustered into three main genetic groups; the landraces grouped with old cultivars, 'Eirete' and 'Morita III', and new cultivars. High (69%) heterozygosity and high (60 to 84%) polymorphism of EST-SSR markers indicated high genetic variability; however, SG phenotypes did not separate based on the genetic cluster, demonstrating high variability in SG phenotype within each genetic cluster (Cosson et al., 2019). Recently, 31 seeded accessions collected near the site of crop origin were genotyped using six published microsatellite markers, including two markers involved in SG biosynthesis. The genotypes were found to be closely related to each other, and the authors suggested introducing exotic germplasm to improve diversity (Ribeiro et al., 2021).

Brandle et al. (2002) identified 70% of the candidate genes for the known steps of the pathway for steviol glycoside production. Totté et al. (2003) have cloned genes that control the isoprenoid biosynthesis pathway. Cloning of seven genes and expression analysis of 15 genes showed different levels of steviol glycoside activity in leaves, one through five, from the growing tip down to the fifth node (Kumar et al., 2012). These results indicated that stevia leaves could have different concentrations of steviol glycosides in other areas of the canopy within the same plant. These advancements in gene identification, gene expression, and the study of genetic inheritance will enable molecular marker development to aid in selecting steviol glycosides for potential sweeteners of interest to processors.

Stevia Germplasm

There are over 90 cultivars of stevia grown around the world. 'Criolla' is one of the most researched cultivars and is believed to be the original one grown in Paraguay (Tavarini et al., 2018). Stevia seeds are available from many US seed company types: garden, heirloom, and commercial. Garden seed sources include Johnny's seeds, Park Seed, Burpee, and Territorial Seeds. Heirloom cultivars are supplied by companies such as Richters, Baker Creek Heirloom, Seed Savers, and Swallowtail Garden. Stevia-Store is an example of a commercial seed company with ‘Morita II’, ‘Morita III’, ‘Eirete I’, ‘Eirete II’, ‘Native’, and ‘Katupyry’ (Stevia-Store, 2021). ‘Eirete’ is a hybrid developed for high productivity, ‘Morita III’ was derived from ‘Morita II’ and selected for its ability to grow without much irrigation, and ‘Katupyry’ was developed for production in arid regions (Tavarini et al., 2018). S&W seed company provides growers with high-quality seeds with improved SG content, including: ‘SW107’, ‘SW129’, ‘SW201’, and ‘SW227’ (swseedco.com, 2020). ‘SW107’ can overwinter, and has high plant vigor, high yield, and sweet taste. ‘SW201’ can overwinter, produces high leaf yield, high SG content, high plant vigor, and late flowering. (Tavarini et al., 2018). Two genotypes derived from Seed Savers, NC-1003 and NC-1022, were identified for superior SG profiles in a trial using 16 genotypes in North Carolina (Huber and Wehner, 2021). Andolfi et al. (2006) reported two genotypes, B1 and B2, derived from a Brazilian seed source that can overwinter successfully for eight years in central Italy and remain highly productive through the fifth or sixth year. A two-year field study was conducted in central Italy to evaluate nine genotypes using two harvest times for agronomic traits (growth cycle, biometric characteristics, leaf yield, and quality traits (content of SG, phenols, flavonoids, and antioxidants) (Clemente et al., 2021). Second-year growth produced the most dried leaves, highest phenol accumulation, and highest antioxidant activity; the first year

SG content was more significant than the second-year crop. Results indicated that genotype and plant age influenced SG profile, which remained consistent over harvest times. Four genotypes were identified (PL, SL, BR5, and SW30) for high performance that will be beneficial for germplasm improvement for Mediterranean regions (Clemente et al., 2021).

Research and breeding programs in China, Japan, India, Brazil, Canada, Italy, France, and the United States examine different stevia cultivars for performance under their conditions. A Brazilian research team identified a new cultivar with a glycoside profile absent of bitter after-taste, UEM-320, with a Reb-A: stevioside ratio of 1: 1 (Dacome et al., 2005). The Chinese Academy of Agriculture Sciences released three cultivars in the 1990s: 'Yunri', 'Yunbin', and 'Zongping'; all have high Reb-A, high stevioside content, and high yield (Shu, 1989; 1995). Another Chinese cultivar released for high yield and high Reb-A: stevioside content is 'SM4' (Weng et al., 1996). In India, a genotype with delayed flowering was identified from the Institute of Himalayan Bioresource Technology, 'CSIR-IHBT-ST-02', which may offer growers the opportunity to harvest multiple times instead of once, and 'CSIR-IHBT-ST-01', which has a 7.34% Reb-A content and 5.87% stevioside content which would be desirable for production (Yadav et al., 2019a; 2019b). In 2000, two cultivars selected for high yield and SG content, 'Madhuguna' and 'Madhuguni', were released from the Institute of Himalayan Bioresource Technology (Yadav et al., 2011). A Romanian breeding program reported two cold-tolerant genotypes developed via recurrent selection; G1 has an erect growth habit, lanceolate leaves, and pubescent stems, while G2 has a globular growth habit, small ovoid leaves, and thin branches (Vînătoru et al., 2019). Both genotypes were selected for their ability to overwinter reliably. Two other genotypes were discovered in central Italy to overwinter successfully for up to 8 years (Andolfi et al., 2006).

In Korea, Lee et al. (1978, 1982) identified two cultivars, ‘Suweon 2’ for high yield and stevioside and ‘Suweon 11’ for high SG content and thick leaves. A Russian cultivar, ‘Ramonskaya Slastena’ was identified in 1996 as having a high yield (Kornienko and Parfenov, 1996; Yadav et al., 2011). Another study reported a lodging resistant, high yielding cultivar: ‘Primorskaya Slastena’, with a yield higher than ‘Ramonskaya Slastena’ (Romashova et al., 2017). Three high-yielding cultivars with improved Reb-A: stevioside ratio, K1, K2, and K3 were identified by Shyu; also, dry leaf yield was correlated with leaf size and leaf thickness, and content of Reb-A was positively correlated with leaf thickness (Shyu, 1994; Yadav et al., 2011). Mass selection experiments in Indonesia led to the creation of the cultivar BPP72 (Suhendi, 1989). The Morita group has developed stevia germplasm that is still grown today; they patented several stevia cultivars with high Reb-A: stevioside ratio of 9.1: 1, high yield, and 10% total SG content (Morita, 1984a; 1984b; 1985; 1986; 1987; Morita and Yucheng, 1998). Pure Circle has several stevia cultivar patents, including a new biparental hybrid cultivar, ‘16265046’, derived from a cross with another patented Pure Circle cultivar, ‘814011’ with an unpatented high Reb-M line ‘44004’, and ‘18136109’ (Pure Circle USA Inc., 2017; 2020a; 2020b).

A stevia breeding project was developed in Canada in 1989 using landraces from China to evaluate germplasm for crop improvement (Sys et al., 1998; Marsolais et al., 1998). Fifteen plants were selected based on SG content and intercrossed to develop half-sibling families. Half-sibling family inheritance for agronomic traits and SG content were evaluated, and two clonal selections were made and maintained; RSIT-94-1306 and RSIT-94-751 from two of the original selections (Brandle and Rosa, 1992; Yadav et al., 2011). Brandle et al. (1998b) developed RSIT-95-166-13 for high Reb-C :stevioside ratio. It was an individual clonal plant selection from a half-sib family plot using intercrossed progeny derived from Brazilian open-pollinated landrace

Zairai under field evaluation at the Delhi research station in Canada (Yadav et al., 2011; Brandle et al., 1998b). Later, the same research team developed AC Black Bird synthetic cultivar because it had 14% total SG and a high ratio of Reb-A: stevioside (Brandle et al., 2001).

Trials conducted in Malaysia by Tan et al. (2008) showed MSR-028 was the best cultivar, with a yield of 10 Mg/ha fresh weight compared to cultivars from China, Canada, U.S., Russia, and India with yields of 1.4 Mg/ha, 2.8 Mg/ha, 3.6 Mg/ha, 5.5 Mg/ha, and 4 Mg/ha, respectively. Cultivar MSR-028 produced 10 Mg/ha fresh weight and 2.8 Mg/ha dry weight (Tan et al., 2008). Environment plays a key role in the performance of cultivars, so MSR-20 may not yield as well in other geographic locations. A regional approach to cultivar improvement is necessary to develop germplasm for specific production areas.

Benefits to Human Health

Stevia consumption has been evaluated for human health benefits and is reportedly anticarcinogenic, antidiabetic, antihyperglycemic, antihypertension, antimicrobial, anti-human rotavirus activities; it also improves renal function and influences glucose metabolism; also, it adds nutritional value, with calcium, phosphorous, and proteins (Singh and Rao, 2005). Several species of *Stevia* have medicinal uses, including *S. balansae*, *S. cardiatica*, *S. connata*, *S. elatior*, and *S. eupatoria*; they can aid in intestinal afflictions, cardiac health, gastric health, dermatological afflictions, and renal function, respectively (Soejarto et al., 1983; 2001).

It is well known that sucrose is detrimental to dental health. Several researchers have evaluated the benefits of stevia extract on oral hygiene. One such study demonstrated microbial inhibition of cariogenic bacteria *Streptococcus* and *Lactobacillus* when stevia hexane extract was

present in *in vitro* assays (Gamboa and Chaves, 2012). The authors reported more than 50% growth inhibition when stevia extract was present in the medium.

Historical use of stevia has been difficult to trace; the Guarani tribe and Mestizos reportedly used the species to sweeten maté, teas, and other foods. Stevia was allegedly sold in local drug stores in Paraguay to treat diabetes once it was recognized for its hypoglycemic property in the late 1800s without any toxicity reported (Lewis et al., 1992; Curry and Roberts, 2008; Momtazi-Borojeni et al., 2017). Stevia has insulinotropic, glucagonostatic, antihyperglycemic and blood-pressure-suppressing effects in diabetes mellitus, the metabolic disease characterized by hyperglycemia from glucose dysregulation (Suanarunsawata et al., 2004; Holvoet et al., 2015; Latha et al., 2017). Stevia has anti-inflammatory activity and could be used to develop safe and cost-effective products for arthritis, inflammatory bowel disease, and atherosclerosis (Shoelson et al., 2006; Audial and Bonnotte, 2015; Zou et al., 2020).

Stevia was shown to have antihyperglycemic effects in an ingestion study with 12 types two diabetic patients, where 1g added stevioside/supplemental meal was compared to 1g added maize starch/supplemental meal. Blood sugar levels were evaluated before and after the test and it revealed stevioside treatment decreased glucagon levels and postprandial blood glucose levels, showing improved glucose metabolism (Gregersen et al., 2004). Hsieh et al. (2003) conducted a two-year clinical study to examine the effect of stevioside consumption on hypertension in 168 patients and found systolic and diastolic blood pressure significantly lower in patients that consumed stevioside compared to the control group. Anti-human rotavirus activities of stevia extract have been discovered from *in vitro* inhibition and binding assays using four serotypes of anti-human rotavirus. Anionic polysaccharides were identified as the inhibiting agent in stevia extract that prevented the virus from attaching to cells usually (Takahashi et al., 2001). A

preliminary clinical study conducted in on chronic kidney disease patients in Bangladesh suggests that consuming stevia helps reduce serum uric acid and microalbumin production for patients suffering from kidney disease; they suggested stevia can be used with a regular treatment program to halt the progress of the disease (Rizwan et al., 2018).

Other Agricultural Uses

Stevia has many botanic properties beyond its sweet taste. Stevia has antifungal, antimicrobial, and nematocidal activity against several important plant pathogens and parasites. For example, Ramirez et al. (2020) discovered that *Fusarium oxysporum* mycelial growth could be inhibited by applying stevia leaf extract at a rate of 833 ppm to prevent Fusarium wilt in tomato production in Mexico. Increased plant height and biomass was overserved in the untreated control and inoculated treatment, indicating the application of stevia leaf extract stimulated plant growth as well as inhibited disease (Ramirez et al., 2020). Shukla et at. (2013) found *F. oxysporum* and *Botrytis cinerae* to be particularly sensitive to both aqueous and ethanol-based stevia leaf extracts at a rate of 1 mg/disc in *in vitro* assays. *B. cinerae* was inhibited 64.2 and 67.5%, and *F. oxysporum* showed 65.3 and 73.6% reduced mycelial growth for aqueous and ethanolic stevia leaf extracts, respectively.

Ntalli et al. (2020) conducted *in vitro* and *in vivo* studies using dried, ground stevia stems and leaves as a biocontrol for Fusarium wilt disease in tomatoes. *In vivo*, they incorporated either the ground stems or leaves into nematode-inoculated soil media prior to transplanting tomatoes. Ground stevia leaves proved more effective than ground stems at reducing the number of nematode females infesting tomato roots (Ntalli et al., 2020). *In vitro*, water-based extracts of both plant parts were used to test the parasites' dosage and time response to paralysis. Both

extracts demonstrated efficacy in inducing paralysis of the second-stage juveniles of *Meloidogyne incognita* and *Meloidogyne javanica* (Ntalli et al., 2020). Analysis of the essential oil extracts revealed many different chemical groups, including amino and fatty acids, flavonoids, phenols, and terpenes. Two major compounds, terpinene-4-ol and B-caryophyllene show promise in causing paralysis of *M. incognita.* These experiments demonstrated that stevia essential oil contains compounds that exhibit nematocidal activity against *Meloidogyne incognita* and *Meloidogyne javanica,* which may be useful in developing new biological control products for tomato production (Ntalli et al., 2020).

Future Research

The SG extraction and purification methods should be evaluated for cost, yield, and sustainability. Optimizing stevia production is of importance for the crop to become economical. There has been limited research to standardize plant traits and plant production guidelines for researchers and growers to evaluate the yield more effectively. There are few reports in the literature on conventional and molecular breeding methods to improve agronomic traits, as well as SG content. A standardized method for seed germination and transplant production is needed for seed testers so growers can obtain high-quality seeds to establish the crop. Seed coating may be helpful to improve germination rates and plant spacing. Disease resistance needs to be identified and introgressed into elite germplasm to develop new cultivars for specific production regions. Pesticide registration and evaluation will be an ongoing area of research since there are few products available for disease and weed control in stevia. Pesticide management programs will significantly benefit growers interested in improving yield and reducing inputs. Finally, research is needed in cytogenetics, nutrient optimization, enhanced glycoside production, use of

growth promoters in micropropagation, stress tolerance, alternative glycoside production using microorganisms, optimizing the breeding process, and seed technology.

References

Ahmed, M.B., Salahin, M., Karim, R., Razvy, M.A., Hannan, M.M., Sultana, R., Hossain, M., & Islam, R. (2007). An efficient method for *in vitro* clonal propagation of newly introduced sweetener from plant *Stevia rebaudiana* Bertoni in Bangladesh. *Am-Eurasian J Sci Res*, 2(2): 121–125.

Akita, M., Shigeoka, T., Koizumi, Y., & Kawamura, M. (1994). Mass propagation of shoots of *Stevia rebaudiana* using large scale bioreactor. *Plant Cell Rep. Jpn.,* 13: 180-183.

Al Amrani, H.A., Al-Taweel, S.K., & Abdul-Qader, Z.M. (2018). Steviosides composition in *Stevia rebaudiana* Bertoni: Effect of drying methods. *Plant Archives*, 18 (2): 2025-2030.

Ali, A., Gull, I., Naz, S., & Afghan, S. (2010). Biochemical investigation during different stages of *in vitro* propagation of *Stevia rebaudiana*. *Pak J Bot,* 42(4): 2827–2837.

Al-Taweel, S.K., Abdel-Aziz, R.M., Rabea, K.M., & Khaled, K.A.M. (2019). Studying cDNA Scot in response to salinity stress in *Stevia rebaudiana* Bertoni. *SABRAO J of Breeding and Gen.,* 51(3): 281-294.

Alvarado-Orea, I.V., Paniagua-Vega, D., Capataz-Tafur, J., Torres-López, A., Vera-Reyes, I., García-López, E., & Huerta-Heredia, A.A. (2020). Photoperiod and elicitors increase steviol glycosides, phenolics, and flavonoid contents in root cultures of *Stevia rebaudiana*. *In Vitro Cell Dev Biol- Plant*, (56): 298–306.

Anami, E., Poletine, J.P., Gonçalves-Vidigal, M.C., Filho, P.S., Lacanallo, G.F., Kvitschal, M.V., & Gonela, A. (2010). Characterization and genetic divergence in *Stevia rebaudiana* (Bert.) Bertoni clones based in agronomical and morphological characteristics. *Journal of Food Agriculture & Environment,* 8: 463-469.

Anbazhagan, M., Kalpana, M., Rajendran, R., Natarajan, V., & Dhanavel, D. (2010). *In vitro* production of *Stevia rebaudiana* Bertoni. *Emir J Food Agric,* 22(3): 216–222.

Andolfi, L., Macchia, M., & Ceccarini, L. (2006). Agronomic-productive characteristics of two genotypes of *Stevia rebaudiana* in Central Italy. *Ital. J. Agron. / Riv. Agron,* 2: 257-262.

Angkapradipta, P., Warsito, T., & Faturachim, P. (1986). The N, P, and K requirements of *Stevia rebaudiana* on latosolic soil. Menara Perkebunan, 54: 1-6.

Ashwell, M. (2015). Stevia, Nature's Zero-Calorie Sustainable Sweetener: A New Player in the Fight Against Obesity. *Nutrition today, 50*(3): 129–134.

Audial, S., & Bonnotte, B. (2015). Inflammation. *Rev Prat*, 65(3): 403–408.

Basuki, S. (1990). Effects of black plastic mulch and plant density on the growth of weeds and stevia. *Biotrop special publication*, 38: 107–113.

Behera, M.S., Verma, O.P., Mahapatra, P.K., Singandhupe, R.B., Kumar, A., Kannan, K., & Brahmanand, P.S. (2013). Effect of fertigation on Stevia (*Stevia rebaudiana*) under drip irrigation. *Indian J of Agronomy*, 58(1): 72-79.

Benhmimou, A., Ibriz, M., Faïz, C.A., Gaboun, F., Douaik, A., Amchra, V.Z., Khiraoui, A., & Lage, M. (2017). Effects of Planting Density and Harvesting Time on Productivity of Natural Sweetener Plant (*Stevia rebaudiana* Bertoni.) in Larache Region, Morocco. *International Journal of Plant Research,* 7 (4): 83-89.

Benhmimou, A., Ibriz, M., Douaik, A., Lage, M., Al Faïz, C., & Zouahri, A. (2018). Effect of organo-mineral fertilizers on growth, biomass, and mineral nutrition of natural non-calorie sweetener plant (*Stevia rebaudiana* Bertoni) in northwestern Morocco. *Intl. J of Sci & Engin. Res*, 9(7): 1785- 1799.

Bertoni, M.S. (1905). Le kaà-heê-ê: Sa nature et ses propriétiés. *An. Cientificos Parag. Ser. I.,* 5: 1-14.

Bovanová, L., Brandšteterová, E., & Baxa, S. (1998). HPLC determination of stevioside in plant material and food samples. *Z Lebensm Unters Forsch.,* 207: 352–355.

Brandle, J.E., & Rosa, N. (1992). Heritability for yield, leaf: stem ratio and stevioside content estimated from landrace cultivar of *Stevia rebaudiana*. *Can. J. Plant Sci.,* 72: 1263-1266.

Brandle, J.E., Starratt, A.N., & Gijzen, M. (1998a).*Stevia rebaudiana*: its agricultural, biological, and chemical properties. *Can. J. Plant Sci.,* 78: 527-536.

Brandle, J., Sys, E.A., & Marsolais, A.A. (1998b). Stevia plant named 'RSIT95-166-13' United States Patent PP10563.

Brandle, J. (1999). Genetic control of Reb-A and C concentration in leaves of the sweet herb, *Stevia rebaudiana*. *Can. J. Plant Sci*., 79: 85–92.

Brandle, J. (2001). *Stevia rebaudiana* with altered steviol glycoside composition. U.S. Patent no. US 6255557 B1.

Brandle, J. E., Richman, A., Swanson, A. K., & Chapman, B. P. (2002). Leaf ESTs from *Stevia rebaudiana*: a resource for gene discovery in diterpene synthesis. *Plant Mol. Biol.*, 50: 613-622.

Brandle, J. (2004). FAQ - Stevia, Nature's Natural Low-Calorie Sweetener. Agriculture and Agri-Food Canada. Accessed Nov. 01, 2017.

Brandle, J. E., & Telmer, P. G. (2007). Steviol glycoside biosynthesis. *Phytochemistry,* 68: 1855-1863.

Bridel, M., & Lavielle, R. (1931). Sur le principesucre des feuilles de kaa-he-e (*Stevia rebaudiana* B). *Academie des Sciences Paris Comptes Rendus* 192: 1123–1125.

Btru, N.T., Gebere, A., Melkamu, H., & Belistie, L. (2017). Influence of plant population density on growth and yield of Stevia (*Stevia rebaudiana* Bertoni L.). *IJABBR*, 5(1): 19-26.

Carneiro, J.W.P. (1990). Maringá: Fundação Universidade Estadual de Maringá. P 65.

Carneiro, J.W.P., & Guedes, T.A. (1992a). Influence of the contact of stevia seeds with the substrate, evaluated by means of the Wiebull function. *Rev. Brasil. Sementes*, 14: 65-68.

Carneiro, J.W.P., & Martins, E.N. (1992b). The performance of Stevia crops transplanted in different densities and double spacing. *Pesq. Agropec. Bras. Brasilia,* 27 (9): 1273-1282.

Carneiro, J.W.P., Muniz, A.S., & Guedes, T.A. (1997). Greenhouse bedding plant production of *Stevia rebaudiana* (Bert) Bertoni. *Can. J. Plant Sci.*, 77: 473-474.

Carneiro, J.W.P. (2007). *Stevia rebaudiana* (Bert.) Bertoni: Stages of plant development. *Can. J. Plant Sci.,* 87: 861-865.

Ceunen, S., & Geuns, J. M. C. (2013). Spatio-temporal variation of the diterpene steviol in *Stevia rebaudiana* grown under different photoperiods. *Phytochemistry,* 89: 32-38.

Chakraborty, R., & Dey, T. (2016). Drying Protocols for Traditional Medicinal Herbs: A Critical Review. *International Journal of Engineering Technology, Management and Applied Sciences,* 4(4): 312-319.

Chalapathi, M.V. (1996). Methods of planting, fertilization, rationing and standardization of vegetative propagation techniques in stevia. Master's thesis, UAS, Bangalore, India.

Chalapathi, M.V., Timmegowda, S., Rama Krishna Prama, V., & Prasas, T.G. (1997a). Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni): A Future crop for India. *Crop Res*.,14: 347-350.

Chalapathi, M.V., Shivaraj, B., Parama, V.R.R., Rama Krishna Prama, V.R. (1997b). Nutrient uptake and yield of Stevia (*Stevia rebaudiana* Bertoni) as influenced by methods of planting and fertilizer levels. *Crop Res*., 14: 205-208.

Chan, P., Tomlinson, B., Chen, Y.J., Liu, J.C., Hsieh, M.H., & Cheng, J.T. (2000). A double-blind placebo—controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *British Journal of Clinical Pharmacology*, 50 (3): 215–220.

Chatsudthipong, V., & Muanprasat, C. (2009). Stevioside and related compounds: therapeutic benefits beyond sweetness. *Pharmacol Ther*., 121(1): 41–54.

Chaturvedula, V. S. P., Clos, J.F., Rhea, J., Milanowski, D., Mocek, U., DuBois, G.E., & Prakash, I. (2011). Minor diterpenoid glycosides from the leaves of *Stevia rebaudiana*. *Phytochemistry Letters*, 4(3): 209-212.

Chen, S. & Shu, S. (1995). Study on Storage Technique of *Stevia rebaudiana* Seed. *Acta Agron. Sin.*, 21: 102–105.

Chester, K., Tamboli, E. T., Parveen, R., & Ahmad, S. (2013). Genetic and metabolic diversity in *Stevia rebaudiana* using RAPD and HPTLC analysis. *Pharmaceutical biology*, *51*(6): 771–777.

Cimpeanu, M., Toma, I., Zbughin, G., Cimpeanu, C., & Capraru, G. (2005). Cytogenetics and morpho-anatomy in *Stevia rebaudiana* Bertoni. *Proc. 3rd CMAPSEEC*, 108-112.

Clemente, C., Angelini, L.G., Ascrizzi, R., & Tavarini, S. (2021). *Stevia rebaudiana* (Bertoni) as a multifunctional and sustainable crop for the Mediterranean climate. *Agriculture*, 11: 123.

Collins, T., Dee, M., Korotkin, H., Hensley, D., & Ownley, B. (2016). Fungal Diseases of *Stevia rebaudiana* Grown in Eastern Tennessee. APS Ann. Meeting, Diseases of Plants: Disease Detection and Diagnosis, 450-P.

Colombus, M. (1997). The cultivation of stevia, "nature's sweetener". Ontario Ministry of Agriculture, Food and Rural Affairs, Toronto, Ontario, p. 4.

Cosson, P. Hastory, C., Errazzu, L.E., Budeguer, C.J., Boutié, P., Rolin, D., & Schurdi-Levraud, V. (2019). Genetic diversity and population structure of sweet leaf herb, *Stevia rebaudiana* B., cultivated and landraces germplasm assessed by EST-SSRs genotyping and steviol glycoside phenotyping. *BMC Plant Biology*, 19: 436.

Curry, L.L., & Roberts, A. (2008). Subchronic toxicity of Reb-A. *Food Chem Toxicol,* 46(7): 11–20.

Dacome, A.S., daSilva, C.C., daCosta, C.E.M., Fontana, J.D., Adelmann, J., & daCosta, S.C. (2005). Sweet diterpenic glycosides balance of a new cultivar of *Stevia rebaudiana* (Bert) Bertoni: isolation and quantitative distribution by chromatographic, spectroscopic and electrophoretic methods. *Process. Biochem*., 44: 3587-3594.

Das, A., Gantait, S., & Mandal, N. (2011). Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. *Int J Agric Res*, 6: 40–48.

Debnath, M. (2008). Clonal propagation and antimicrobial activity of endemic medicinal plant *Stevia rebaudiana*. *J of Med. Plant. Res*., 2(2): 45-52.

Debnath, M., Ashwath, N., Hill, C.B., Callahan, D.L., Dias, D.A., Jayasinghe, N.S., Midmore, D.J., & Roessner, U. (2018). Comparative metabolic and ionomic profiling of two cultivars of *Stevia rebaudiana* Bert. (Bertoni) grown under salinity stress. *Plant physiology and biochemistry: PPB*, *129*: 56–70.

Debnath, M., Ashwath, N., & Midmore, D.J. (2019). Physiological and morphological responses to abiotic stresses in two cultivars of *Stevia rebaudiana* (Bert.) Bertoni. *South African J of Bot.,* 123: 124-132.

Díaz-Gutiérrez, C., Trillos, A-T., Villa, V., Silva, Z., Acevedo, L., Arroyave, C., Poschenrieder, C., & Peláez, C. (2021). Altitude and fertilization type: concentration of nutrients and production of biomass in *Stevia rebaudiana* Bertoni. *J Plant Nutr*., 44 (3): doi.org/10.1080/01904167.2020.1822402

Dieterich, K. (1909). Composition of *Eupatorium Reb-audianum*. *Chemist & Druggist*, June 5.

Donalisio, M.G., Duarte, F.R., & Souza, C.J. (1982). Estevia (*Stevia rebaudiana*). *Agronômico, Campinas* (brazil), 34: 65-68.

Ermakov, E.I., & Kochetov, A.A. (1994). Growth and productivity of stevia under regulated conditions depending on the photoperiod and light intensity. *Russ Agric Sci.,* 11: 11-14.

Fazeli Kakhki, S. F., Goldani, M., & Arian, A. G. (2019). Evaluation response of photosynthesis of stevia plant (*Stevia rebaudiana* Var. Bertoni) to potassium humate and photoperiod. *Indian Journal of Agricultural Research*, *53*(3): 348–352.

Feekes, W. (1941). De tarwe en haar milieu. *Versl. Techn. Tarwe. Comm*., 12: 523-888.

Ferrazzano, G.F., Cantile, T., Alcidi, B., Coda, M., Ingenito, A., Zarrelli, A., Di Fabio, G., & Pollio, A. (2015). Is *Stevia rebaudiana* Bertoni a noncariogenic sweetener? *A review. Molecules*, 21(1): E38.

Ferreira, C.M., & Handro, W. (1988). Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. *Plant Cell Rep,* 7: 123-126.

Filho, L.O.F., Malavilta, E., De Sena, J. O. A., & Carneiro, J. W. P. (1997a). Uptake and accumulation of nutrients in Stevia (*Stevia rebaudiana*). II. Micronutrients. *Scientia Agricola,* 54: 23–30.

Filho, L.O.F., Malavolta, E., and Filho, O. F. D. L. (1997b). Symptoms of nutritional disorders in stevia (*Stevia rebaudiana*). *Scientia Agricola*, 54: 53–61.

Fisher, L.R., & Vann, M.C. (2019). Producing healthy transplants in a float system. Flue-cured tobacco guide, *North Carolina State Univ. Ext. Bulletin,* 187: 39-55.

Food Insights. (2021). Everything you need to know about stevia sweeteners. Accessed July 14, 2021.

Frederico, A.P., Ruas, P.M., Marin-Morales, M.A., Fuas, C.F. & Nakajima, J.N. (1996). Chromosome studies in some Stevia Cav. (Compositae) species from Southern Brazil. *Braz. J. Genet*, 19: 605-609.

Gamboa, F., & Chaves, M. (2012). Antimicrobial potential of extracts from *Stevia rebaudiana* leaves against bacteria of importance in dental caries. *Acta Odontol. Latinoam*, 25: 171-175.

Gardana, C., Scaglianti, M., & Simonetti, P. (2010). Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. *Journal of chromatography. A*., *1217*(9): 1463–1470.

Gaurav, S. S., Y. P. Singh, & Sirohi, S. P. S. (2008). Genetic variability for yield and quality traits in *Stevia rebaudiana* (Bertoni). *Progressive Res*., 3: 95-96.

Geuns, J.M.C. (2003). Molecules of interest: Stevioside. *Phytochemistry*, 64: 913–921.

Goettemoeller, J., & Ching, A. (1999). Janick, J. ed. Perspectives on new crops and new uses: Seed germination in *Stevia rebaudiana*. ASHS Press, Alexandria, VA, 510-511.

Grashoff, J.L. (1972). A systematic Study of the North and Central American Species of *Stevia*. Ph.D. dissertation, University of Texas.

Gregersen, S., Jeppesen, P.B., Holst, J.J., & Hermansen, K. (2004). Antihyperglycemic effects of stevioside in type 2 diabetic subjects. *Metabolism,* 53 (1): 73-76.

Grenby, T.H. (1991). Prospects for sugar substitutes. *Chem. Br.,* 27: 342-345.

Guang Xi, R., & Yan, S. (2018). Correlation between glycoside in leaves and agronomic traits of *Stevia rebaudiana* Bertoni. *Bangladesh Bot Soc.,* 47 (2): 337-342.

Gutiérrez, D.G., Muñoz-Schick, M., Grossi, M.A., Rodrí- Guezcravero, J.F., Morales, V., & Moreira-Muñoz, A. (2016). The genus Stevia *(Eupatorieae, Asteraceae*) in Chile: a taxonomical and morphological analysis. *Phytotaxa.*, 282(1): 1–18.

Handro, W., Ferreira, C.M., & Floh, E.I.S. (1993). Chromosomal variability and growth rate in cell suspension cultures of *Stevia rebaudiana*. *Plant Sci*., 93: 1-2.

Hastory, C., Cosson, P., Sclaunich, E., Jannot, P., Boutié, P., Rolin, D., & Schurdi-Levraud, V. (2015). in Stevia*: Growth in Knowledge and Taste*, ed. S. Ceunen and J. M. C. Geuns, Proceedings of the 8th Stevia Symposium of European Stevia Association, p. 225.

Hellfritsch, C., Brockhoff, A., Stahler, F., Meyerhof, W., & Hofmann, T. (2012). Human psychometric and taste receptor responses to steviol glycosides. *Agric. Food Chem.,* 60: 6782-6793.

Hilal, A.A., & Baiuomy, M.A. (2000). First report of fungal diseases of stevia (*Stevia rebaudiana* Bertoni) in Egypt. *Egyptian J. Agri. Res.,* 78(4): 1435-1448.

Hossain, M.F., Islam, M.T., Islam, M.A., & Akhtar, S. (2017). Cultivation and uses of Stevia (*Stevia rebaudiana* Bertoni): A review. *Afr. J Food Agric. Nutr. Dev*., 17(4): 12745- 12757.

Hossain, M.A., Siddique, A.B., Mizanur Rahman, S.M., & Hossain, M.A. (2010). Chemical composition of the essential oils of *Stevia rebaudiana* Bertoni leaves. *Asian J of Traditional Medicine,* 5(2): 56-61.

Holvoet, P., Rull, A., Garcia-Heredia, A., Lopez-Sanroma, S., Geeraert, B., Joven, J., & Camps, J. (2015). Stevia-derived compounds attenuate the toxic effects of ectopic lipid

accumulation in the liver of obese mice: a transcriptomic and metabolomic study. *Food Chem Toxicol*., 77: 22–33.

Huang, Y.S., Guo, A.G., Qian, Y., Chen, L.Y. & Gu, H.F. (1995). Studies on the variation of steviosides content and selection of type R-A in *Stevia rebaudiana*. *J. Plant Res. Environ.*, 4: 2832. [In Chinese, English summary.]

Huber, B., & Wehner, T. (2021). Performance of 16 *Stevia rebaudiana* seed cultigens for glycosides and yield in North Carolina. *Scientia Horticulturae, 277*: 109803.

Hsieh, M., Hsieh, M.H., Chan, P., Sue, Y.M., Liu, J.C., Liang, T.H., Huang, T.Y., Tomlinson, B., Chow, M.S., Kao, P.F., & Chen, Y.J. (2003). Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: a two-year, randomized, placebo-controlled study. *Clinical Therapeutics*, 25(11): 2797-808.

International Plant Genetic Resources Institute (IPGRI). (1996). IPGRI, Rome, Italy. Descriptors for Tomato (*Lycopersicon spp*.).

Ishiba, C., Tani, T., & Yokoyama, T. (1982). Leaf spot disease of stevia caused by *Septoria steviae. Ann. Phytopathol. Soc. Jpn.,* 48: 34-43.

Jaitak, V., Bikram Singh, B., & Kaul, V.K. (2009). An efficient microwave-assisted extraction process of stevioside and Reb-A from *Stevia rebaudiana* (Bertoni). *Phytochem Anal.,* 20(3): 240–245.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2008). Compendium of Food Additive Specifications: Joint FAO/WHO Expert Committee on Food Additives, 69th Meeting. *FAO Food Nutr Pap.,* 52(2): 783–1589.

Katayama, O., Sumida, T., Hayashi, H., & Mitsuhashi, H. (1976). The practical application of Stevia and research and development data (English translation). I.S.U. Company, Japan, p747.

Kaur, H. (2012). Micropropagation of *Stevia rebaudiana* and Tylophora indica and extraction of secondary metabolites. Department of Biotech & Environ. Sci. Thapar University, Patiala, Punjab, India.

Kaur, R., Sharma, N., & Raina, R. (2015). Identification and Functional Annotation of expressed sequence tags based SSR markers of *Stevia rebaudiana*. *Turk J Agric For.,* 39: 439-450.

Kawatani, T., Kaneki, Y., & Tanabe, T. (1977). The cultivation of kaa he-e (*Stevia rebaudiana*). II Seed germination with special reference to the optimum temperature and light sensitivity. *Jpn. J. Trop. Agric.*, 20: 137-142.

Khan, A., Jayanthi, M., Gantasala, N.P., Bhooshan, N., & Roa, U. (2016). A rapid and efficient protocol for *in vitro* multiplication of genetically uniform *Stevia rebaudiana* (Bertoni). *Indian J. Exp. Biol*., 54: 477-481.

Kienle, U. (1990). Method of making a natural sweetener based on *Stevia rebaudiana* and use thereof. United States patent US 5,112,610.

Kinghorn, A.D., & Soejarto, D.D. (1985). Current status of stevioside as a sweetening agent for human use. In: Economic and medical plant research. Academic Press, London, UK. H. Wagner, H. Hikino, and N.R. Farnsworth, eds.

Kinghorn, A.D. (2002). Stevia: the genus Stevia. London, UK: Taylor & Francis.

Koehler, A.M., & Shew, H. (2014). First report of stem and root rot of Stevia caused by *Sclerotium rolfsii* in North Carolina. *Plant Dis.,* 98 (7): 1005.

Koehler, A.M., Brown, J.A., Huber, B., Wehner, T.C., & Shew, H.D. (2016). First report of Tomato spotted wilt virus in *Stevia rebaudiana* in North Carolina. *Plant Dis.,* 100 (6): 1251-1251.

Koehler, A.M., & Shew, H. (2017a). Enhanced overwintering survival of stevia by Qol fungicides used to manage *Sclerotium rolfsii*. *Plant Dis*.**,** 101: 1417-1421.

Koehler, A.M., Lookabaugh, E.C., Shew, B.B., & Shew, H.D. (2017b). First report of Pythium root rot of stevia caused by *Pythium myriotylum*, *Pythium irregulare*, *Pythium aphanidermatum* in North Carolina. *Plant Dis.,* 101:1331.

Koehler, A.M. (2018a). Etiology and Management of Stevia Diseases. Doctoral Dissertation North Carolina State University, USA.

Koehler, A.M., & Shew, H. (2018b). Field efficacy and baseline sensitivity of *Septoria steviae* to fungicides used for managing Septoria leaf spot of stevia. *Crop Prot.,* 109: 95-101.

Koehler, A.M., & Shew, H.D. (2018c). First report of charcoal rot of stevia caused by *Macrophomina phaseolina* in North Carolina. *Plant Dis.,* 102: 241.

Koehler, A.M., & Shew, H. (2019). Effects of fungicide applications on root-infecting microorganisms and overwintering survival of perennial stevia. *Crop Prot.,* 120: 13-20.

Kornienko, A.V., & Parfenov, A.M. (1996). Some results of work at the All-Russian Sugarbeet and Sugar Institute. *Sakharnaya Svekla*. 5: 6-7.

Kovylyaeva, G.I., Bakaleinik, G.A., Strokina, I.Y., Gubskaya, V.I., Sharipova, R.R., Alfonsov, V.A., Kataev, V.E., & Tolstikov, A.G. (2007). Glycosides from *Stevia rebaudiana. Chem Nat Compd*., 43(1): 81–85.

Kozik, E.U., Yücesan, B., Saravitz, C., Wehner, T.C. (2020). Cold tolerance of diverse stevia cultigens under controlled environment conditions. *Agrosystems, Geosciences & Envi*r. 3(1): https://doi.org/10.1002/agg2.20120

Kumar, H., Kaul, K., Bajpai-Gupta, S., Kaul, V. K., & Kumar, S. (2012). A comprehensive analysis of fifteen genes of steviol glycosides biosynthesis pathway in *Stevia rebaudiana* (Bertoni). *Gene*, 492: 276-284.

Lata, H., Chandra, S., Wang, Y.H., El Sohly, M.A., & Khan, I.A. (2014). *In vitro* germplasm conservation of elite *Stevia rebaudiana* Bertoni. *Acta Hortic*., 1039: 303-308. DOI: 10.17660/ActaHortic.2014.1039.38

Latha, S., Chaudhary, S., & Ray, S.R. (2017). Hydroalcoholic extract of *Stevia rebaudiana* Bert. leaves and stevioside ameliorates lipopolysaccharide induced acute liver injury in rats. *Biomed Pharmacother*, 95: 1040–1050.

Lavini, A., Riccardi, M., Pulvento, C., De Luca, S., Scamosci, M., & d'Andria, R. (2008). Yield, quality, and water consumption of *Stevia rebaudiana* Bertoni grown under different irrigation regimes in southern Italy. *Ital. J. Agron. / Riv. Agron*., 2: 135-143.

Le Bihan, Z., Cosson, P., Rolin, D., & Schurdi-Levraud, V. (2020). Phenological growth stages of stevia (*Stevia rebaudiana* Bertoni) according to the BBCH scale. *Annals of Applied Biology*, 177. 10.1111/aab.12626.

Lee, J.I., Kang, K.H., & Ham, Y.S. (1978). The high yielding Stevia variety Suweon 2. *S. Korea, Office of Rural Development. Annu. Res. Rep*., 21:21.

Lee, J.I., Kang, K.H., Park, H.W., Ham, Y.S., & Park, C.H. (1980). Studies on the new sweetening source plant, *Stevia rebaudiana* in Korea. II. Effects of fertilizer rate and plant density on dry leaf yields and various agronomic characteristics of *Stevia rebaudiana*. *Research reports of the office of Rural development (crop Suwon)*, 22: 138-144.

Lee, J.I., Kang, K.H., & Park, H.W. (1982). New high Reb-A stevia variety 'Suweon 11'. *Res. Rept. ORD 24*, 186-188.

Lemus-Mondaca, R., Ah-Hen, K., Vega-Gálvez, A., Honores, C., & Moraga, N.O. (2016). *Stevia rebaudiana* Leaves: Effect of Drying Process Temperature on Bioactive Components, Antioxidant Capacity and Natural Sweeteners. *Plant Foods Hum Nutr.,* 71**:** 49–56.

Lester, T. (1999). *Stevia rebaudiana* (sweet honey leaf). *Aust. New Crops News Lett. 11. Nat. Prod. Radiance*, 2:120.

Lewis, W.H., Rawat, A.S., Pharswan, A.S., Nautiyal, M.C., & Kostermans, A.J.G.H. (1992). Notes on economic plants. *Economic Botany*, 46: 336-340.

Liu, J., Li, J.W., & Tang, J. (2010). Ultrasonically assisted extraction of total carbohydrates from *Stevia rebaudiana* Bertoni and identification of extracts. *Food Bioprod. Process*, 88 (2–3): 215–221.

Loving, N.M., & Reeleder, R.D. (1996). First report of *Septoria steviae* on stevia (*Stevia rebaudiana*) in North America. *Plant Dis.,* 80: 959.

Machado, R.M., & Serralheiro, R.P. (2017). Soil salinity: effect on vegetable crop growth. Management practices to prevent and mitigate soil salinity. *Horticulturae*, 3:1-13.

Madhav, H., Bhasker, S., & Chinnamma, M. (2013). Functional and structural variation of uridine diphosphate glycosyltransferase (UGT) gene of *Stevia rebaudiana*- UGTSr involved in the synthesis of Reb-A. *Plant Physiology and Biochem.,* 63: 245-253.

Mahajan, M., Sharma, S., Kumar, P., & Pal, P.K. (2020). Foliar application of KNO_3 modulates the biomass yield, nutrient uptake and accumulation of secondary metabolites of *Stevia rebaudiana* under saline conditions. *Industrial Crops and Products*, 145: 112102.

Maiti, C.K., Sen, S., Acharya, R., & Acharya, K. (2007). First report of *Alternaria alternata* causing leaf spot on *Stevia rebaudiana*. *Plant Path*., 56 (4): 723.

Mahmoud, A.W.M., El-Attar, A.B.S., Esmail, S.E.A., & Sany, H. (2020). Boosting biochemical composition of stevia plant using biochar loaded with beneficial microorganism strains. *Bulgarian J of Agri Sci*., 26(4): 748-760.

Marín, S.B.A., Jimenez, L.A.L., García, F.E.M., & Barrera, C.F. (2016). Development of a protocol for the *in vitro* establishment of *Stevia rebaudiana* Bertoni Morita II variety**.** *Revista de Investigación Agraria y Ambiental*, 7(2): 99-106.

Markovic, I.S., Darmati, Z.A., & Abramovic, B.F. (2008). Chemical composition of leaf extracts of *Stevia rebaudiana* Bertoni frown experimentally in Vojvodina. *J. Serb. Chem. Soc*., 73(3): 283-297.

Marsolais, A.A., Brandle, J., & Sys, E.A. (1998). Stevia plant named 'RSIT 94-751'. United States Patent PP10564.

Mengesha, B., GeJa, W., Damtew, Z. 2014. Ethiopian Institute of Agricultural Research. Stevia production Guideline.

Metivier, J., & Viana, A.M. (1979). The effect of long and short-day length upon the growth of whole plants and the level of soluble proteins, sugars and stevioside in leaves of *Stevia rebaudiana* Bert. *J. Exp. Bot*., 30: 1211-1222.

Mitra, A., & Pal, A. (2007). *In vitro* regeneration of *Stevia rebaudiana* Bert. from the nodal explants*. J Plant Biochem Biotechnol*., 16: 59–62.

Momtazi-Borojeni, A.A., Esmaeili, S.A., Abdollahi, E., & Sahebkar, A. (2017). A review on the pharmacology and toxicology of steviol glycosides extracted from *Stevia rebaudiana*. *Curr Pharm Des*., 23(11): 1616–1622.

Monteiro, R. (1980). Taxonomiae biologiada reproducaoda *Stevia rebaudiana* Bert. Ph.D. thesis, Univ. Estadual de Campinas, Brazil.

Moraes, R. M., Donega, M. A., Cantrell, C. L., Mello, S. C., & McChesney, J. D. (2013). Effect of harvest timing on leaf production and yield of diterpene glycosides in *Stevia rebaudiana* Bert: A specialty perennial crop for Mississippi. *Industrial Crops and Products*, 51: 385-389.

Morita, K.K. (1984a). Japanese Patent 59034848.

Morita, K.K. (1984b). Japanese Patent 59034826.

Morita, K.K. (1985). Japanese Patent 60160823.

Morita, K.K. (1986). Japanese Patent 61202667.

Morita, T. (1987). Dried leaves. Japanese Patent 6296025.

Morita, T., & Yucheng, B. (1998). Variety of *Stevia rebaudiana* Bertoni. United States Patent 6031157.

Murayama, S., Kayano, R., Miyazato, K., & Nose, A. (1980). Studies on the cultivation of *Stevia rebaudiana*. II. Effects of fertilizer rates, planting density, and seedling clones on growth and yield. *Science Bulletin of the College of Agriculture, University of the Ryukyus, Okinawa*, 27: 1-8.

Nakamura, S., & Tamura, Y. (1985). Variation in the main glycosides of stevia. *Jpn. J. Trop. Agric*., 29: 109-115.

Nesom, G.L. (2006). Stevia. Flora of North America. FNA Vol 21: 483. Accessed Nov. 07, 2017.www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=131515#KEY-1-2.

Ntalli, N., Kasiotis, K. M., Baira, E., Stamatis, C.L., & Machera, K. (2020). Nemacidal activity of *Stevia rebaudiana* (Bertoni) Assisted by Phytochemical Analysis. *Toxins*, 12: 319-336.

Oddone, B. (1997). How to grow stevia. *Technical manual.* Guarani Botanicals, Inc., Pawtucket, CT.

Oddone, B. (1999). How to grow stevia. Guarani Botanicals, Inc., Pawtucket, CT. pp. 1-30.

de Oliveira, V.M., Forni-Martins, E.R., Magalhães, P.M., & Alves, M.N. (2004). Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (Eupatorieae, Asteraceae). *Genetics and Molecular Biology*, 27(2): 215-222.

Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). (2021). The cultivation of Stevia, 'Nature's Sweetener". Accessed July 27, 2021. www.omafra.gov.on.ca/english/crops/facts/stevia.htm

Othman, H.S., Osman, M., & Zainuddin, Z. (2018). Genetic variabilities of *Stevia rebaudiana* Bertoni cultivated in Malaysia as revealed by morphological, chemical, and molecular characterization. *Agrivita j Agri Sci*., 40(2): 267-283.

Pal, P.K., Kumar, R., Guleria, V., Mahajan, M., Prasad, R., Pathania, V., Gill, B.S., Singh, D., Chand, G., Singh, B., Singh, R.D., & Ahuja, P.S. (2015). Crop-ecology and nutritional variability influence growth and secondary metabolites of *Stevia rebaudiana* Bertoni. *BMC Plant Biol*., 15*:* 67.

Pande S.S., & Gupta, P. (2013). Plant tissue culture of *Stevia rebaudiana* (Bertoni): a review. *J Pharmacogn Phytother*, 5(1): 26–33.

Pasquel, A., Meireles, M.A.A., Marques, M.O.M., & Petenate, A.J. (2000). Extraction of stevia glycosides with CO2 + water, CO2 + ethanol, and CO2 + water + ethanol. *Braz J Chem Eng.,* 17(3): 271–282.

Periche, A., Castelló, M.L., Heredia, A., & Escriche, I. (2015). Influence of drying method on steviol glycosides and antioxidants in *Stevia rebaudiana* leaves. *Food Chem*., 172: 1–6.

Phillips, K.C. (1987). Stevia: steps in developing a new sweetener. *In: Developments in sweeteners*. Grenby, T.H., ed. London, UK: Elsevier Applied Science, p. 1-43.

Pól, J., Varaďová Ostrá, E., Karásek, P., Roth, M., Benešová, K., Kotlaříková, P., & Čáslavský, J. (2007). Comparison of two different solvents employed for pressurised fluid extraction of stevioside from *Stevia rebaudiana*: methanol versus water. *Anal Bioanal Chem*., 388: 1847–1857**.**

Purecircle USA Inc. (2017). United States Patent 9668451.

Purecircle USA Inc. (2020a). Stevia Cultivar '16265046'. United States Patent 6279871.

Purecircle USA Inc. (2020b). Stevia Cultivar '18136109'. World Intellectual Property Organization WO 2021/084325 A1.

Raina, R., Bhandari, S. K., Chand, R., & Sharma, Y. (2013). Strategies to improve poor seed germination in *Stevia rebaudiana*, a low-calorie sweetener. *J. Medicinal Plants Res*., 7: 1793-1799.

Ramesh, K., Singh, V., & Megeji, N.W. (2006). Cultivation of *Stevia* [*Stevia rebaudiana* (Bert.) Bertoni]: a comprehensive review. *Adv Agro*., 89(5): 137–177.

Ramirez, P.G., Ramirez, D.G., Meija, E.Z., Ocampo, S.A., Diaz, C.N., & Rojas Martinez, R.I. (2020). Extracts of *Stevia rebaudiana* against *Fusarium oxysporum* associated with tomato cultivation. *Sci. Hortic*., 259: 108683.

Rashid, Z., Rashid, M., Inamullah, S., Rasool, S., & Bahar, F.A. (2013). Effect of different levels of farmyard manure and nitrogen on the yield and nitrogen uptake by stevia (*Stevia rebaudiana* Bertoni). *Afri J of Agric Res*., 8(29): 3941-3945.

Reeleder, R. (2001). Septoria leaf spot of *Stevia rebaudiana* in Canada and methods for screening for resistance. *J. Phytopathology,* 147(10): 605-613.

Ribeiro, M.M., Diamantino, T., Domingues, J., Montanari Jr, Í., Alves, M.N., & Gonçalves, J.C. (2021). *Stevia rebaudiana* germplasm characterization using microsatellite markers and steviol glycosides quantification by HPLC. *Mol Biol Reports*, 48: 2573-2582.

Richman, A.S., Gijzen, M., Starratt, A.N., Yang, Z., & Brandle, J.E. (2002). Diterpene synthesis in *Stevia rebaudiana*: Recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway. *Plant J,* 19: 411-421.

Richman, A., Swanson, A., Humphrey, T., Chapman, R., McGarvey, B., Pocs, R., & Brandle, J. (2005). Functional genomics uncovers three glucosyltransferases involved in the synthesis of the major sweet glucosides of *Stevia rebaudiana*. *Plant J.,* 41 (1): 56-67.

Rizwan, F., Rashid, H. U., Yesmine, S., Monjur, F., & Chatterjee, T. K. (2018). Preliminary analysis of the effect of Stevia *(Stevia rebaudiana*) in patients with chronic kidney disease (stage I to stage III). *Contemp. clin. trials comm.*, 12: 17–25.

Robinson, R.L. (1930). Contributions from the Gray Herbarium of Harvard University XC. The Gray Herbarium of Harvard University, Cambridge, MA. p 78-91.

Romashova, M. V., Barsukova, Ye. N., Gorpenchenko, T. Yu., Khrolenko, & Yu. A. (2017). New variety of *Stevia rebaudiana* Bertoni PRIMORSKAYA SLASTENA. *Selection and Seed growing*, 4: 9.

Sairkar, P., Chandravanshi, M.K., Shukla, N.K., Mehrotra, M.N. (2009). Mass propagation of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation technique. *J Med Plants Res*., 3(4): 266–270.

Samadpourrigani, E., Tansi, L.S., & Kafkas, N.E. (2019). The effect of planting density, cutting stage and cutting number on yield and quality of *Stevia rebaudiana* B. *JAFAG*, 36(1): 81-90.

Schneiter, A.A., & Miller, J.F. (1981). Description of sunflower growth stages. *Crop Sci.*, 21: 901-903.

Schilling, E. E., Panero, J. L., & Cox, P. B. (1999). Chloroplast DNA restriction site data support a narrowed interpretation of Eupatorium (Asteraceae). *Plant Syst. Evol*., 219: 209-223.

Shafii, B., Vismeh, R., Beaudry, R., Warner, R., & Jones, A.D. (2012). Large-scale profiling of diterpenoid glycosides from *Stevia rebaudiana* using ultrahigh performance liquid chromatography / tandem mass spectrometry. *Anal. Bioanal. Chem*., 403: 2683-2690.

Shock, C.C. (1982a). Rebaudi's stevia: natural non-caloric sweeteners. *California Agric*. 36: 4-5.

Shock, C.C. (1982b). Experimental cultivation of Rebaudi's Stevia in California. *Agronomy progress report, UC Davis Coop. Ext. No. 122*.

Shoelson, S.E., Lee, J., & Goldfine, A.B. (2006). Inflammation and insulin resistance. *J Clin. Invest.*, 116(7): 1793–1801.

Shu, S. Z. (1995). A study on good variety selection in *Stevia rebaudiana*. *Sci. Agric. Sin.* 28: 37-42.

Shu, S. (1989). *Stevia rebaudiana* variety trials. *Zuowu Pinzhong Ziyuan*, 1: 17-18.

Shukla, S., Mehta, A., & Bajpai, V.K. (2013). Phytochemical Screening and Anthelmintic and Antifungal Activities of Leaf Extracts of *Stevia rebaudiana*. *J Biol. Act Prod from Nat.*, 3(1): 56-63.

Shyu, Y.T. (1994). Effects of harvesting dates on the characteristics, yield, and sweet. *J. Agric. Res. China*, 43: 29-39.

Singh, D.P., Kumari, M., Prakash, H.G., Rao, G.P., & Solomon, S. (2019). Phytochemical and Pharmacological Importance of Stevia: A Calorie-Free Natural Sweetener. *Sugar Tech*, 21: 227–234.

Singh S.D., & Rao G.P. (2005). *Stevia*: the herbal sugar of 21st century. *Sugar Technol*., 7: 17–24.

Singh, P., & Dwivedi, P. (2014). Two-stage culture procedure using thidiazuron for efficient micropropagation of *Stevia rebaudiana*, an anti-diabetic medicinal herb. *Biotech*. 4: 431- 437.

Singh, M., Saharan, V., Rajpurohit, D., Sen, Y., Sharma, A., & Joshi, A. (2017). Criteria for selection of superior *Stevia rebaudiana* plant for propagation establishment. *J Pharmacognosy and Phytochem.,* 6(4): 1362-1365.

Sivaram, L., & Mukundan, U. (2003). *In vitro* culture studies on *Stevia rebaudiana*. *In Vitro Cell Dev Biol Plant*, 39: 520–523.

Slamet, I.H., & Tahardi, S. (1988). The effect of shading and nitrogen fertilization on the flowering of *Stevia rebaudiana* Bertoni. *Menara Perkebunan*, 56: 34-37.

Soejarto, D., Compadre, C.M., & Kinghorn, A.D. (1983). Ethnobotanical Notes on Stevia; *Botanical Museum Leaflets*. Harvard University: Cambridge, MA, USA, p 1–25.

Soejarto, D.D. (2001). Ethnobotany of Stevia and *Stevia rebaudiana*. In: Stevia: The Genus Stevia; Kinghorn, A.D., Ed. CRC Press: London, UK; New York, NY, USA. p. 40–67.

Soufi, S., Rezgui, S., & Bettaeib, T. (2015). Early effects of chilling stress on morphological and physiological statute of pretreated *Stevia rebaudiana* Bert. seedlings. *Agri & BioTech,* 14 (5): 467-472.

Soufi, S., D'Urso, G., Pizza, C., Rezgui, S., Bettaieb, T., & Montoro, P. (2016). Steviol glycosides targeted analysis in leaves of *Stevia rebaudiana* (Bertoni) from plants cultivated under chilling stress conditions. *Food chem*., 190: 572–580.

Soufi, S., Aabaoui, S., Jelleli, S., Rezgui, S., & Bettaieb, T. (2019). High Photochemical Efficiency and Nutrient Homeostasis in Stevia (*Stevia rebaudiana*) Plant Leaf Increases Tolerance to Saline Irrigation water. *Intl. J. Agric. Biol.*, 22: 1059-1064.

SteviaLand Paraguay. Mar. 9, 2011. Successful Farming. Stevia leaves| Paraguay| www.Stevia-Store.com. Accessed Nov. 11, 2017. https://www.agriculture.com/farm-management/technology/stevia-leaves-paraguay_322-ar15272

Stevia-Store. (2015). Stevia seeds. Stevia-Store.com. Updated 2021. Accessed Nov. 11, 2017. http://steviaeirete.com/manual.html

Suanarunsawata, T., Klongpanichapaka, S., Rungseesantivanona, S., & Chaiyabutrb, N. (2004). Glycemic effect of stevioside and *Stevia rebaudiana* in streptozotocin-induced diabetic rats. *East J Med*., 9(2): 51–56.

Suhendi, D. (1989). Mass selection of *Stevia rebaudiana* Bertoni. *Menara Perkebunan*, 56: 93-95.

swseedco.com. Updated 2020. Accessed July 14, 2021. https://swseedco.com/stevia/

Sys, E.A., Marsolais, A.A., & Brandle, J. (1998). Stevia plant named 'RSIT 94-1306' United States Patent PP10562.

Takahashi, K., Matsuda, M., Oashi, K., Yaniguchi, K., Nakagomi, O., Abe, Y., Mori, S., Sato, N., Okuyani, K., & Shigeta, S. (2001). Analysis of anti-rotavirus activity of extract from *Stevia rebaudiana. Antiviral Res*., 49(1): 15-24.

Taiariol, D.R. (2004). Characterization of the *Stevia rebaudiana* Bert. Accessed July 27, 2021. www.monografias.com/trabajos 13/stevia/stevia html.

Tamura, Y., Nakamura, S., Fukui, H., & Tabata, M. (1984). Comparison of Stevia plants grown from seeds, cuttings and stem tip cultures for growth and sweet diterpene glycosides. *Plant Cell Rep*., 3: 180-182.

Tan, S., Shibuta, Y., Tanaka, O. (1988). Isolation of sweetener from *Stevia rebaudiana Jpn. Kokai,* 63 (177): 764.

Tan, S. L., Ghawas, M. M., Najib, M. Y. M., & Zawayi, M. (2008). Preliminary evaluation and selection of stevia under Malaysian conditions. *J. Trop. Agric. Food Sci.* 36: 171-177.

Tanaka, O. (1985). Application of C-nuclear magnetic resonance spectrometry to structural studies on glycosides: saponins of Panax spp. and natural sweet glycosides. *Yakugaku Zasshi,* 105: 323-351.

Tateo, F., Mariotti, M., Bononi, M., Lubian, E., Martello, S., & Cornara, L. (1998). Stevioside content and morphological variability in a population of *Stevia rebaudiana* (Bertoni) Bertoni from Paraguay. *Italian J. Food Sci*., 10: 261-267.

Tavakoli Hasanaklou, N., Sedghi, M., Moradi, F., Ghadim, A.E.K., & Ghodehkahriz, S.J. (2020). Greenhouse evaluation of branching, leaf yield and biochemical compositions of *Stevia rebaudiana* Bertoni to decapitation and foliar application of abscisic acid and fluridone. *Functional Plant Biol.,* 47: 1083-1097.

Tavarini, S., & Angelini, L.G. (2013). *Stevia rebaudiana* Bertoni as a source of bioactive compounds: the effect of harvest time, experimental site and crop age on steviol glycoside content and antioxidant properties. *J Sci Food Agric*., 93: 2121-2129.

Tavarini, S., Passera, & Angelini, L.G. (2018). Crop and Steviol Glycoside Improvement. In: Stevia by Breeding, in *Steviol Glycosides: Cultivation, Processing, Analysis and Applications in Food.* p 1-31.

Thiyagarajan, M., & Venkatachalam, P. (2012). Large scale *in vitro* propagation of *Stevia rebaudiana* Bert. for commercial application: pharmaceutically important and antidiabetic medicinal herb. *Ind Crops Prod,* 37(1): 111–117.

Thiyagarajan, M., & Venkatachalam, P. (2015). Assessment of genetic and biochemical diversity of *Stevia rebaudiana* Bertoni by DNA fingerprinting and HPLC analysis. *Ann of Phytomed*., 4(1): 79-85.

Thomas, S. C. L. (2000). Medicinal Plants-Culture, Utilization and Phytopharmacology. Technomic Publishing Co., Inc., Lancaster, Basel. p. 517.

Tirtoboma, Z. B. (1988). The effect of cutting material and internode number on the growth and yield of *Stevia rebaudiana*. *Menara Perkebunan***,** 56(4), 96-101.

Tolba, S.T.M, Ibrahim, M., Amer, E.A.M., & Ahmed, D.A.M. (2019). First insight into salt tolerance improvement of stevia by plant growth promoting Streptomyces species. *Archives of Microbiology*. 201: 1295-1306.

Totté, N., VandenEnde, W., VanDamme, E.J.M., Compernolle, F., Baboeuf, I., & Geuns, J.M.C. (2003). Cloning and heterologous expression of early genes in gibberellin and steviol biosynthesis via the methylerythritol phosphate pathway in *Stevia rebaudiana*. *Can. J. Bot*., 81: 517-522.

Tzortzakis, N.G. (2010). Potassium and calcium enrichment alleviates salinity-induced stress in hydroponically grown endives. *Hort. Sci., 37*: 155–162.

U.S. Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition. (2019). Import Alert 45-06. Silver Spring, MD: U.S. Food and Drug Administration. Accessed July 14, 2021.

Verma, P.P.S, Kumar, A., Padalia, R.C., Singh, V.R. 2020. Influence of NPK level on growth and yield of *Stevia rebaudiana* Bertoni under the hills of Uttarakhand. *Indian J of Nat Prod and Res*., 11(1): 66-72.

Viana, A.M., & Metivier, J. (1980). Changes in the levels of total soluble proteins and sugars during leaf ontogeny in *Stevia rebaudiana* Bert. *Annal Bota*., 45(4): 469-74.

Valio, I.F.M., & Rocha, R.F. (1977). Effect of photoperiod and growth regulators on growth and flowering of *Stevia rebaudiana* Bertoni. *Jpn. J. Crop Sci*., 46: 243-248.

Vaněk, T., Nepovı&́m, A., & Valı&́ček, P. (2001). Determination of stevioside in plant material and fruit teas. *J Food Comp Anal*., 14(4): 383–388.

Vînătoru, C., Mușat, B., Bratu, C., & Tsoktouridis, G. (2019). New genotypes of sweetleaf (*Stevia rebaudiana* Bertoni) acclimated and bred at Buzau. *Bulletin UASVM Hort*., 76 (2): 271-272.

Watanabe, K., Yahara, T., Soejima, A., & M. Ito. (2001). Mexican species of the genus Stevia (Eupatorieae, Asteraceae): Chromosome numbers and geographical distribution. *Plant Species Biol*., 16: 49-68.

Well, C., Frank, O., & Hofmann, T. (2013). Quantitation of sweet steviol glycosides by means of a HILIC-MS / MS-SIDA approach. *J. Agric. Food Chem*., 61: 11312-11320.

Weng, X.Y., Sun, J.Y., & Zang, R.C. (1996). Study on the growth and physiological characteristics of *Stevia rebaudiana* SM4. *J. Zhejiang Agric. Univ.,* 22(5): 538-540.

Wölwer-Rieck, U. (2012). The leaves of *Stevia rebaudiana* (Bertoni), their constituents and the analyses thereof: a review. *J of agri. and food chem., 60*(4): 886–895.

Yadav, A.K., Singh, S., Dhyani, D., & Ahuja, P.S. (2011). A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)] *Can. J. Plant Sci*., 91: 1-27.

Yadav, A. K., Sanatsujat, S., Bikram, S., & Sanjay, K. (2019a). CSIR-IHBT-ST-02 (IC0624506; INGR17053), a Stevia (*Stevia rebaudiana*) Germplasm with Delayed flowering by 120 days. Prolonged Vegetative Phase with more number of harvests per year. *Indian J Plant Genetic res*. 32 (2): 260-261.

Yadav, A. K., Sanatsujat, S., Kumar, S.R., Bikram, S., Vijaylata, & Singh, A.P. (2019b). Him Stevia (CSIR-IHBT-ST-01) (IC0624505; INGR17052), a Stevia (*Stevia rebaudiana*) Germplasm with Reb-A/Stevioside ratio=1.25; Reb-A content (%) =7.34; Stevioside content (%)=5.87. *Indian J Plant Gen. res.*, 32 (2): 259-260.

Yang, Y.-h, Huang, S.-z, & Tong, H.-y. (2012). Comparisons of setting rate of different cross combinations of *Stevia rebaudiana* and germination, growth and NaCl resistance of their F1 hybrids. *J. Plant Resour. & Environ.,* 21(2): 73-78.

Yao, Y., Ban, M.M., & Brandle, J. (1999). A genetic linkage map for *Stevia rebaudiana*. *Genome*, 42: 657-661.

Zadoks, J.C., Chang, T.T., & Konzak, C.F. (1974). A decimal code for the growth stages of cereals. *Weed Research*, 14: 415-421.

Zaidan, L.B.P., Dietrich, S.M.C., & Felippe, G.M. (1980). Effect of photoperiod on flowering and stevioside content in plants of *Stevia rebaudiana*. *Jpn. J. Crop Sci*., 49: 569-574.

Zou, X., Tan, Q., Goh, B., Lee, L., Tan, K., & Ser, H. (2020). 'Sweeter' than its name: anti-inflammatory activities of *Stevia rebaudiana*. *All Life,* (13)1: 286-309.

CHAPTER 2

Effect of Storage Temperature and Duration on Seed Longevity of *Stevia rebaudiana*

Abstract

Stevia rebaudiana is a small herbaceous shrub native to Paraguay that is found between 22º to 24º south and 53º to 56º west. It is grown around the world for its steviol glycosides, that are sweet and non-caloric and can be used to make drinks and foods (Pande and Gupta, 2013; Hossain et al., 2017). A major limiting factor for expanding stevia production is poor seed germination. Seed viability depends on the genotype, initial seed quality, and environmental conditions during seed production (Goettemoeller and Ching, 1999; Lester, 1999; Shock, 1982; Carneiro et al., 1997). Black seeds are fertile and visually distinguishable from non-fertile, tan seeds (Oddone, 1997; Monteiro, 1980; Goettemoeller and Ching, 1999).

A seed longevity experiment on *Stevia rebaudiana* was initiated to observe genotype response under two different storage conditions (4ºC and 21ºC) for 53 months. This study used seed collected from ten hybrid isolation field cages (20.39m3) and two commercial seed sources (Botanical Interest and Jung Seed Co.). Germination assays were evaluated with three replications of 100 seeds, and a subset of 20 seeds was used for viability assays using tetrazolium chloride (1%) in routine intervals. Genotype response varied greatly, with higher germination observed in seed lots stored at 4ºC. Cold room storage-maintained viability longer. A significant drop in viability was observed at 498 days in storage under room temperature conditions and 862 days in cold room storage. Seeds should be monitored annually for loss of viability and regenerated within two years to prevent loss of viability of 5 to 15% in refrigerated (4°C) storage and 15 to 45% in ambient storage (21°C).

Introduction

Stevia rebaudiana is native to the highlands of northeastern Paraguay between 22 to 24º south and 53º to 56º west, where temperatures range from -16ºC to 43ºC (Strauss, 1995; Pande and Gupta, 2013; Hossain et al., 2017). The Guarani Indians reportedly used it for medicinal teas and for sweetening foods (Shock, 1982). *S. rebaudiana* is an adaptable plant grown worldwide for its sweet leaves that accumulate steviol glycosides 300 times sweeter than sugar (Strauss, 1995). *S. rebaudiana* is an herbaceous shrub with a large, shallow root system able to perennialize reliably in sub-tropical and tropical regions. The branches are brittle and easily lodged in storms (Tavarini et al., 2018). The small, lanceolate-elliptical shaped leaves are oppositely arranged on the stem (Ferrazzano et al., 2015; Gutiérrez et al., 2016). Stevia is a short-day plant with a critical day length of 13 hours (Singh and Rao, 2005; Tavarini et al., 2018; Macchia et al., 2011). Flower time is dictated by the response of genotypes to day length and temperature in a specific environment (Brandle et al., 1998). Environmental conditions play a key role in plant growth and steviol glycoside content as well as flowering time (Ramesh et al., 2006).

Stevia flowers are hermaphroditic, with anthers recessed and surrounding the bi-lobed stigma that is receptive when visible above the five-petaled corolla (Goettemoeller and Ching, 1999; de Oliveira et al., 2004). Five flowers are borne on each corymb within an inflorescence, and flowering is asynchronous (Taiariol, 2004). A large, mature stevia plant produces thousands of flowers over a six to eight-week period. Stevia is an outcrossing species requiring insect pollination to transfer pollen among plants. Seed producers should provide beehives for sufficient pollination and seed set (Oddone, 1999). Natural pollinators in Italy were *Apidae* and *Syrphidae,* and were more attracted to plants with more flowers in early autumn when

environmental conditions were favorable for seed ripening (Martini et al., 2017). Sporophytic self-incompatibility has been reported in stevia (Chalapathi et al., 1997). Crossing of two genetically divergent genotypes is needed to produce fertile seeds. Controlled cross-pollination has been facilitated using bumblebees in screen cages (Strauss, 1995).

Seeds will mature 32 days after pollination; they have a persistent pappus, composed of 20 bristles, that changes position on the seed plate to aid in wind dispersal as seeds mature. Seeds are small (1 x 3 mm) with little endosperm contained in the seed coat (Goettemoeller and Ching, 1999). The average seed weight of the small, slender achenes is 300 mg per 1,000 seeds (Uçar et al., 2016). It is important to collect anthesis data to schedule seed harvest immediately after the bloom period ends to avoid collecting unripe seeds and prevents loss of fertile seeds due to wind dispersal (Colombus, 1997). Seed quality and quantity varies with genotype and environment, and properly timing seed harvest with phenological maturity is crucial (Carneiro and Guedes, 1992). Reduced seed set was observed when plants were subjected to heavy rain during flowering, and shade has been shown to delay the time of flowering (Slamet and Tahardi, 1988). Harvest time, plant density, and a number of flowers per plant impact seed production (Macchia et al., 2011).

Stevia seeds are photoblastic, requiring light to germinate (Kawatani et al., 1977; Brandle et al., 1998; Goettemoeller and Ching, 1999). Large plants have more flowers which attract more pollinators and produce more seeds (Martini et al., 2017). The germination rate of stevia is inconsistent and generally low. Seed viability depends on the genotype and environment (Goettemoeller and Ching, 1999; Lester, 1999; Shock, 1982; Carneiro et al., 1997). Dark-colored seeds are fertile and easy to distinguish from tan-colored seeds that are non-fertile (Oddone, 1997; Monteiro, 1980; Goettemoeller and Ching, 1999). Most seeds germinate within a 4 to 14

day period (Shock, 1982; Carneiro and Guedes, 1992). Germination was highest at temperatures of 20 to 25 ºC, the optimum for stevia (Kawatani et al., 1977; Tanaka, 1985; Takahashi et al., 1996; Uçar et al., 2016).

Asexual propagation is used for commercial production because of seed germination problems (Taware et al., 2010). A single-plant selection is made by cloning the desired genotype and maintaining it *in vitro* using nodal segments in tissue culture to ensure it is disease-free before mass multiplication (Abdullateef and Osman, 2011). Unfortunately, clonal propagation requires high labor input, the space for stock plant nurseries, and the operation of propagation greenhouses. Resources are needed to multiply a single-plant selection into large quantities of plants for field production. Weeds, pests, and diseases can occur in perennial stock plant nurseries but can be managed by integrated practices.

Seeded crops are generally easier to manage than clonal crops because they need less storage space, use less labor, and take less time from seeding to transplanting. However, a low germination rate may require more seeds to be used to reach the desired plant density for optimum yield (Goettemoeller and Ching, 1999). Seed production and handling are variable among seed companies. For instance, Stevia Store supplies raw seeds, with pappus attached (Stevia Store, 2015), and a mix of tan and black seeds, resulting in a low germination rate (personal experience; Rank and Midmore, 2006). Other companies, such as Everstevia Co., provide debearded seeds with a higher germination rate (personal observation; Everstevia.com, 2008). Debearded seeds imbibe more readily than raw seeds because of increased contact with the soil medium, resulting in higher germination rates (Carneiro et al., 1997). A guide for seed production, seed handling, and germination is needed for seed producers and growers for high-quality, viable seeds that will germinate consistently under favorable conditions.

Stevia research has expanded since the FDA approved stevia as a food additive in the US (FDA, 2019). Seed management practices are currently being investigated. An important question is the storage life of stevia seeds before there is a significant loss of viability. Seed moisture content has an enormous impact on seed longevity. If the moisture content is too high, seeds will deteriorate at a faster rate (Ellis and Roberts, 1980a). Seed moisture content requirements have not been established in stevia.

The International Seed Testing Association does not list a germination protocol for *S. rebaudiana* (Macchina et al., 2007; Martini et al., 2017). Several researchers have reported optimum temperature conditions, but photoperiod, germination medium, containers, and methods were variable and not standard. The lack of established protocols has led researchers to seek methods used for different crops that may not be compatible with stevia. We hope the germination protocol in this study might help unify stevia seed research for a more effective comparison of seed lots.

Seed longevity has yet to be determined for *S. rebaudiana*. No viability was lost when seeds were stored in a facility that was cool, dry, and dark for up to three years (Kawatani et al., 1977). They also concluded that the optimum temperature for germination was 20ºC to 25ºC with emphasis on the need for light to germinate. Seed viability declined 50% when stored in paper envelopes at room temperature for one year. In contrast, viability remained stable when seeds were stored at room temperature in a desiccator (to maintain low humidity) for one year (Kawatani et al., 1977).

Seed viability after three years of storage in airtight containers was 63% for seeds stored at 0ºC, 68% for seeds stored at -10°C, and 74.5% for seeds stored at -18ºC (Chen and Shu, 1995). Seeds are susceptible to deterioration during storage. Seed moisture content, temperature,

and initial seed quality influence seed deterioration rate (Walters, 1998; Walters et al., 2005a). Seed longevity is a quantitative trait associated with maintaining seed viability after dry storage, the inheritance of which is not well understood (Nguyen et al., 2012).

Cabanillas and Díaz (1997) conducted an 11-month study on stevia seeds, storing them in plastic bags in a refrigerator at 4ºC, and compared with a subset stored moist at room temperature conditions to evaluate seed longevity in a seed lot with 63% mean germination at harvest. It was concluded that 4ºC refrigerator storage was sufficient to maintain germinability and seed vigor for up to 11 months with final mean germination of 61%, compared with 51% for seeds stored moist at room temperature (Cabanillas and Díaz, 1997). However, this study was not robust, covering only 11 months of storage with no viability evaluation. An Australian guide for the production of stevia by Rank and Midmore (2006) stated that seeds should be stored at 4ºC and used within 6 to 12 months because of high loss of viability; that contradicts reports of Chen and Shu (1995) and Kawatani et al. (1977).

Stevia genetic preservation has yet to be optimized. It is essential to determine the length of time seeds will maintain viability and germinability before regenerating. Researchers need methods for the long-term storage of seeds to maintain genetic resources and to regenerate germplasm that has lost viability. Information is needed to determine regeneration intervals and the frequency of monitoring germplasm that is required to determine viability losses (Ellis and Roberts, 1980b). Effective management requires monitoring of germplasm to avoid reduced germination and to avoid loss of seed resources (including frequent evaluation of seed lots). Routine germination and seed viability tests are used to determine the decline in seed lifespan. Seed longevity equations can be used to estimate the frequency of regeneration from seeds in long-term storage. Recommendations from the International Board of Plant Genetic Resources

(1976) state that seeds in long-term storage should be kept at -20ºC with 5% moisture content and regeneration after a 5% loss in variability is detected. Seed viability is determined prior to storage, and if under 80% initially, the seeds are recommended to be regenerated before storage (International Board of Plant Genetic Resources, 1976).

Most seed-propagated crop species show orthodox storage characteristics where they tolerate being dried to a low moisture content without damage (Roberts, 1973; Dickie et al., 1990). Seed production environment and storage conditions play an important role in the survivability of seed lots. Ecological aspects of seed longevity and germination are correlated: species originating in warm, arid environments produce seeds with a longer lifespan than seeds originating from cold environments (Walters et al., 2005b). Three main factors affect seed deterioration: moisture content, time, and temperature; seed lots with low seed quality have higher losses in viability (Ellis and Roberts, 1980b). There is a relationship between seed longevity, temperature, and moisture that influences the rate of seed aging during storage (Ellis and Roberts, 1980a). Storage time influences seed viability more than the production region and geographical location of seed storage. Ellis (1991) reported that tropical regions are generally poor for storing seeds at ambient temperature, followed by temperate regions. Seed longevity can be quantified and decreases with increased seed moisture content and storage temperature (Ellis, 1991). Orthodox seeds can be stored at low temperatures and low relative humidity for extended periods. Storage conditions influence seed longevity and can be manipulated to improve seed lifespan. Modifications in seed storage can be evaluated to determine proper conditions to maintain seed viability to benefit seed producers (Ellis, 1991). Even so, seed lots vary in their ability to maintain viability under standard storage conditions.

The first objective of this research was to develop standard, reproducible germination and viability protocols to evaluate stevia seed longevity. The hypothesis was that seeds stored at low temperature and low humidity conditions remain viable for longer than seeds stored at room temperature conditions and that seed viability and germinability decrease with the increasing length of time in storage. This study contributes to understanding the effect of storage temperature and duration on stevia seed longevity. Germination and viability were measured for 53 months, significantly longer than in previous studies. Our results address the longevity of stevia seeds stored at ambient or cold conditions. We hope to aid seed processors in understanding how long seeds will last before regeneration is necessary.

Materials and Methods

Growth Chamber Conditions

Germination assays were performed in a growth chamber having the facilities requirement published by Davies et al. (2015). The growth chamber was set to 25ºC +/-1ºC (Takahashi et al., 1996), and was equipped with 5000-lumen, cool white fluorescent T5 lamps. The photoperiod was 16 hours light / 8 hours dark. Sensors were placed in seed storage areas to monitor temperature and relative humidity throughout the experiments.

Germination Experiment

Seeds were sorted based on color, tan seeds were discarded, and black seeds were used for all germination tests (Abdullateef et al., 2015). Seeds were surface-sterilized with 20% Clorox bleach solution (5% sodium hypochlorite) by soaking them for 15 minutes, followed by rinsing with sterile distilled water (Simlat et al., 2016). Seeds were dried on paper towels in a laminar flow hood for 24 hours before storing them in low temperatures and ambient conditions. Three replications of 100 seeds each were used for each germination assay (Goettemoeller and

Ching, 1999; Takahashi et al., 1996; Macchina et al., 2007). Petri dishes, 100 x 15 mm, were lined with one layer of sterile Whatman filter paper #3 and wetted with sterile distilled water before sowing the seeds. Seeds were placed on top of the moistened filter paper and arranged to contact moistened filter paper. Germination was monitored every 48 hours and rated based on radical emergence, and then seeds were removed from the experiment (Davies et al., 2015). The filter paper was hydrated as needed with sterile distilled water. At the end of each assay, the remaining non-germinated seeds were dissected under a microscope to determine if they were empty, moldy, or infested by pests (Davies et al., 2015). Germination assays were terminated after 21 days of incubation (Simlat et al., 2016). The experiment was started after 72 days in storage. Twenty-four germination and viability assays were conducted from fall 2016 to spring 2021 over the 1,591 days of the experiment.

Viability Test

Viability assays were conducted on a subset of 20 black seeds from each genotype stored in either cold or ambient temperature (Goettemoeller and Ching, 1999). The tetrazolium chloride test evaluated seed viability and vigor (AOSA, 2000). Seeds were soaked in a 1% tetrazolium chloride solution and placed in a dark cabinet overnight (AOSA, 2000). Seeds were rinsed with sterile distilled water before bisecting longitudinally under a dissection microscope to observe tissue staining. Seeds were rated as viable if the entire embryo was stained red while partially stained (<80%), and non-stained embryos were rated non-viable (Goettemoeller and Ching, 1999; AOSA, 2000; de Oliveira et al., 2004).

Seed Production

Seeds were produced in field isolation cages (20.39m^3) at the Horticultural Crops Research Station in Clinton, NC. Each cage contained two genotypes for cross-pollination to

produce full-sib, hybrid seeds. Cages had two raised beds covered with 1.25mm black plastic on 1.52m row centers. Two genotypes (20 plants per row) were transplanted for each genotype with 0.15m between-plant spacing. Drip irrigation was used to water field isolation cage plants.

Clones were propagated at the Horticulture Field Laboratory, North Carolina State University. Two node stem segments were rooted using indole-3-butyric acid (IBA) rooting hormone (Hormodin 3, 0.8% IBA) in Fafard 4p soilless potting media in 1020-72 cell trays (each cell contained 129.05cm^3 volume of soilless media). Clones were misted 1 min/hour for 12 hours/day for 14 days until numerous roots were observed and new growth was evident. Clones were overhead watered, then fertilized two to three times/week with synthetic 20:20:20 water-soluble N:P:K fertilizer before field transplant in late May 2016.

Plants were hand-weeded as needed. Flower initiation occurred in early September. Bluebottle flies were used for insect pollination (Forked Tree Ranch, Idaho, USA). Flies were released into the cages in mid-September and again 14 days later. Seeds were harvested on October 26, 2016, in a once-over harvest. Seeds were extracted manually by shaking seeds into a gallon (4.5 L) Ziplock bag until they detached from the plant. Seeds were harvested from each genotype before collecting seed weights and drying seeds. Two debearded commercial seed lots (Botanical Interest and Jung Seed Co.) were purchased for this study. Commercial seed lots were not weighed, and moisture content was not evaluated.

Initial seed weights were collected before drying. Seed weights were calculated based on 1,000 seeds = 300 mg (Uçar et al., 2016). Percentage seed moisture was calculated using the formula:

$$\frac{\text{Initial seed weight} - \text{Final seed weight}}{\text{Initial seed weight}} \times 100$$

Seeds were dried at 60ºC for 24 to 48 hours. Dry seeds were weighed before storage to calculate moisture content. After three days, seeds were sown in a preliminary germination assay to identify genotypes with high initial germination rates and viability (data not shown). Ten field cage genotypes were used in this study (Table 1). Seeds were stored under two different conditions: ambient, room temperature (21ºC and 70%, +/-5% relative humidity) and controlled, cold room temperature (4ºC and 25%, +/-3% relative humidity).

Statistical Analysis

A split-plot treatment arrangement was used in a randomized complete block design, with storage duration as a whole plot, storage temperature, and genotype as a subplot. Independent variables were genotype (n = 12), temperature (n = 2), and storage duration (72 to 1,591 days). Dependent variables evaluated were final germination percent, germination speed, germination rate, mean germination rate, mean germination time, germination time variance, the standard deviation of germination, coefficient of germination velocity, time to 50% germination, time spread of germination, first germination time, last germination time, emergence rate index, and seed viability. Storage durations 72, 498, 862, 1,226, and 1,591 days were used for statistical analysis to simplify pair-wise comparisons.

Final germination percentage, germination speed, germination rate, mean germination rate, mean germination time, germination time variance, standard deviation of germination, and coefficient of germination velocity were analyzed using the GerminaR package, R version 4.1.1 (Lozano-Isla et al., 2021; R Core Team, 2021). Means were separated using Tukey's honestly significant difference (HSD) test. Germination parameters time to 50% germination, time spread of germination, first germination time, last germination time, and emergence rate index were analyzed using the germinationmetrics R package (Aravind et al., 2021). Means were separated

using Tukey's HSD test, 95% confidence level. Aravind et al. (2021) described equations used to measure germination parameters listed above excluding standard deviation of germination which was described by Ranal et al. (2006).

Seed survival curves were created using the *fitsigma* function in the viability metrics R package to estimate seed viability (Aravind et al., 2019). Seed survival curves were calculated using equations reported by Ellis et al. (1980b).

Results

Seed Traits

Seed fresh weight ranged from 3.70g to 5.20g, and seed dry weight ranged from 3.10g to 4.90g, respectively. We estimated that 9,000 to 16,000 seeds were produced from cross-pollinated genotypes in each isolation cage in the field. Seed moisture content ranged from 9.26% to 18.92%. An initial germination test was carried out at three days in storage, and ten genotypes were identified with high germination percent and viability to use for this experiment (Table 1). The final germination percent ranged from 64.3% to 87.0% for the ten selected genotypes. Botanical Interest and Jung Seed Co. seed lots had a final germination percent of 75.6% and 78.6%, respectively. Genotypes 10 and 9 had the lowest viability of 60.0% and 65.0%, respectively, while genotypes 4 and 12 had the highest viability of 90.0% and 90.0% (Table 2.1).

Final Germination Percent

The final germination percent was significantly different among genotypes ($F=9.433$, $p<0.05$). Storage temperature significantly impacted the final germination percent ($F=497.05$, $p<0.05$). There was an interaction between genotype and storage temperature for final germination percent ($F=2.33$, $p<0.05$) (Table 2.2). Genotypes with the highest final

germination percent in cold storage were genotypes 11, 4, and 5, with 75.2%, 74.4%, and 72.8%, respectively. The genotypes with the lowest final germination percent in 4ºC storage were 8, 9, and 7, with 67.6%, 67.7%, and 68.5%, respectively. The final germination percent was lower for genotypes 8 and 7 in ambient conditions, with 51.7% and 53.0% final germination percent, respectively. Genotypes 12, 4, and 5 had the highest final germination percent in room temperature storage, with 64.14%, 61.53%, and 60.14%, respectively. Genotypes 11, 5, and 4 had the highest final germination percent regardless of storage temperature, and genotypes 7, 8, 9, and 10 had the lowest final germination percent of all genotypes under both conditions (Figure 2.1).

Genotypes 10 and 12 had the smallest drop in final germination percent of 2.33% and 5.33% from 72 to 498 days in cold room storage (Table 2.3). Genotypes 4 and 5 had the largest difference in final germination percent from 72 to 498 days in cold room storage with 16.0% and 20.0%, respectively. Genotypes 10 and 2 also had the lowest difference in final germination percent in room temperature storage with 8.67% and 11.67%, and genotypes 4, 5, and 1 had the largest difference in final germination percent with 31.0%, 34.33%, and 35.67%, respectively, during the 426 day period (Table 2.3). After 862 days in cold room storage, genotypes 10, 9, and 12 only dropped 3.67%, 9.0%, and 9.33% in final germination percent, and genotypes 4 and 5 had the most significant difference in final germination percent of 21.67% and 28.33%, respectively (Table 2.3). Genotypes 12, 9, and 10 also had the smallest drop in final germination percent under room temperature conditions between 72 and 498 days in storage, with 17.0%, 21.0%, and 24.67%, respectively. Genotypes 5 and 1 had the highest loss in final germination percent in room temperature storage after 498 days in storage, with 49.67% and 47.0%, respectively (Table 2.3). Genotype 10 had the least difference in final germination percent from

72 days to 1,591 days in cold room storage with 7.33%, and genotypes 4, 3, and 5 had the largest difference of 30.33%, 35.00% and 36.33%, respectively. Genotypes 10 and 12 had the lowest difference in final germination percent under room temperature conditions from 72 to 1,591 days, with 28.0% and 29.0% (Table 2.3). Genotypes 3 and 5 had the largest difference in final germination percent in room temperature storage between 72 and 1,591 days in storage, with 62.67% and 59.0%, respectively.

Final germination percent is a valuable measurement because it informs researchers of how many seeds within the seed lot were capable of germination at the beginning, middle, and end of an experiment. Binary data (germinated/non-germinated) is a qualitative trait transformed into a quantitative measurement of the germination process to provide the final germination percent. It is relied on heavily by seed scientists due to the simplicity of data transformation, statistical analysis, and its ability to discriminate differences in the germination response of seeds (Ranal et al., 2006). However, it is only one of many traits that can be assessed from a germination experiment.

Mean Germination Rate

The mean germination rate was significantly different among genotypes ($F=9.54$, $p<0.05$) and significantly different for storage temperatures ($F=19.74$, $p<0.05$). The interaction between storage temperature and genotype was significant as well ($F=2.66$, $p<0.05$) (Table 2.4). Genotype 10 had the highest mean germination rate in ambient storage temperatures at 8.40 day^{-1} and refrigerator storage at 8.20 day^{-1}. Genotypes 7 and 11 had the lowest mean germination rate in cold room storage, with 7.80 day^{-1} and 7.80 day^{-1}, respectively (Figure 2.2). The mean germination rate ranged from 6.70 day^{-1} to 10.50 day^{-1}. Germination rate is a function of time; although statistically significant differences were detected among genotypes, the range of mean

germination rate was 7.80 to 8.40 day^{-1}, averaging 8.00 day^{-1} in the study. These results are consistent with other reports that the majority of seeds germinated in four to 14 days (Shock, 1982; Carneiro and Guedes, 1992).

Mean Germination Time

Mean germination time was significantly different among genotypes ($F=9.04$, $p<0.05$). Storage temperature had a significant impact on mean germination time ($F=16.68$, $p<0.05$). There was an interaction between genotype and storage temperature that was significant ($F=2.61$, $p<0.05$) (Table 2.5). In this experiment, the mean germination time ranged from 9.57 to 14.88 days. Genotype 7 had the longest mean germination time of 12.91 days under cold storage, and genotype 10 had the shortest mean germination time of 11.93 under room temperature conditions (Figure 4). Notably, 10 of 12 seed lots stored in the cold room took longer to germinate than seed lots stored at room temperature, excluding genotypes 5 and 12 (Figure 4). Germination time is a function of speed and velocity (Ranal et al., 2006). Those results showed that seeds stored at room temperature take longer to reach their mean germination time than seeds stored at cold temperatures.

Germination Rate

Genotypes were significantly different regarding germination rate ($F=9.43$, $p<0.05$) (Table 2.6). Germination rate was highest in cold room storage, with the highest Germination rate observed in genotypes 11, 4, and 5 demonstrating 75.2%, 74.4%, and 72.8%, respectively ($F=497.05$, $p<0.05$). Genotypes 7 and 8 had the lowest germination rate under room temperature conditions, with 51.74% and 51.22%, respectively (Figure 2.4). Cultigen 1 had a significantly higher germination rate in cold room storage than at room temperature with 71.4% compared to 58.6% at room temperature. Germination rate varied greatly for genotype 2, with 69.1% in cold

storage and 54.3% in room temperature conditions. Genotype 3 also had a high germination rate in cold storage (70.2%) compared to room temperature (57.7%). A 13% difference in germination rate was observed in genotype 4 under both storage conditions (Figure 2.4). germination rate ranged from 72.75% in cold room conditions to 60.1% in room temperature storage for genotype 5, a significant difference. Genotype 6 had a higher germination rate in cold storage compared to room temperature, with 69.7% compared to 54.6%, respectively. A significant difference in germination rate was observed in genotypes 7 and 8, with 68.5% and 67.6% in cold room storage versus 51.2% and 51.7% in room temperature storage, respectively. Genotype 9 exhibited a higher germination rate in cold room storage at 67.7%, while room temperature storage had a reduced germination rate of 53%. Genotype 10 had a 10% spread in germination rate in cold room and room temperature storage conditions with 63.9% and 53.2%, respectively. Genotype 11 had a 19% difference in germination rate spread in cold room versus room temperature conditions with 75.2% and 56.8%. Finally, genotype 12 showed significant differences in germination rate in cold and room temperature storage with 70% and 64.1%, respectively (Figure 2.4).

Germination rate is a commonly studied measurement for comparing seed lots (Ranal et al., 2006). Germination rate provides researchers with an easy way to differentiate seed lots. Germination rate is an accumulative measure of the number of seeds germinated each day for the duration of a study and then divided by the time intervals during which germination was scored. Previous research on stevia has focused mainly on this measure for evaluating seed lots (Kawatani et al., 1977; Alvarez et al., 1994; Chen and Shu, 1995; Cabanillas Díaz, 1997; Macchia et al., 2011; Martini et al., 2017) because it provides a quick comparison with little statistical effort.

Germination Speed

Germination speed was significantly different among genotypes ($F=9.54$, $p<0.05$). The temperature was a significant factor in germination speed ($F=19.74$, $p<0.05$). There was an interaction of genotype and temperature that was significant ($F=2.66$, $p<0.05$) (Table 2.7). Genotype 10 had the highest germination speed under both storage temperatures, compared to the rest of the genotypes in the study, with a germination speed of 8.23 days in cold room conditions and 8.41 days in room temperature conditions (Figure 6). Genotypes 7 and 11 had the shortest germination speed in cold room storage of 7.76 and 7.78 days, respectively. Germination speed ranged from 6.78 to 9.62 days in cold room storage and 6.51 to 10.47 days in room temperature storage. Similar to mean germination time, germination speed was generally lower in cold room stored seed lots than at room temperature, excluding genotypes 12 and 5. Mean germination time and germination speed measure velocity and rate of germination between time intervals, which explains the similarity in results observed for genotypes 10, 5, 12, 7, and 11 (Figures 2.3 and 2.5). These two traits have an inverse relationship; a higher germination speed indicates that a seed lot can germinate in less time.

Germination speed varied over time, with higher losses observed after 862 and 1,591 days in storage. Germination speed declined by 0.33 days to 2.77 days in cold temperature storage from 72 to 498 days, 0.33 days to 2.94 days at 862 days in storage, and by the end of the experiment, the decline in germination speed was 1.40 days to 4.56 days across all seed lots (Table 10). At room temperature conditions, germination speed dropped by 0.37 days to 3.17 days at 498 days in storage, 0.89 days to 4.45 days at 862 days in storage, and 2.44 days to 7.44 days by 1,591 days in storage (Table 10). The drop in germination speed indicated that storage time influenced how quickly seeds germinated, and room temperature storage had a more

significant drop in germination speed than cold room storage. The delay in germination was due to seed aging at prolonged storage.

Variance of Mean Germination Time

Germination time variance was significant among genotypes ($F=9.23$, $p<0.05$). The temperature was significant for germination time variance ($F=17.44$, $p<0.05$). There was an interaction between genotype and temperature ($F=2.80$, $p<0.05$) (Table 2.8). Genotype 10 had the lowest germination time variance in cold storage and room temperature storage, with 54.83% and 52.77%, respectively. Genotype 11 had a significantly higher germination time variance of 60.38% in cold room storage (Figure 2.6). Germination time variance ranged from 60.38% to 54.83% in cold room stored seed lots and 58.72% to 52.77% in room temperature stored seed lots. Germination time variance is used to measure the distribution of mean germination time and see statistical differences in germination response among seed lots (Ranal et al., 2006). Germination time variance is a function of mean germination time and germination speed, and similar trends are observed for seeds for all three traits. Genotypes 5 and 12 had higher germination time variance and mean germination time and lower germination speed under room temperature conditions. Genotypes 7 and 11 had the highest mean germination time and germination time variance and lowest germination speed under cold room storage conditions. Genotype 10 had the lowest mean germination time and germination time variance and the highest germination speed under room temperature conditions.

Germination Standard Deviation

The standard deviation of germination was significantly different among genotypes ($F=9.53$, $p<0.05$). Significant differences in the standard deviation of germination were observed in storage temperature as well ($F=18.81$, $p<0.05$), and the interaction of these two factors was

significant ($F=2.83$, $p<0.05$) (Table 2.9). Genotypes 11 and 7 had the highest standard deviation of germination of all genotypes, with a 7.77 standard deviation of germination and a 7.76 standard deviation of germination in cold room temperatures (Figure 2.7). Genotype 10 had the lowest standard deviation of germination of all seed lots exposed to room temperature conditions with a 7.26 standard deviation of germination and cold room conditions with a standard deviation of 7.40 (Figure 2.7). The measure of standard deviation in mean germination time is another trait used to assess variability and determine the accuracy of mean germination time calculations (Ranal et al., 2006). Moreover, we see the same trend in mean germination time, germination speed, germination time variance, and standard deviation of germination for specific genotypes, indicating that traits are highly correlated with each other, and that genotype response is variable under different storage temperature conditions.

Coefficient of Germination Velocity

The coefficient of germination velocity was significantly different among genotypes ($F=6.85$, $p<0.05$). The temperature was also a significant factor for the coefficient of germination velocity ($F=8.71$, $p<0.05$). A significant interaction between genotype and storage temperature was observed ($F=1.92$, $p<0.05$) (Table 2.10). Under room temperature storage, genotype 10 had a high coefficient of germination velocity of 60.86%. In cold room storage, genotypes 10 and 12 had a similar coefficient of germination velocity rates of 60.64% and 60.63%, respectively (Figure 2.8). The genotypes stored at cold room storage with the lowest coefficient of germination velocity were genotypes 7 and 4, with 60.1% and 60.12% coefficient of germination velocity, respectively (Figure 2.8). The coefficient of germination velocity is the reciprocal calculation of the mean length of incubation time and is subject to increase with seed number increases (Ranal et al., 2006). Since it is a weighted measure of the number of germinated seeds,

it is not influenced by sample size or treatment, which may explain the lack of correlation with other traits related to rate and speed (mean germination time, germination speed, germination time variance, and standard deviation of germination) in this experiment.

Time to 50% Germination

Time to 50% germination is the measure of median germination time and was affected primarily by storage duration for genotypes 1, 3, 6, 10, 11, and 12 (Table 2.3). Genotype 1 had significantly different time to 50% germination at storage times of 72 and 1,226 days, 862 and 1,226 days, and 1,226 to 1,591 days in room temperature conditions ($F=6.73$, $p<0.05$). Genotype 3 had significant differences in time to 50% germination at several time intervals in both storage treatments ($F=20.82$, $p<0.05$). Differences in time to 50% germination of genotype 3 were observed between 72 and 498 days, 72 and 862 days, 72 and 1,226 days, and 72 and 1,591, 498 compared to 1,226 days, and finally, 862 compared to 1,226 days in storage (Table 2.3). Genotype 6 showed a significant difference in time to 50% germination when comparing time intervals 72 to 1,226 days and 862 compared to 1,226 days in room temperature storage ($F=4.83$, $p<0.05$). Differences were detected in time to 50% germination of genotype 10 when comparing storage durations of 72 to 1,226 days, 498 to 1,226 days, 862 to 1,226 days, and 1,226 to 1,591 days ($F=7.56$, $p<0.05$). A significant difference in time to 50% germination of genotype 10 was identified in cold room storage when comparing 72 and 1,226 days in storage, and differences in time to 50% germination were observed in room temperature storage at 862 and 1,226 days in storage (Table 2.3). Genotype 11 showed differences in time to 50% germination at 72 and 1,226 days in cold room storage; differences in time to 50% germination were discovered between 72 and 1,226 days and 862 compared to 1,226 days in room temperature storage ($F=14.69$, $p<0.05$).

Genotypes 4, 5, and 9 were observed to have differences in time to 50% germination based on storage temperature and duration (Table 2.3). Differences in time to 50% germination were also observed in genotype 4 subjected to cold room temperatures ($F=6.01$, $p<0.05$). Storage time significantly affected time to 50% germination in genotype 4 when comparing 72 and 1,226 days, 498 and 1,226 days, 862 and 1,226 days, and 72 compared to 1,591 days in storage ($F=9.81$, $p<0.05$). Genotype 5 also had significant differences in time to 50% germination when stored at room temperature compared to cold temperature storage ($F=5.43$, $p<0.05$). Differences in time to 50% germination were observed comparing storage durations of 72 to 498 days, 72 to 1,226 days, 72 to 1,591 days, and 862 to 1,226 days for genotype 5 ($F=8.45$, $p<0.05$). Storage duration was an important factor influencing time to 50% germination in genotype 6, with significant differences observed between 72 and 1,226 days and 862 compared to 1,226 days in storage ($F=4.83$, $p<0.05$). Genotype 9 had significantly different time to 50% germination at room temperature storage between 72 days and 1,226 days in storage ($F=5.64$, $p<0.05$), and temperature showed slight differences in time to 50% germination ($F=4.61$, $p=0.044$). The temperature was a significant factor for time to 50% germination of genotype 7, with differences detected at 862 days in storage ($F=10.89$, $p<0.05$). Time to 50% germination was significantly different when comparing storage duration of 862 to 1,226 days for room and cold temperature conditions in genotype 7 ($F=3.32$, $p<0.05$).

Time Spread of Germination

Time spread of germination describes the distribution of germination between the time of first and last germination (Kader, 2005). Time spread of germination was significantly different for genotypes 1, 2, 6, and 11 under different storage temperatures (Table 10). Genotype 1 had a significant different in the time spread of germination when subjected to room temperature

storage compared to cold room storage conditions ($F=6.86$, $p<0.05$). Genotype 2 showed significant differences in the time spread of germination in response to storage temperature ($F=5.54$, $p<0.05$). The time spread of germination was significantly different for genotype 6 when subjected to room temperature versus cold room storage conditions ($F=9.78$, $p<0.05$). The time spread of germination was significantly different for genotype 11 subjected to the room and cold temperature storage conditions ($F=25.6$, $p<0.05$). Storage time was also a significant factor for cultigen 11, time spread of germination at 498 days in storage and 1,226 days in storage between a room and cold temperature conditions ($F=2.10$, $p<0.05$). The time spread of germination was significantly different for genotype 12 at 72 and 498 days in storage ($F=3.21$, $p<0.5$). However, the temperature was not an important factor in the time spread of germination in this seed lot.

The time spread of germination was significantly different for genotypes 3, 5, and 8 under different storage times and over storage time (Table 2.3). Genotype 3 showed significant differences in the time spread of germination under room and cold temperature storage ($F=13.0$, $p<0.05$). Time was an important factor for the time spread of germination in genotype 3, with differences observed between 72 and 1,591, 498 and 1,591, and 862 and 1,591 days in storage ($F=6.04$, $p<0.05$). Genotype 5 significantly differed in the time spread of germination at room storage compared to cold storage conditions ($F=6.23$, $p<0.05$). The greatest difference in time spread of germination was observed between 72 and 1,226, and 1,226 and 1,591 days in room temperature storage for genotype 5 ($F=5.08$, $p<0.05$). Genotype 8 showed a difference in the time spread of germination under room temperature and cold temperature at 1,226 days in storage ($F=19.69$, $p<0.05$). Under room temperature storage, there was a significant difference in

the time spread of germination at 498 and 1,226 days in storage observed in genotype 8 ($F=3.92$, $p<0.05$).

First Germination Time

First germination time was significantly different for genotypes 1, 2, and 11 in storage temperature (Table 2.3). Genotype 1 had a significant difference in first germination time at different storage temperatures ($F=9.09$, $p<0.05$). Genotype 2 also significantly responded to storage temperature ($F=9.0$, $p<0.05$). The storage temperature impacted the first germination time in genotype 11 ($F=8.17$, $p<0.05$). First germination time was significantly different from storage time in genotype 10 ($F=3.92$, $p<0.05$), with the most significant difference in first germination time was detected between 72 and 1,226 days in storage under room temperature conditions ($F=8.33$, $p<0.05$).

First germination time was significantly different for storage temperature and storage period for genotypes 3, 5, and 8 (Table 2.3). The first germination time for genotype 3 was significant for storage time and temperature ($F=7.66$ and $F=6.0$, $p<0.05$, respectively). There were significant differences in first germination time for room temperature conditions between 72 and 1,591 days in storage, 498 and 1,591 days in storage, and 862 and 1,591 days in storage ($F=7.67$, $p<0.05$). Genotype 5 had significant differences in first germination time at room temperature storage at 72 and 1,226 days in storage, and 498 and 1,226 days in storage ($F=6.30$, $p<0.05$). Genotype 8 had a significant difference in first germination time between 72 and 1,226 days in storage under room temperature conditions ($F=6.13$, $p<0.05$). There was a significant difference in first germination time for temperature and time for all genotypes ($F=3.44$, $p<0.05$).

Last Germination Time

The last germination time was significantly different for genotypes 1, 2, 9, and 12 for storage duration (Table 10). Storage time was a significant factor for last germination time in genotype 1, with differences observed at 72 and 1,226 days in storage and 862 compared to 1,226 days in storage at room temperature ($F=4.61$, $p<0.05$). Genotype 2 had significant differences in last germination time at 72 and 1226 days in storage under room temperature conditions ($F=3.15$, $p<0.05$). Last germination time was affected by longer storage time under room temperature and cold temperature conditions at 72 and 1,226, and 862 and 1,226 days in storage for genotype 9 ($F=4.17$, $p<0.05$). The last germination time in genotype 12 was significantly different at 72 and 1,226 days in storage under both cold and room temperature storage ($F=4.25$, $p<0.05$).

The last germination time was significantly different for storage temperature for genotypes 6, 7, and 11 (Table 2.3). A significant difference in Last germination time was observed in genotype 6 when comparing storage temperature ($F=7.14$, $p<0.05$). Last germination time was significantly different at room temperature storage compared to cold storage in genotype 7 ($F=4.57$, $p<0.05$). Genotype 11 had significant differences in last germination time at different storage temperatures ($F=9.0$, $p<0.05$). The last germination time was significantly different for genotype 8 at room and cold storage temperatures ($F=5.40$, $p<0.05$). Slight differences in last germination time were observed in genotype 10 subjected to different temperature storage conditions ($F=4.46$, $p=0.048$).

Emergence Rate Index

The emergence rate index was significantly different for all genotypes for storage temperature and duration (Table 2.3). Storage duration was a significant factor for genotype 1

comparing 72 to 862, 72 to 1,226, 72 to 1,591, 498 to 1,226, 498 to 1,591, 862 to 1,226, and 862 to 1,591 days in storage ($F=25.73$, $p<0.05$). The emergence rate index was significantly higher in cold room storage for cultigen 1 ($F=18.75$, $p<0.05$). Cold room storage showed a higher emergence rate index at 72 days compared to 1,226 and 1,591 days, and ambient temperature storage differences were found for 72 days in storage compared to 1,591, 498, and 1,226, and 498 compared to 1,591 days (Table 2.3). Genotype 2 had significant differences in emergence rate index between storage durations 72 and 1,226, 72 and 1,591 days in storage in cold room conditions ($F=67.16$, $p<0.05$), and the temperature was a significant factor at 72 and 862, 72 and 1,226, and 72 and 1,591 days in storage ($F=55.48$, $p<0.05$). Significant differences were observed in the emergence rate index for genotype 3 between storage durations of 72 compared to 498, 498, and 862, 862 and 1,226, and 1,226 and 1,591 days in storage ($F=85.73$, $p<0.05$), and cold temperature storage had a higher emergence rate index than room temperature at each time point ($F=48.02$, $p<0.05$). Genotype 4 had significant differences for the five evaluated storage durations ($F=45.87$, $p<0.05$) and cold room temperature had significantly higher emergence rate index at 72 compared to 498, 72 and 862, 72 and 1,226, 72 and 1,591, 498 and 1,226, 498 and 1,591, 862 and 1,226, and 862 compared to 1,591 days ($F=14.15$, $p<0.05$). Genotype 5 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=48.10$, $p<0.05$).

The emergence rate index in genotype 5 was significantly different when comparing all time points, excluding 498 and 862 days in storage ($F=68.29$, $p<0.05$). Genotype 6 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=60.24$, $p<0.05$). The emergence rate index in genotype 6 was significantly different when comparing all time points, excluding 498 and 862 days in storage ($F=34.36$, $p<0.05$). Genotype 7

had a higher emergence rate index under cold storage conditions ($F=65.90$, $p<0.05$), and there were significant differences in storage durations ($F=34.05$, $p<0.05$), excluding comparisons between 72 and 498 days and between 1,226 and 1,591 days. Genotype 8 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=105.40$, $p<0.05$). The emergence rate index in genotype 8 was significantly different when comparing all time points, excluding 498 and 862 and between 1,226 and 1,591 days in storage ($F=69.01$, $p<0.05$). Interaction of time and temperature was observed in the genotype 8 emergence rate index at 498, 862, 1,226, and 1,591 days in storage ($F=7.20$, $p<0.05$). Genotype 9 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=39.52$, $p<0.05$). The emergence rate index in genotype 9 was significantly different when comparing all time points, excluding 498 and 862 and between 1,226 and 1,591 days in storage ($F=32.92$, $p<0.05$).

An interaction of time and temperature was observed in the genotype 9 emergence rate index at 1,226 and 1,591 days in storage ($F=7.20$, $p<0.05$). Genotype 10 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=40.06$, $p<0.05$). The emergence rate index in genotype 10 was significantly different when comparing all time points, excluding 498 and 862 and between 1,226 and 1,591 days in storage ($F=28.04$, $p<0.05$). An interaction of time and temperature was observed in the genotype 10 emergence rate index at 1,226 and 1,591 days in storage ($F=7.20$, $p<0.05$). Genotype 11 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=93.35$, $p<0.05$). The emergence rate index in genotype 11 was significantly different when comparing all time points, excluding 1,226 and 1,591 days in storage ($F=63.74$, $p<0.05$). An interaction of time and temperature was observed in the genotype 11 emergence rate index at 498, 862, 1,226,

and 1,591 days in storage ($F=6.88$, $p<0.05$). Genotype 12 had a significantly higher emergence rate index in cold room storage than in room temperature storage ($F=15.89$, $p<0.05$). The emergence rate index in genotype 12 was significantly different when comparing 72 and 498, 72 and 1,226, and 72 compared to 1,591 days in storage ($F=23.91$, $p<0.05$). These results suggested that the emergence rate index changed over time, and storage temperature influenced genotype response (Table 2.3).

Seed Viability

The initial seed viability constant, K_i, was highest in seed lots stored at cold temperatures, excluding genotypes 5 and 8, with higher K_i at room temperature conditions. K_i ranged from 0.77 to 1.45 under cold room storage and 0.57 to 1.34 under room temperature storage. Genotypes 11, 1, 12, and 2 had the highest K_i values of 1.45, 1.35, 1.31, and 1.0 under cold room temperature conditions. Genotypes 4 and 8 had the lowest K_i of 0.77 and 0.83 under cold room storage, respectively. Genotypes 11, 12, and 1 had the highest K_i values of 1.34, 1.27, and 0.98, respectively. Genotypes 4 and 9 had the lowest K_i of 0.57 and 0.79 under cold room storage, respectively.

Seed viability decreased over time at a faster rate in room temperature storage compared to cold room storage for all genotypes (Table 2.3). Genotype 1 was predicted to remain viable longer in cold room storage than under room temperature storage (Figure 2.9). Genotype 2 was predicted to remain viable longer under cold room storage than under room temperature storage (Figure 2.10). Genotype 3 was predicted to remain viable for 1,774.20 days under cold room storage and 858.28 days under room temperature storage (Figure 2.11). Genotype 4 was predicted to remain viable for more days under cold room storage than under room temperature storage (Figure 2.12). Genotype 5 was predicted to maintain viability longer under cold room

storage compared to room temperature storage (Figure 2.13). Genotype 6 seeds were predicted to remain viable for 1,897.80 days under cold room storage and 848.84 days under room temperature storage (Figure 2.14). Genotype 7 was predicted to remain viable longer under cold room storage than under room temperature storage (Figure 2.15). Genotype 8 was predicted to remain viable for longer storage duration in cold room storage compared to room temperature storage (Figure 2.16). Genotype 9 was predicted to remain viable for 2,785.57 days under cold room storage and 1,082.54 days under room temperature storage (Figure 2.17). Genotype 10 was predicted to maintain viability for 1,680.41 days in cold room storage and 1,284.51 days in room temperature storage (Figure 2.18). Genotype 11 was predicted to remain viable for longer under cold room storage conditions compared to room temperature storage conditions (Figure 2.19). Genotype 12 was predicted to remain viable for 2,464.36 days under cold room storage and 1,044.65 days under room temperature storage (Figure 2.20).

These results indicated that cold storage helps maintain seed viability longer than room temperature storage for all genotypes. Genotypes 4, 9, 12, 7, and 5 were predicted to maintain seed viability the longest under cold room storage, and genotypes 1 and 11 were predicted to lose viability the fastest despite having the highest initial seed viability of genotypes studied. Genotypes under room temperature storage with the highest viability were 10, 4, and 9, while genotypes 7, 6, and 5 lost viability more rapidly. Genotypes 4 and 9 had the lowest initial viability in this study but had higher predicted viability than all genotypes under room temperature. These results indicate that the seed viability of genotypes 4 and 9 were less effected by temperature treatment. The rate of viability loss varied by genotype, storage temperature, and storage duration. Initial seed viability varied among genotypes, and genotype response to storage temperature was not necessarily predictable based on initial viability.

Discussion

In 2016, a seed longevity experiment on *S. rebaudiana* was initiated to observe differences in seed viability and germination traits at two different storage conditions for 53 months. Radical emergence was used to score seed germination in this study because radical emergence is the first step in the germination process (Bewley et al., 2013; Baskin and Baskin, 2014). Germination percentage, rate, time to the first germination, and germination speed vary greatly between and among individuals, populations, and seed lots and aid in the comparison of diverse germplasm (Soltani et al., 2015). The relationship between seed survival and storage conditions (time and temperature) was quantified using the seed viability equation reported by Ellis and Roberts (1980a; 1980b), which predicts the length of time a seed lot takes to lose one probit percent of viability at a known moisture content, known initial viability and specified temperature. The seed viability equation has proven helpful for many species and provides insight into the lifespan of seed lots. The seed viability equation was successfully fit to the stevia seed lots stored in cold room and room temperature conditions in this study. The current study determined that seed lots stored in the cold room had higher predicted seed viability compared to those stored at room temperature. Overall, seeds in cold room conditions remained viable twice as long as seeds at room temperature. The shape of the survival curves under cold room storage were similar among all seed lots and in contrast to survival curves from seed lots stored at room temperature. Differences in seed viability were observed among seed lots which may be attributed to seed moisture content and initial viability. In lettuce, seed storage at high moisture content showed lower viability with higher moisture content (Ellis, 1991).

Species within the same genus and further within the same family are similar in seed longevity (Walters et al., 2005b). Relative longevity can be defined by evaluating the general

response of species to seed storage conditions (Mira et al., 2015). Seed longevity is reported to be variable in species of the Asteraceae family, to which *Stevia* belongs (Walters et al., 2005b). Lettuce, *Lactuca sativa* (also a member of the Asteraceae), has similar seed characteristics as stevia, having small, orthodox seeds with an attached, persistent pappus. Lettuce seeds have been evaluated for seed longevity and shown to tolerate dehydration to 7 to 12% moisture content with a critical moisture content of 15% (Roberts and Ellis, 1989). Roberts and Ellis (1989) also stated that imbibition injury could occur when orthodox seeds take up water rapidly, especially when seeds are stored at low temperatures with low moisture content. The 10 field-produced seed lots used in this study had moisture content ranging from 9.26% to 18.92%, which may not have been adequate for longer-term storage for stevia. Also, it is plausible that some seeds may have been damaged during imbibition, causing them not to germinate after hydration; that could have been due to a lack of humidification prior to conducting germination tests (Roberts and Ellis, 1989). Under normal distribution, σ, the time it takes for seeds to lose one probit percent of viability declines from 97.7% to 84.1%, or from 84.1% to 50.0% (Roberts and Ellis, 1989). For example, genotype 12 had initial viability of 95%. It fell to 45% after 1591 days in room temperature storage, indicating initial viability was 2σ above 50%, meaning longevity decreased twofold over the storage period under ambient air conditions.

Macchia et al. (2011) demonstrated that stevia seeds germinated at a higher rate when pre-chilled compared to non-chilled seeds, while alternating temperatures without pre-chilling treatment resulted in higher germination rates. An early flowering accession showed higher germination than an accession that exhibited delayed flowering and seed set, indicating seed production environment, specifically temperature, influenced germinability. The researchers concluded that the high variability in germination response was due to the lack of domestication

and genetic improvement. Martini et al. (2017) reported that early flowering accessions produced more seeds because of favorable weather conditions during seed set, and cross-pollination of stevia by insects positively affected seed yield and quality. Early seed harvest resulted in higher yields and germination rates, ranging from 34.7% to 85.3%; late harvest resulted in a reduced germination rate (as low as 17.3%) among the 36 genotypes they evaluated (Martini et al., 2020).

Flowering time, seed harvest timing, and pollination technique can affect stevia seed production. Heavy rainfall during flowering can reduce seed set; shade also reduces biomass, hinders flowering time, and reduces the rate of flowering and subsequent seed production (Slamet and Tahardi, 1988). Seeds were harvested at one event before conducting this experiment, which may account for variability in initial seed viability and quality. Seed harvest timing can affect seed quality, with late harvests resulting in fewer black, fertile seeds. Inadequate pollination also resulted in lower seed production. Bluebottle flies were released twice during flowering to ensure entomological pollination; however, low temperatures may have reduced their mobility and reduced the production of fertile seeds. Heavy rain and lower temperatures during seed development and ripening can negatively impact the number of viable seeds produced from a plant (Macchia et al., 2011). Flowering time also affects seed harvest timing, with late flowering germplasm producing fewer fertile seeds during suboptimum environmental conditions. Flowering time is a phenological trait that can be selected against, and improvements can be made to adapt different germplasm to non-native regions.

High germination percentages are possible in stevia (Carneiro and Guedes, 1992). This study used two commercial seed lots (Botanical Interest and Jung seed Co.), both with pappus removed. Those seed lots lost viability more slowly and germinated at a higher rate under cold room conditions than seeds having their pappus. Enhanced germination and viability in those

seed lots may be due to a lack of pappus, ensuring the seeds had adequate contact with moist filter paper for imbibing (Macchina et al., 2007; Carneiro and Guedes, 1992). Some techniques for pappus removal can damage the seeds and result in an increased number of abnormal seedlings; those are noted by stunted rootlets, dark apex, and cotyledons separating from the growing tip (Carneiro, 1990). Abnormal seedlings were not observed in this study, and the debearded seeds did not appear damaged when seeds were counted prior to the start of the experiment. The pappus attached to raw seeds prevented complete seed contact with the medium, which may have affected seed imbibition and germination for the 10 field-produced seed lots.

Chen and Shu (1995) reported that low-temperature storage (0ºC, -10ºC, and -18ºC) using airtight containers was preferable to maintain seed viability for up to three years in three seed samples produced from three different geographical regions. The researchers noted that seeds stored at room temperature declined drastically in germination by the end of the first year. Seed production location and temperature significantly influenced the germinability of stevia seeds in their study (Chen and Shu, 1995). A separate study conducted in Japan reported stevia seed lifespan to be three years (Kawatani et al., 1977). In examining storage conditions, viability was unchanged when stored seeds were in a desiccator in a cool, dark place for one year. A 50% decline in viability was observed when stored at room temperature without humidity control (Kawatani et al., 1977). Seed viability dropped 45% to 70% in seed lots in room temperature storage and 15% to 45% for seed lots stored in cold room conditions over 53 months in storage (Table 10). The results of our study agree with those of Chen and Shu (1995), with low germination and viability observed after a year of room temperature storage, but it was not the larger change reported by Kawatani et al. (1977). However, a loss of viability was observed in

the present study under cold room conditions when seeds were kept in a cool, dark, humidity-controlled room.

Marcvillaca (1985) was of the opposite opinion, that stevia seeds have a small window of germination capacity and need to be used soon after harvest. Alvarez et al. (1994) stated that stevia seeds should not be sown directly following harvest. They suggested seeds could be stored outside during cold weather in temperate regions as an economical method of maintaining seed viability. Regardless, they reported that storing seeds after harvest in airtight containers at 4ºC was preferable because seeds lose viability quickly at room temperature (Alvarez et al., 1994). Cabanillas and Díaz (1997) also concluded that 4ºC refrigerator storage was optimum to maintain stevia seed viability, but only for an 11-month duration (Cabanillas and Díaz 1997). Further, Rank and Midmore (2006) suggested 4ºC was optimum for seed storage. However, our results do not agree with the high loss of viability in 6 to 12 months. In fact, stevia seeds remain viable for longer than previous reports, and 4ºC is preferred for medium-term storage compared with room temperature storage.

Bojimirov et al. (2020) reported a decrease in germination percent of 15.8% after one year of seed storage, with high variability among genotypes evaluated; however, researchers did not report storage conditions before germination assays. Bebawi et al. (2018) examined a related species, *Stevia ovata,* in Australia, which was reported to lose complete viability within 18 months at seeding depths of 2.5, 10, and 20cm under wet-tropic conditions, while dry-tropic climate helped to maintain viability for 24 to 42 months when seeds were stored underground at depths of 2.5, 10, and 20cm. Buried seeds were not able to germinate above 39ºC, but *S. ovata* could remain viable for longer and germinate readily at lower temperatures in dry conditions (Bebawi, et al., 2018). Though seed burial was not evaluated in this study, it does agree with the

long-term seed survival of stevia species under lower temperatures when seeds are stored in conditions of low temperature and low humidity.

Seed production in North Carolina occurred during fall when temperatures were lower during the reproductive phase, and this may have had a negative impact on the collection of viable seeds. The final germination percent was highest at the beginning of the study and dropped significantly after 498 days, with a range of 8.67% to 35.67% in room temperature storage and 2.33% to 20% in cold room storage. The difference in final germination percent ranged from 3.67% to 28.33% in seed lots stored at cold temperature conditions and 17% to 49.67% in room temperature after 862 days in storage. Overall, the final germination percentage dropped by 7.33% to 36.33% in cold room conditions and 29% to 54% in room temperature conditions over 1,591 days in storage. The final germination percent was greatest at the start of the experiment and declined by 3.33% to 27.70% in cold room storage and 3.71% to 31.10% in room temperature storage after 498 days. Germination speed dropped by 3.33% to 29.40% in cold room conditions and 8.90% to 44.5% in room temperature storage after 862 days in storage. By the end of the 1,591-day experiment, germination speed ranged from 14.00% to 45.6% in cold room conditions and 24.40% to 74.4% in room temperature storage. The emergence rate index was also affected by storage time and temperature and showed a decrease after 498 days in storage from 21.67 to 135.33 seedling days under cold room conditions and 16.67 to 159.0 seedling days. By 862 days in storage, the emergence rate index ranged from 28.0 to 151.67 seedling days in cold temperature storage and 46.67 to 226.33 seedling days in room temperature storage. By the end of the 1,591 days in storage, the emergence rate index ranged from 64.33 to 236.33 seedling days in cold room conditions and 130.0 to 380.0 seedling days in room

temperature storage. Germination declined at a faster rate in room temperature storage than in cold room storage, and significant differences were observed within 862 days in storage.

Seed moisture content was highest in genotypes 4 and 7 (18.92% and 18.42%, respectively); genotype 4 lost 15% and 40% viability at the cold and room temperature, and genotype 7 lost 30% and 55% viability at cold and room temperature within 1,591 days. Within the 498 days in storage, viability decreased by 0% to 25% in room temperature storage and 0% to 10% in cold room storage in all genotypes. Further, viability decreased by 862 days in storage by 15% to 45% in room temperature storage and 5% to 15% in cold room storage across all genotypes. Overall, seed viability loss was greatest in room temperature storage (40% to 70%) compared to cold room storage (15% to 45%) over 1,591 days in storage. Time played a significant factor in viability losses, with large differences observed at 862 days in storage. These results indicate that 21ºC is suboptimal for maintaining seed viability and that seed storage duration should not exceed 862 days due to loss of viability. Seed viability declined at a slower rate under cold room storage than at room temperature storage.

Stevia seed longevity showed wide variation among 12 seed lots for the storage conditions studied. Storage duration and temperature had a profound effect on the germination and vigor parameters evaluated. Genotypes subjected to room temperature storage resulted in reduced germination with a faster loss of viability compared to cold room storage. Results indicated that cold room seed storage maintained viability longer than ambient temperature storage and that seeds should be used within two years of storage since there were losses of the viability of 5% to 15 % in refrigerator storage and 15% to 45% in ambient storage conditions during that time.

References

Abdullateef, R.A., & Osman, M. (2011). Effects of Visible Light Wavelengths on Seed Germinability in *Stevia rebaudiana* Bertoni. *International Journal of Biology*, 3(4): 83-91.

Alvarez, L., Casaccia, R., & Lopez, G. (1994). Producción de ka'a he'é. Instituto Agronómico Nacional. S.E.A. Ministerio de Agricultura y Ganadería. Asunción. Paraguay: 1-47.

AOSA. (2000). Tetrazolium Testing Handbook. Ed. Jack Peters. Contribution No. 29. Association of Official Seed Analysts. New Mexico 88003.

Aravind, J., Vimala Devi, S., Radhamani, J., Jacob, S.R., & Srinivasan, K. (2021). The Germinationmetrics Package: A Brief Introduction. R package version 0.1.5. ICAR-National Bureau of Plant Genetic Resources, New Delhi.

Aravind, J., Radhamani, J., Vimala Devi, S., Jabob, S.R., & Srinivasan, K. (2019). Viabilitymetrics: Seed Viability Calculations and Curve Fitting. R package version 0.0.0.9100, https://aravind-j.github.io/viabilitymetrics/. Accessed Aug. 31, 2021.

Baskin, C.C., & Baskin, J.M. (2014) Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination. 2nd ed. Elsevier/Academic Press, San Diego, CA.

Bebawi, F.F., Campbell, S.D., Mayer, R.J., Setter, M.J., & Setter, S.D. (2018). Effects of Temperature and Burial on Seed Germination and Persistence of the Restricted Invasive *Stevia ovata* in Northern Queensland. *Australian J of Bot*., 66(5): 388-397.

Bewley, J.D., Bradford, K.J., Hilhorst, H.W.M., & Nonogaki, H. (2013). Seeds: Physiology of Development, Germination, and Dormancy. 3rd ed. Springer, New York.

Bojimirov, S., Kikindonov, T., & Mehmed, A. (2020). Assessment of Stevia (*Stevia rebaudiana*) Seed Vitality in the Conditions of Bulgaria. *Horticulture*, 64(2): 177-181.

Brandle, J.E., Starratt, A.N., & Gijzen, M. (1998). *Stevia rebaudiana*: Its Agricultural, Biological, and Chemical Properties. *Can. J. Plant Sci.,* 78: 527-536.

Cabanillas, C. & Díaz, M.P. (1997). Influence of the Conditions of Storage on the Seed Quality of *Stevia rebaudiana* (Bertoni) Bertoni. *Acta Hort*., 502: 255-259.

Carneiro, J.W.P. (1990). Maringá: Fundação Universidade Estadual de Maringá. p 65.

Carneiro, J.W.P., & Guedes, T.A. (1992). Influence of the Contact of Stevia Seeds with the Substrate, Evaluated by Means of the Wiebull Function. *Rev. Brasil. Sementes*, 14: 65-68.

Carneiro, J.W.P., Muniz, A.S., & Guedes, T.A. (1997). Greenhouse Bedding Plant Production of *Stevia rebaudiana* (Bert) Bertoni. *Can. J. Plant Sci.*, 77: 473-474.

Chalapathi, M.V., Timmegowda, S., Rama Krishna Prama, V., & Prasas, T.G. (1997). Natural Non-Calorie Sweetener Stevia (*Stevia rebaudiana* Bertoni): A Future Crop for India. *Crop Res*., 14: 347-350.

Chen, S. & Shu, S. (1995). Study on Storage Technique of *Stevia rebaudiana* Seed. *Acta Agron. Sin.*, 21: 102–105.

Colombus, M. (1997). The Cultivation of Stevia, "Nature's Sweetener". Ontario Ministry of Agriculture, Food and Rural Affairs, Toronto, Ontario, p 4.

Davies, R., Di Sacco, A., & Newton, R. (2015). Germination Testing: Procedures and Evaluation. *Technical Information Sheet_13a*. Royal Botanic Gardens, Kew.

Dickie, J.B., Ellis, R.H., Kraak, H.L., Rydes, K., & Tompsett, P.B. (1990). Temperature and Seed Storage Longevity. *Annals of Botany*, 65: 197-204.

Ellis, H.E. (1991). The Longevity of Seeds. *HortScience*, 26(9): 1119-1125.

Ellis, H.E., & Roberts, E.H. (1980a). The Influence of Temperature and Moisture on Seed Viability Period in Barley (*Hordeum distichum* L.). *Ann. Bot*., 45: 31-37.

Ellis, H.E., & Roberts, E.H. (1980b). Improved Equations for the Prediction of Seed Longevity. *Ann. Bot*., 45: 13-30.

Everstevia.com. (2008). Sweetener of the Millennium. Stevia Seeds. Accessed July 17, 2021. www.everstevia.com/stevia-seeds.html

Ferrazzano, G.F., Cantile, T., Alcidi, B., Coda, M., Ingenito, A., Zarrelli, A., Di

Goettemoeller, J., & Ching, A. (1999). Perspectives on New Crops and New Uses: Seed Germination in *Stevia rebaudiana*. Janick, J. ed. ASHS Press, Alexandria, VA, 510-511.

Gutiérrez, D.G., Muñoz-Schick, M., Grossi, M.A., Rodrí- Guezcravero, J.F., Morales, V., & Moreira-Muñoz, A. (2016). The Genus *Stevia (Eupatorieae, Asteraceae*) in Chile: a Taxonomical and Morphological Analysis. *Phytotaxa.*, 282(1): 1–18.

Hossain, M.F., Islam, M.T., Islam, M.A., & Akhtar, S. (2017). Cultivation and Uses of Stevia (*Stevia rebaudiana* Bertoni): A Review. *Afr. J Food Agric. Nutr. Dev*., 17(4): 12745- 12757.

International Board of Plant Genetic Resources. (1976). Report of the IBPGR Working Group on Engineering, Design and Cost Aspects of Long-term Seed Storage Facilities. IBPGR, Rome. p 19

Kader, M. A. (2005). A Comparison of Seed Germination Calculation Formulae and the Associated Interpretation of Resulting Data. *Journal and Proceedings of the Royal Society of New South Wales,* 138: 65-75.

Kawatani, T., Kaneki, Y., & Tanabe, T. (1977). The Cultivation of kaa he-e (*Stevia rebaudiana*). II Seed Germination with Special Reference to the Optimum Temperature and Light Sensitivity. *Jpn. J. Trop. Agric.*, 20: 137-142.

Lester, T. (1999). *Stevia rebaudiana* (Sweet honey leaf). *Aust. New Crops News Lett. 11. Nat. Prod. Radiance*, 2: 120.

Lozano-Isla, F., Benites-Alfaro, O.E., & Pompelli, M.F. (2021). GerminaR: An R Package for Germination Analysis with the Interactive Web Application "GerminaQuant for R". *Ecological Research*, 34(2): 339-346.

Macchia, M., Andolfi, L., Ceccarini, L., & Luciana, A. (2007). Effects of Temperature, Light and Pre-Chilling on Seed Germination of *Stevia rebaudiana* (Bertoni) Bertoni Accessions. *Italian Journal of Agronomy,* 2 (1): 55-62.

Marcavillaca, C. (1985). Micropropagation "in vitro" de *Stevia rebaudiana* por Medio de Segmentos Nodales y Meristemas. *Anales de SAIPA*, 6:241-243.

Martini, A., Tavarini, S., Macchia, M., Benelli, G., Canale, A., Romano, D., & Angellini, L.G. (2017). Influence of Insect Pollinators and Harvest Time on the Quality of *Stevia rebaudiana* (Bert.) Bertoni Seeds. *Plant biosystems*, 151(2): 341-351.

Mira, S., Estrelles, E., & González-Benito, M.E. (2015). Effect of Water Content and Temperature on Seed Longevity of Seven Brassicaceae Species After 5 Years of Storage. *Plant Biology*, 17: 153-162.

Monteiro, R. (1980). Taxonomiae Biologiada Reproducaoda *Stevia rebaudiana* Bert. Ph.D. thesis, Univ. Estadual de Campinas, Brazil.

Nguyen, T. P., Keizer, P., van Eeuwijk, F., Smeekens, S., & Bentsink, L. (2012). Natural Variation for Seed Longevity and Seed Dormancy are Negatively Correlated in Arabidopsis. *Plant physiology*, 160(4): 2083–2092.

Oddone, B. (1997). How to Grow Stevia. *Technical manual.* Guarani Botanicals, Inc., Pawtucket, CT.

Oddone, B. (1999). How to Grow Stevia. Guarani Botanicals, Inc., Pawtucket, CT. p 1-30.

de Oliveira, V.M., Forni-Martins, E.R., Magalhães, P.M., & Alves, M.N. (2004). Chromosomal and Morphological Studies of Diploid and Polyploid Cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (Eupatorieae, Asteraceae). *Genetics and Molecular Biology*, 27(2): 215-222.

Pande S.S., & Gupta, P. (2013). Plant Tissue Culture of *Stevia rebaudiana* (Bertoni): a Review. *J Pharmacogn Phytother*, 5(1): 26-33.

R Core Team. (2021). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/, accessed Sept 1, 2021.

Ramesh, K., Singh, V., & Megeji, N.W. (2006). Cultivation of *Stevia* [*Stevia rebaudiana* (Bert.) Bertoni]: a Comprehensive Review. *Adv Agro*., 89(5): 137-177.

Ranal, M., & De Santana, D.G. (2006). How and Why to Measure the Germination Process? *Revista Brasil. Bot*., 29(1): 1-11.

Rank, A.H., & Midmore, D.J. (2006). Stevia- An Intense, Natural Sweetener, Laying the Groundwork for a New Rural Industry. Rural Industries Research and Development Corporation. RIRDC publication No 06/020.

Roberts, E.H. (1973). Predicting the Storage Life of Seeds. *Seed Science and Technology*, 1: 499-514.

Roberts, E.H., & Ellis, H.R. (1989). Water and Seed Survival. *Ann Bot*., 63: 39-52.

Slamet, I.H., & Tahardi, S. (1988). The Effect of Shading and Nitrogen Fertilization on the Flowering of *Stevia rebaudiana* Bertoni. *Menara Perkebunan*, 56: 34-37.

Shock, C.C. (1982). Rebaudi's Stevia: Natural Non-Caloric Sweeteners. *California Agric*. 36: 4-5.

Simlat, M., Ślęzak, P., Moś, M., Warchoł, M., Skrzypek, E., & Ptak, A. (2016). The Effect of Light Quality on Seed Germination, Seedling Growth and Selected Biochemical Properties of *Stevia rebaudiana* Bertoni. *Scientica Horticulturae*, 211: 295-304.

Singh S.D., & Rao G.P. (2005). *Stevia*: the Herbal Sugar of 21st Century. Sugar *Technol*., 7: 17-24.

Soltani, E., Ghaderi-Far, F., Baskin, & Baskin, J.M. (2015). Problems with Using Mean Germination Time to Calculate Rate of Seed Germination. *Australian J of Botany*, http://dx.doi.org/10.1071/BT15133. Accessed Sept. 3, 2021.

Stevia-Store. (2015). Stevia Seeds. Stevia-Store.com. Updated 2021. Accessed Nov. 11, 2017. http://steviaeirete.com/manual.html

Strauss, S. (1995). The Perfect Sweetener? *Technol. Rev*., 98(8): 18-20.

Taiariol, D.R. (2004). Characterization of the *Stevia rebaudiana* Bert. Accessed July 27, 2021. www.monografias.com/trabajos 13/stevia/stevia html.

Takahashi, L., Melges, E., & Carneiro, J.P. (1996). Germination Performance of Stevia Seeds Under Different Temperatures. *Revista Brasileira de Sementes*. 18: 1-5.

Tanaka, O. (1985). Application of C-nuclear Magnetic Resonance Spectrometry to Structural Studies on Glycosides: Saponins of *Panax* spp. and Natural Sweet Glycosides. *Yakugaku Zasshi,* 105: 323-351.

Tavarini, S., Passera, & Angelini, L.G. (2018). Crop and Steviol Glycoside Improvement. In: Stevia by Breeding, in *Steviol Glycosides: Cultivation, Processing, Analysis and Applications in Food.* p 1-31.

Taware, A.S., Harke, S.N., Mukadam, D.S., Chavan, A.M., & Taware, S.D. (2010). Effect of Different Extracts of Callus and Plantlets of *Stevia rebaudiana* (Bertoni) on Seed Germination of Some Agricultural Crops. *African Journal of Biotechnology*, 9(40): 6675-6683.

Uçar, E., Özyiğit, Y., & Turgut, K. (2016). The Effects of Light and Temperature on Germination of Stevia (*Stevia rebaudiana* BERT.) seeds. *Türkiye Tarımsal Araştırmalar Dergisi*, 3(1): 37-40.

U.S. Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition. (2019). Import Alert 45-06. Silver Spring, MD: U.S. Food and Drug Administration. Accessed July 14, 2021.

Walters, C. (1998). Understanding the Mechanisms and Kinetics of Seed Aging. *Seed Science Research*, 8(2): 223-244. doi:10.1017/S096025850000413X

Walters, C., Hill, L.M., & Wheeler, L.J. (2005a). Dying While Dry: Kinetics and Mechanisms of Deterioration in Desiccated Organisms. *Integr Comp Biol,* 45: 751-758.

Walters, C., Wheeler, L.J., & Grotenhuis, J.M. (2005b). Longevity of Seeds Stored in a Genebank: Species Characteristics. *Seed Science Research*, 15: 1-20.

Table 2.1. Seed traits of 12 genotypes of stevia.

	Preliminary germination and viability					
Genotype	**Wet weight (g)**	**Dry weight (g)**	**% MC**	**Seed Estimate**	**Final Germ Percent**	**Viability Percent**
1	5.20	4.70	9.62	15510	81.67	85.00
2	5.30	4.50	15.09	14850	75.00	80.00
3	4.80	4.20	12.50	13860	81.67	80.00
4	3.70	3.00	18.92	9900	86.67	90.00
5	5.40	4.90	9.26	16170	86.33	85.00
6	5.10	4.40	13.73	14520	72.33	70.00
7	3.80	3.10	18.42	10230	72.67	75.00
8	4.20	3.70	11.90	12210	73.67	80.00
9	5.50	4.80	12.73	15840	68.67	65.00
10	4.40	3.80	13.64	12540	63.67	60.00
11	N/A	N/A	N/A	8000	75.67	85.00
12	N/A	N/A	N/A	8000	78.67	90.00

Data are means of two replications of 12 genotypes tested at 72 days post-harvest.

Table 2.2. Analysis of variance for seed germination percentage in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	16768	1524	9.433	***
Temperature	1	80319	80319	497.05	***
Genotype: Temperature	11	4146	377	2.33	**
Residuals	1704	275353	162		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.3. Means for seed germination traits of 12 genotypes of stevia. Abbreviations: cold temperature (CT), room temperature (RT), germination (germ.), coefficient of velocity (coeff. of vel.) and emergence (emerg.) rate index.

Genotype	Storage Temp	Days in Storage	Final Germ. %	Mean Germ %	First Germ. Time	Last Germ. Time	Time Spread of Germ.	T50	Mean Germ. Time	Mean Germ. Rate	Germ. Speed	Coeff. of Vel. of Germ.	Emerg. Rate Index	Viability %
		72	80.33	3.83	2.33	10.00	7.67	10.74	11.94	8.38	7.57	8.38	404.33	95.00
		498	73.33	3.49	2.00	10.33	8.33	11.86	12.61	7.93	6.63	7.93	348.00	85.00
		862	72.00	3.43	2.33	9.33	7.00	11.36	12.34	8.12	6.48	8.12	344.00	80.00
		1226	60.33	2.87	2.33	10.33	8.00	12.83	13.59	7.37	4.98	7.37	254.00	65.00
	CT	1591	53.00	2.52	2.67	10.67	8.00	11.92	12.88	7.76	4.63	7.76	241.00	55.00
		72	80.33	3.83	2.67	9.33	6.67	10.82	11.86	8.48	7.60	8.48	412.33	90.00
		498	70.67	3.37	2.67	10.00	7.33	11.46	12.37	8.09	6.35	8.09	340.33	65.00
		862	44.67	2.13	3.33	9.67	6.33	11.23	12.34	8.11	3.93	8.11	216.00	45.00
		1226	30.67	1.46	3.33	11.00	7.67	13.06	13.83	7.24	2.44	7.24	124.67	35.00
1	RT	1591	26.33	1.25	3.00	10.00	7.00	11.33	12.42	8.11	2.39	8.11	127.00	20.00
		72	76.33	3.63	2.33	9.00	6.67	11.27	12.19	8.20	6.87	8.20	374.67	70.00
		498	73.33	3.49	2.33	10.67	8.33	11.53	12.53	7.99	6.54	7.99	347.33	80.00
		862	70.00	3.33	2.33	9.67	7.33	11.03	12.10	8.27	6.54	8.27	346.67	80.00
		1226	60.33	2.87	2.67	11.00	8.33	12.40	13.34	7.52	5.04	7.52	261.00	60.00
	CT	1591	50.33	2.40	2.33	10.00	7.67	12.06	12.88	7.78	4.36	7.78	230.33	50.00
		72	74.00	3.52	3.00	9.33	6.33	11.21	12.24	8.17	6.64	8.17	361.00	60.00
		498	64.00	3.05	2.67	9.67	7.00	11.44	12.38	8.08	5.73	8.08	307.67	60.00
		862	49.00	2.33	2.33	10.00	7.67	11.57	12.56	7.96	4.32	7.96	231.00	45.00
		1226	32.67	1.56	3.33	10.33	7.00	11.98	13.07	7.69	2.72	7.69	145.00	25.00
2	RT	1591	20.67	0.98	3.67	10.00	6.33	11.89	12.98	7.73	1.73	7.73	93.33	15.00
		72	83.33	3.97	2.00	9.67	7.67	10.32	11.27	8.90	8.69	8.90	446.67	85.00
		498	72.33	3.44	2.33	10.33	8.00	11.80	12.59	7.95	6.49	7.95	341.00	75.00
		862	71.67	3.41	2.33	10.00	7.67	11.14	12.16	8.23	6.65	8.23	352.67	75.00
		1226	70.33	3.35	2.00	10.33	8.33	12.13	13.05	7.67	6.09	7.67	314.67	60.00
	CT	1591	48.33	2.30	3.00	10.00	7.00	11.86	12.75	7.84	4.14	7.84	223.33	45.00
		72	80.67	3.84	2.00	9.33	7.33	9.32	10.48	9.54	8.96	9.54	464.33	80.00
		498	70.33	3.35	2.33	9.67	7.33	10.98	11.91	8.41	6.65	8.41	354.33	60.00
		862	51.33	2.44	2.33	10.33	8.00	11.97	12.73	7.86	4.51	7.86	238.00	40.00
		1226	36.33	1.73	3.33	9.67	6.33	13.01	13.51	7.43	2.96	7.43	155.67	30.00
3	RT	1591	18.00	0.86	3.67	9.00	5.33	11.67	12.66	7.90	1.52	7.90	84.33	15.00
		72	85.33	4.06	2.00	10.00	8.00	10.64	11.76	8.50	8.17	8.50	436.33	65.00
		498	79.33	3.78	2.67	10.33	7.67	12.15	13.00	7.71	6.80	7.71	356.00	85.00
		862	69.33	3.30	2.33	10.00	7.67	11.04	12.11	8.27	6.40	8.27	343.33	85.00
		1226	63.67	3.03	2.33	10.67	8.33	13.37	13.94	7.18	5.22	7.18	257.33	65.00
	CT	1591	55.00	2.62	2.33	10.67	8.33	12.65	13.46	7.44	4.59	7.44	235.33	50.00
		72	83.33	3.97	2.00	10.00	8.00	10.24	11.22	8.92	8.63	8.92	450.00	65.00
		498	66.00	3.14	2.33	9.67	7.33	10.74	11.78	8.50	6.28	8.50	338.33	55.00
		862	52.33	2.49	2.33	10.33	8.00	12.04	12.88	7.79	4.54	7.79	238.67	45.00
		1226	43.00	2.05	2.67	10.00	7.33	11.98	12.73	7.86	3.76	7.86	199.33	35.00
4	RT	1591	33.67	1.60	2.33	9.67	7.33	11.86	12.60	7.97	3.01	7.97	158.00	25.00

Table 2.3. (Continued).

Genotype	Storage Temp	Days in Storage	Final Germ. %	Mean Germ %	First Germ. Time	Last Germ. Time	Time Spread of Germ.	T50	Mean Germ. Time	Mean Germ. Rate	Germ. Speed	Coeff. of Vel. of Germ.	Emerg. Rate Index	Viability %
		72	89.33	4.25	2.00	10.33	8.33	10.25	11.40	8.77	9.11	8.77	473.33	80.00
		498	72.67	3.46	3.00	10.00	7.00	11.76	12.73	7.86	6.33	7.86	338.00	75.00
		862	69.33	3.30	2.33	11.00	8.67	11.59	12.71	7.88	6.16	7.88	321.67	75.00
		1226	61.00	2.90	2.67	10.00	7.33	12.03	13.06	7.66	5.15	7.66	272.67	65.00
	CT	1591	53.00	2.52	2.33	10.67	8.33	12.08	13.02	7.69	4.54	7.69	237.00	50.00
		72	87.33	4.16	2.00	10.67	8.67	11.42	12.30	8.15	8.19	8.15	423.00	75.00
		498	62.00	2.95	2.00	10.00	8.00	11.92	12.77	7.83	5.51	7.83	286.33	65.00
		862	53.00	2.52	2.67	9.67	7.00	11.28	12.30	8.13	4.74	8.13	257.00	45.00
		1226	40.33	1.92	3.67	10.00	6.33	13.00	13.92	7.19	3.09	7.19	163.00	25.00
5	RT	1591	28.33	1.35	3.00	9.67	6.67	12.52	13.08	7.66	2.37	7.66	127.00	20.00
		72	75.67	3.60	2.00	9.67	7.67	10.95	12.00	8.36	7.18	8.36	379.33	85.00
		498	79.00	3.76	2.33	10.33	8.00	12.13	12.94	7.73	6.82	7.73	357.67	75.00
		862	67.67	3.22	2.67	10.33	7.67	11.38	12.59	7.95	5.92	7.95	318.33	80.00
		1226	63.67	3.03	2.67	10.67	8.00	12.32	13.23	7.56	5.36	7.56	279.00	70.00
	CT	1591	55.00	2.62	2.33	10.33	8.00	12.09	12.96	7.73	4.73	7.73	249.00	40.00
		72	70.00	3.33	2.33	9.67	7.33	11.48	12.36	8.12	6.38	8.12	337.67	70.00
		498	61.33	2.92	3.00	10.00	7.00	11.73	12.60	7.94	5.33	7.94	288.00	60.00
		862	51.00	2.43	3.00	9.67	6.67	11.29	12.38	8.08	4.46	8.08	245.33	50.00
		1226	39.33	1.87	2.67	9.67	7.00	12.43	12.97	7.71	3.32	7.71	177.33	30.00
6	RT	1591	19.67	0.94	2.67	9.00	6.33	11.11	11.91	8.40	1.83	8.40	99.33	15.00
		72	72.33	3.44	2.33	10.00	7.67	12.23	12.92	7.76	6.38	7.76	329.00	80.00
		498	76.00	3.62	2.67	10.33	7.67	11.71	12.66	7.90	6.72	7.90	354.67	80.00
		862	65.33	3.11	2.67	10.67	8.00	12.27	13.19	7.59	5.48	7.59	288.00	75.00
		1226	59.00	2.81	2.33	9.67	7.33	11.51	12.34	8.11	5.33	8.11	284.33	65.00
	CT	1591	57.67	2.75	2.67	10.33	7.67	11.97	12.82	7.81	4.98	7.81	264.67	50.00
		72	68.33	3.25	2.33	9.33	7.00	11.91	12.60	7.94	6.07	7.94	321.33	70.00
		498	61.33	2.92	2.33	10.33	8.00	10.89	12.07	8.31	5.70	8.31	304.67	60.00
		862	45.00	2.14	2.33	9.00	6.67	10.17	11.14	8.99	4.53	8.99	244.33	35.00
		1226	30.33	1.44	2.67	10.33	7.67	12.01	12.75	7.87	2.67	7.87	140.33	20.00
7	RT	1591	18.33	0.87	3.33	9.33	6.00	10.78	11.85	8.48	1.69	8.48	93.67	15.00
		72	76.33	3.63	2.00	10.33	8.33	11.47	12.29	8.14	7.13	8.14	370.67	85.00
		498	70.67	3.37	2.33	9.33	7.00	11.31	12.27	8.15	6.38	8.15	343.33	70.00
		862	70.33	3.35	2.00	9.33	7.33	11.56	12.35	8.10	6.44	8.10	339.00	70.00
		1226	58.67	2.79	2.67	10.33	7.67	12.30	13.23	7.56	4.92	7.56	257.33	60.00
	CT	1591	52.00	2.48	2.33	10.33	8.00	11.47	12.52	8.01	4.70	8.01	246.00	45.00
		72	74.33	3.54	2.33	9.00	6.67	11.19	12.07	8.31	6.92	8.31	369.33	85.00
		498	55.33	2.63	2.67	10.00	7.33	11.87	12.80	7.82	4.70	7.82	254.00	65.00
		862	51.00	2.43	2.67	9.67	7.00	11.40	12.40	8.08	4.52	8.08	244.33	50.00
		1226	32.33	1.54	3.67	9.00	5.33	12.14	12.91	7.76	2.64	7.76	146.67	30.00
8	RT	1591	21.33	1.02	2.33	9.00	6.67	10.60	11.31	8.91	2.12	8.91	113.33	20.00

Table 2.3. (Continued).

Genotype	Storage Temp	Days in Storage	Final Germ. %	Mean Germ %	First Germ. Time	Last Germ. Time	Time Spread of Germ.	T50	Mean Germ. Time	Mean Germ. Rate	Germ. Speed	Coeff. of Vel. of Germ.	Emerg. Rate Index	Viability %
		72	73.67	3.51	2.00	9.00	7.00	10.64	11.64	8.60	7.31	8.60	381.67	80.00
		498	70.00	3.33	2.67	9.67	7.00	11.80	12.65	7.91	6.12	7.91	326.67	65.00
		862	67.33	3.21	2.33	9.67	7.33	11.21	12.19	8.21	6.26	8.21	330.67	75.00
		1226	64.67	3.08	2.67	10.67	8.00	12.19	13.24	7.56	5.43	7.56	283.00	70.00
	CT	1591	58.33	2.78	2.00	10.33	8.33	12.15	12.91	7.76	5.13	7.76	265.00	55.00
		72	68.33	3.25	2.00	9.67	7.67	10.27	11.40	8.77	6.99	8.77	362.33	75.00
		498	62.00	2.95	2.67	10.33	7.67	11.17	12.34	8.11	5.56	8.11	299.33	55.00
		862	52.67	2.51	2.33	9.33	7.00	11.16	12.07	8.31	4.88	8.31	261.33	55.00
		1226	43.67	2.08	2.33	10.33	8.00	12.28	12.94	7.75	3.76	7.75	198.33	40.00
9	RT	1591	30.00	1.43	2.67	9.33	6.67	10.53	11.55	8.68	2.87	8.68	156.00	25.00
		72	66.33	3.16	2.00	9.33	7.33	10.50	11.30	8.88	6.82	8.88	354.33	75.00
		498	64.33	3.06	3.00	11.00	8.00	11.69	12.74	7.85	5.56	7.85	297.33	75.00
		862	64.00	3.05	2.33	9.67	7.33	11.38	12.19	8.23	5.87	8.23	313.67	70.00
		1226	62.67	2.98	2.67	10.00	7.33	12.70	13.50	7.42	5.14	7.42	265.67	60.00
	CT	1591	59.00	2.81	2.00	10.00	8.00	11.28	12.38	8.08	5.37	8.08	283.67	50.00
		72	63.33	3.02	2.33	9.67	7.33	10.71	11.86	8.45	5.99	8.45	322.00	80.00
		498	59.67	2.84	2.33	10.00	7.67	11.05	12.05	8.30	5.56	8.30	296.67	60.00
		862	51.67	2.46	2.00	9.33	7.33	10.27	11.34	8.82	5.10	8.82	275.33	60.00
		1226	42.33	2.02	3.33	9.67	6.33	12.34	13.10	7.64	3.47	7.64	188.33	50.00
10	RT	1591	35.33	1.68	2.67	9.00	6.33	11.36	11.96	8.37	3.25	8.37	177.00	30.00
		72	84.00	4.00	2.00	10.33	8.33	10.68	11.77	8.50	8.32	8.50	429.67	95.00
		498	74.67	3.56	2.33	10.67	8.33	11.91	12.93	7.74	6.50	7.74	338.67	90.00
		862	75.00	3.57	2.00	10.00	8.00	11.94	12.79	7.82	6.71	7.82	345.33	80.00
		1226	70.00	3.33	2.33	11.00	8.67	12.81	13.47	7.43	5.88	7.43	297.67	75.00
	CT	1591	65.00	3.10	2.33	10.00	7.67	11.67	12.76	7.84	5.82	7.84	300.33	60.00
		72	79.00	3.76	2.33	9.67	7.33	10.27	11.23	8.95	8.09	8.95	425.67	90.00
		498	56.67	2.70	3.00	9.67	6.67	11.68	12.60	7.95	4.92	7.95	266.67	80.00
		862	53.67	2.56	2.33	10.33	8.00	11.42	12.40	8.06	4.87	8.06	257.33	65.00
		1226	42.67	2.03	3.00	10.00	7.00	13.29	13.81	7.25	3.35	7.25	174.33	50.00
11	RT	1591	34.00	1.62	2.67	9.33	6.67	11.06	12.03	8.32	3.10	8.32	169.33	35.00
		72	76.00	3.62	2.33	9.33	7.00	11.25	12.09	8.28	7.09	8.28	376.67	90.00
		498	70.00	3.33	2.00	10.00	8.00	12.38	13.08	7.66	6.01	7.66	311.67	85.00
		862	70.67	3.37	2.00	10.00	8.00	11.28	12.31	8.12	6.46	8.12	342.33	85.00
		1226	66.67	3.17	2.33	10.67	8.33	13.00	13.63	7.34	5.51	7.34	278.67	80.00
	CT	1591	60.67	2.89	2.33	10.00	7.67	11.35	12.50	8.00	5.49	8.00	288.00	70.00
		72	74.00	3.52	2.33	9.33	7.00	11.19	12.11	8.29	6.83	8.29	366.67	90.00
		498	66.00	3.14	2.00	10.33	8.33	12.17	12.95	7.73	5.82	7.73	298.67	80.00
		862	65.33	3.11	3.00	10.67	7.67	12.19	13.28	7.53	5.41	7.53	284.33	70.00
		1226	57.00	2.71	2.67	10.33	7.67	12.35	13.33	7.51	4.74	7.51	247.00	55.00
12	RT	1591	45.00	2.14	2.00	9.33	7.33	10.42	11.49	8.72	4.39	8.72	236.67	40.00

Table 2.4. Analysis of variance for seed germination rate in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	0.00225	0.0002	9.541	***
Temperature	1	0.00042	0.00042	19.74	***
Genotype: Temperature	11	0.00063	0.00005	2.66	**
Residuals	1704	0.03653	0.0002		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.5. Analysis of variance for seed germination time in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	51.4	4.67	9.04	***
Temperature	1	8.6	8.62	16.68	***
Genotype: Temperature	11	14.8	1.348	2.61	**
Residuals	1704	880.3	0.52		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.6. Analysis of variance for seed germination rate in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	16768	1524	9.43	***
Temperature	1	80319	80319	497.05	***
Genotype: Temperature	11	4146	377	2.33	**
Residuals	1704	275353	162		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.7. Analysis of variance for seed germination speed in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	22.5	2.05	9.54	***
Temperature	1	4.2	4.23	19.74	***
Genotype: Temperature	11	6.3	0.57	2.66	**
Residuals	1704	365.3	0.21		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.8. Analysis of variance for seed germination time in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	2976	270.5	9.23	***
Temperature	1	511	511.1	17.44	***
Genotype: Temperature	11	901	81.9	2.80	**
Residuals	1704	49938	29.3		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.9. Analysis of variance for seed germination standard deviation in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	13.35	1.21	9.53	***
Temperature	1	2.40	2.40	18.81	***
Genotype: Temperature	11	3.97	0.36	2.83	**
Residuals	1704	217.03	0.13		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Table 2.10. Analysis of variance for seed germination coefficient of velocity in 12 genotypes of stevia.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square Error	F Value	P(>F)
Genotype	11	36.6	3.32	6.85	***
Temperature	1	4.2	8.71	8.71	**
Genotype: Temperature	11	10.2	0.93	1.92	*
Residuals	1704	827.1	0.49		

Significance: codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05'

Figure 2.1. Final germination percent for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

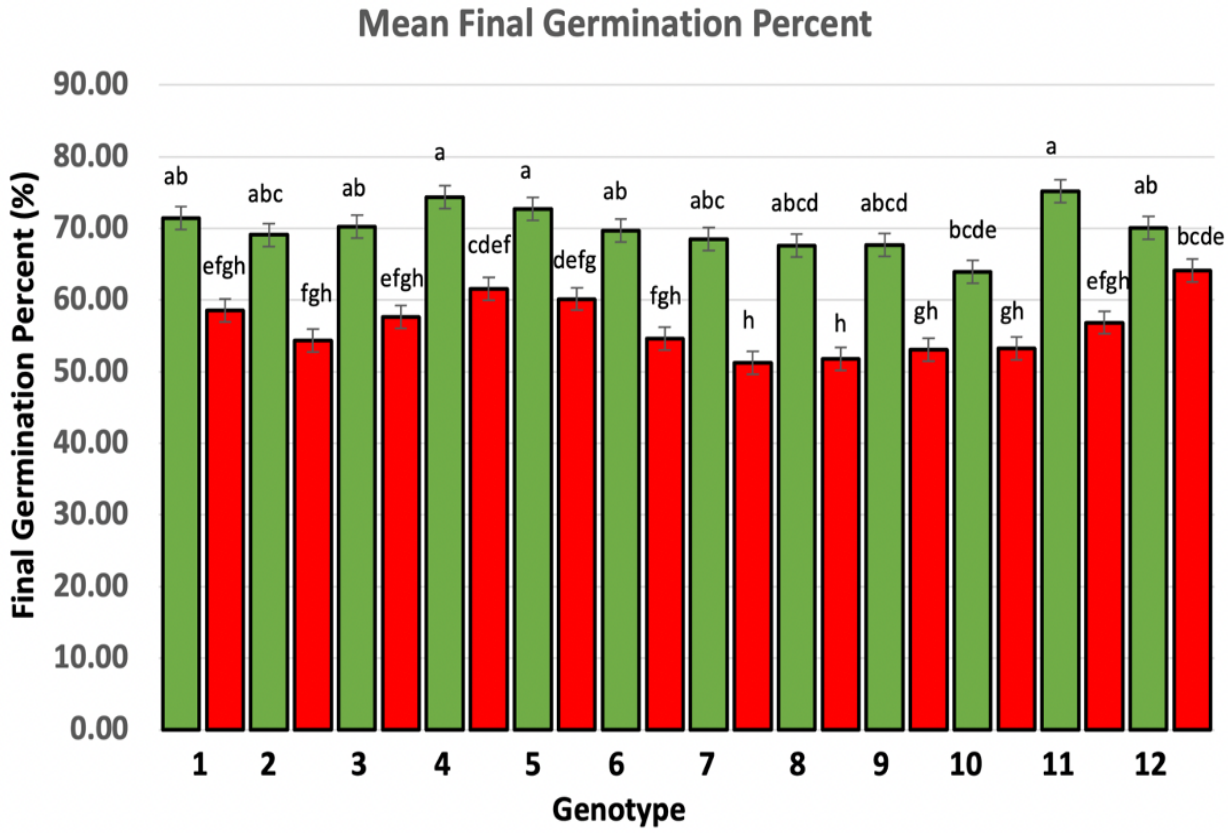


Figure 2.2. Mean germination rate for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

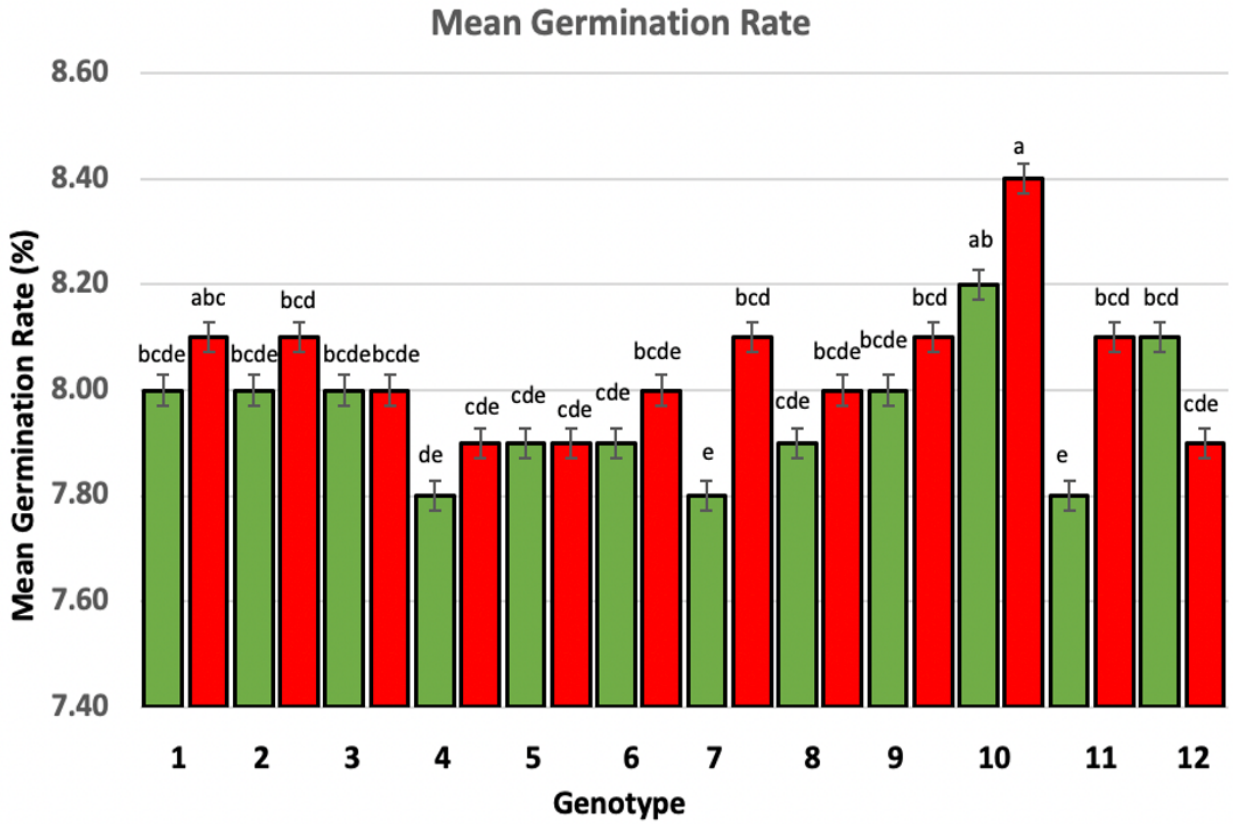


Figure 2.3. Mean germination time for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

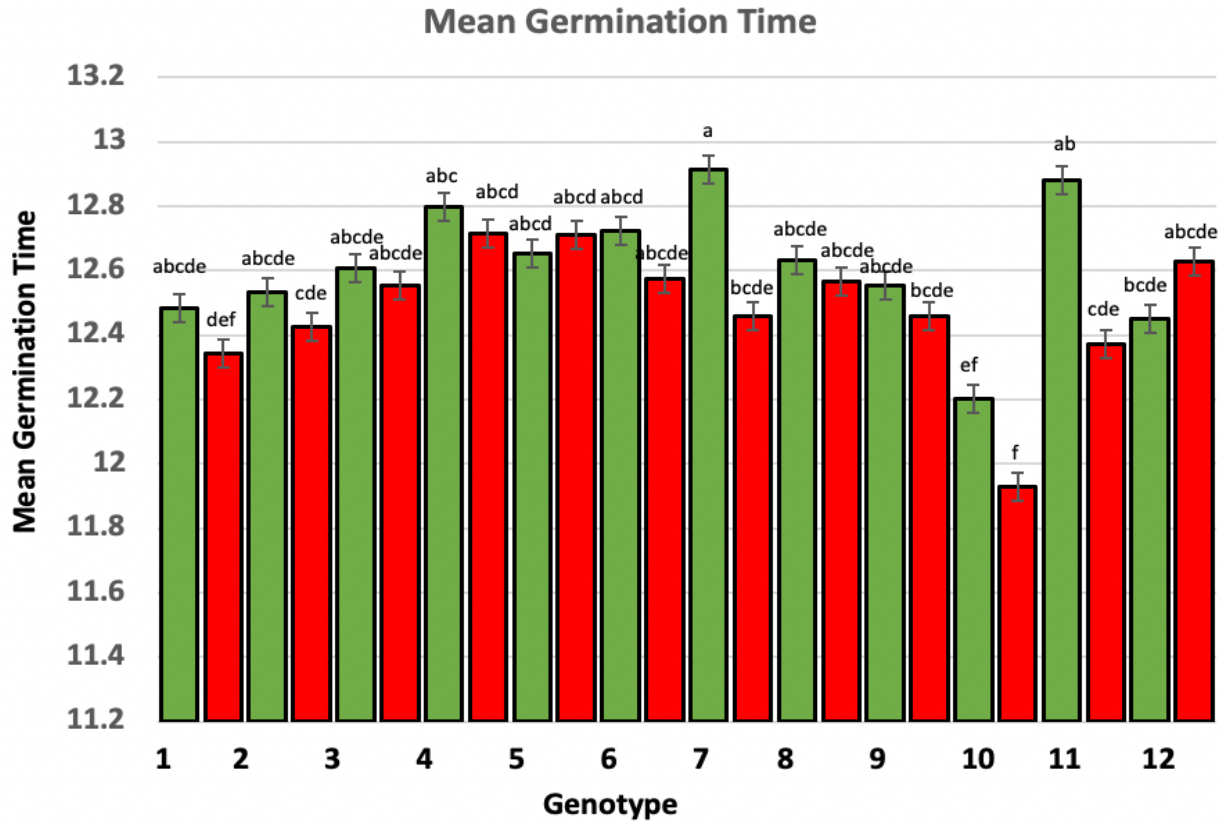


Figure 2.4. Germination rate for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

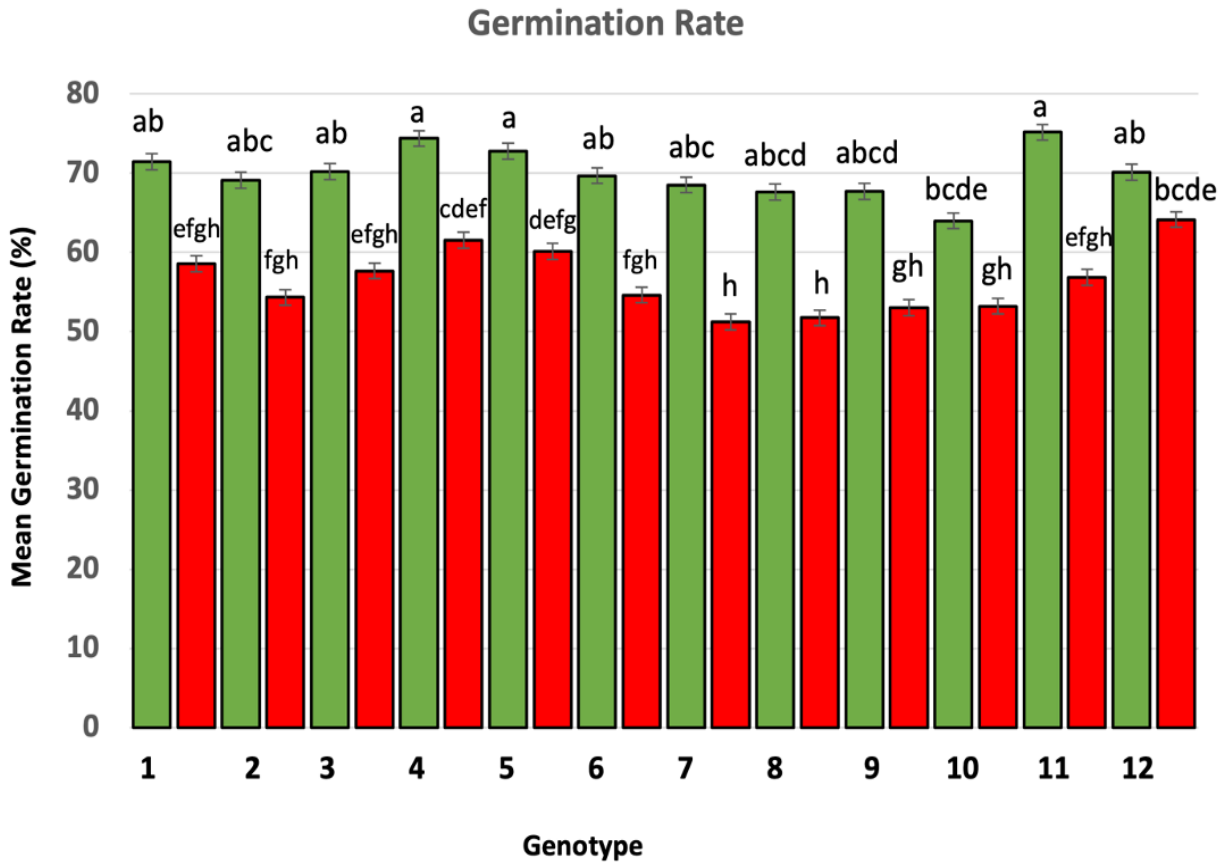


Figure 2.5. Germination speed for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

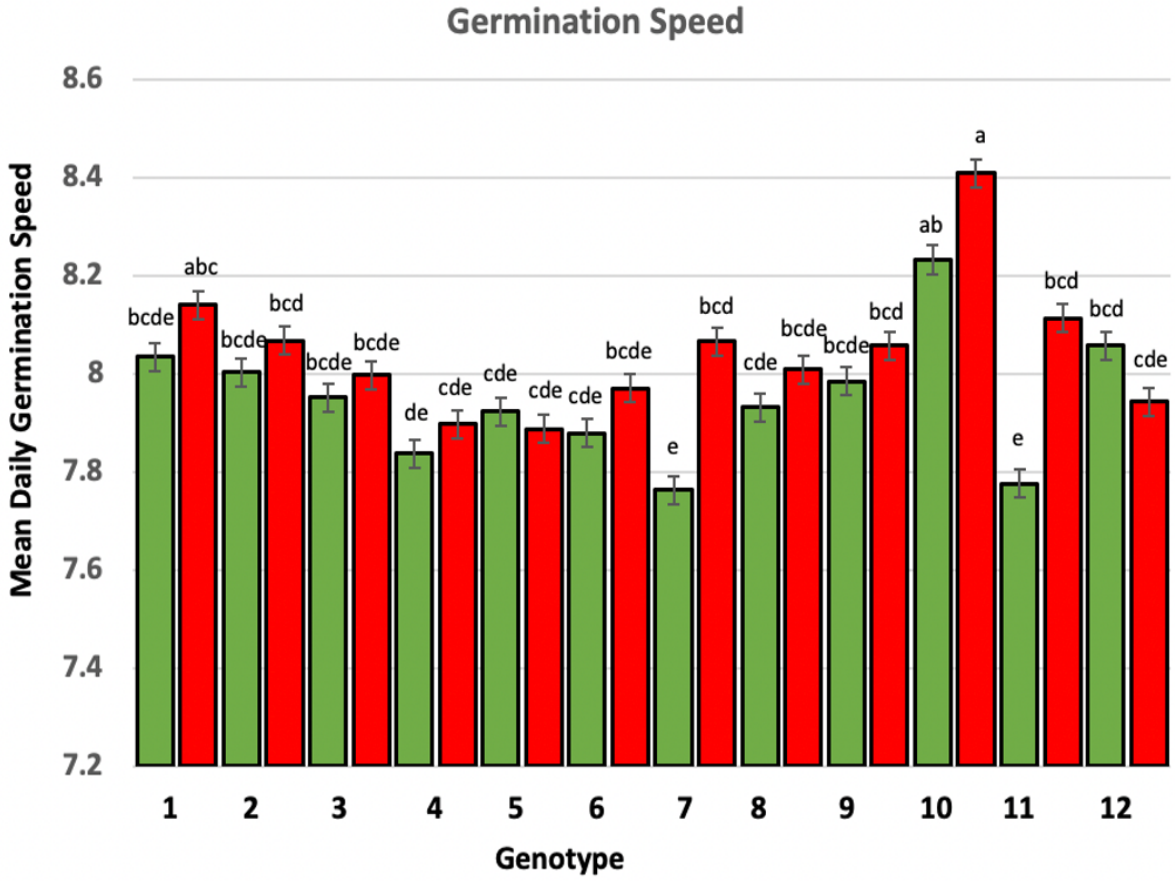


Figure 2.6. Variance of mean germination time for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

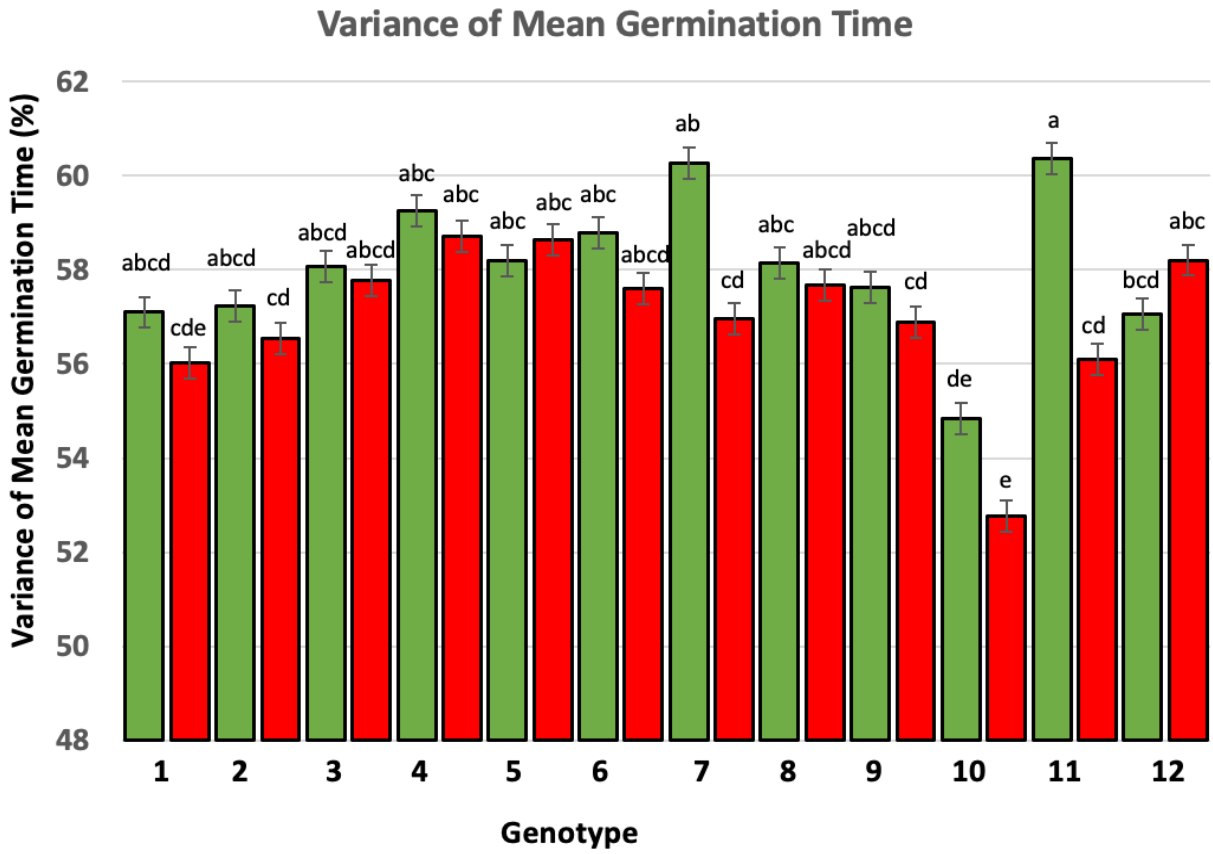


Figure 2.7. Germination standard deviation for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

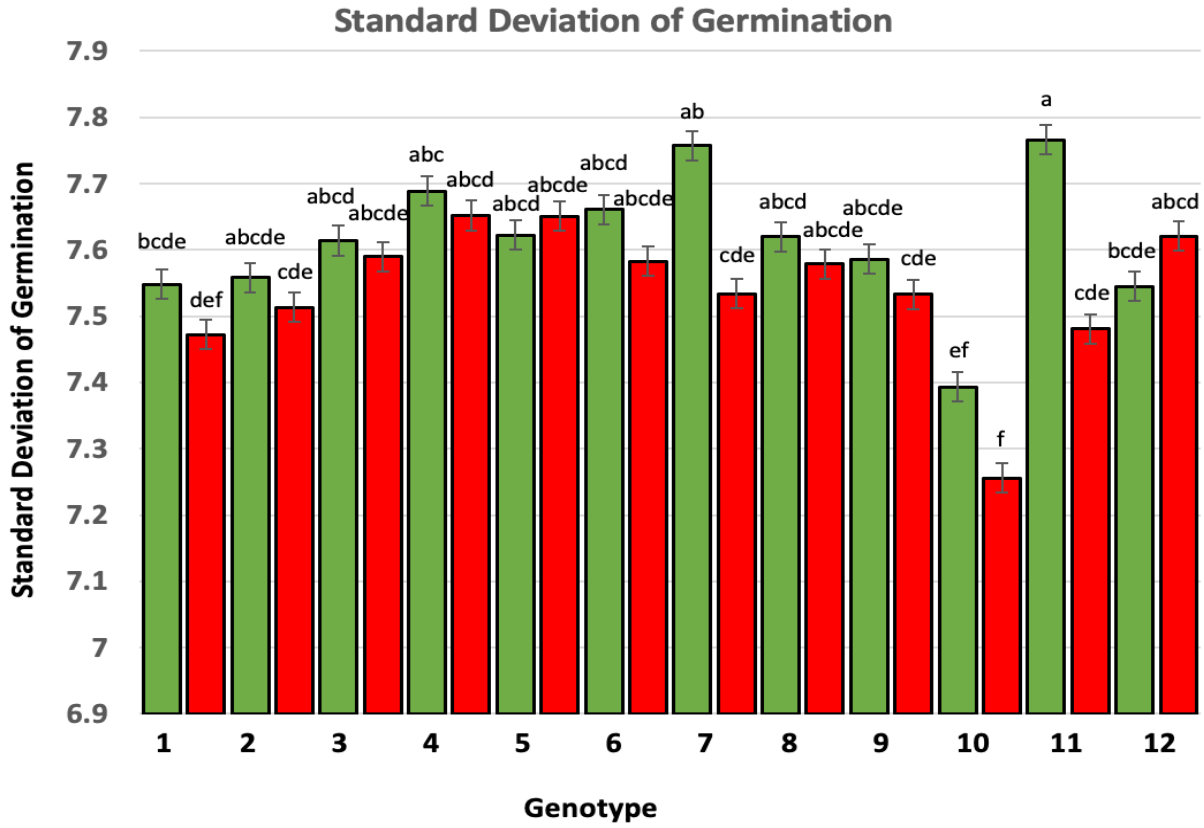


Figure 2.8. Coefficient of germination velocity for genotype 1-12 subjected to two temperatures. Cold room temperature (green) and room temperature (red), standard error bars are present for each reported genotype mean. Letters indicate significance level analyzed via Tukey's HSD test, $p<0.05$.

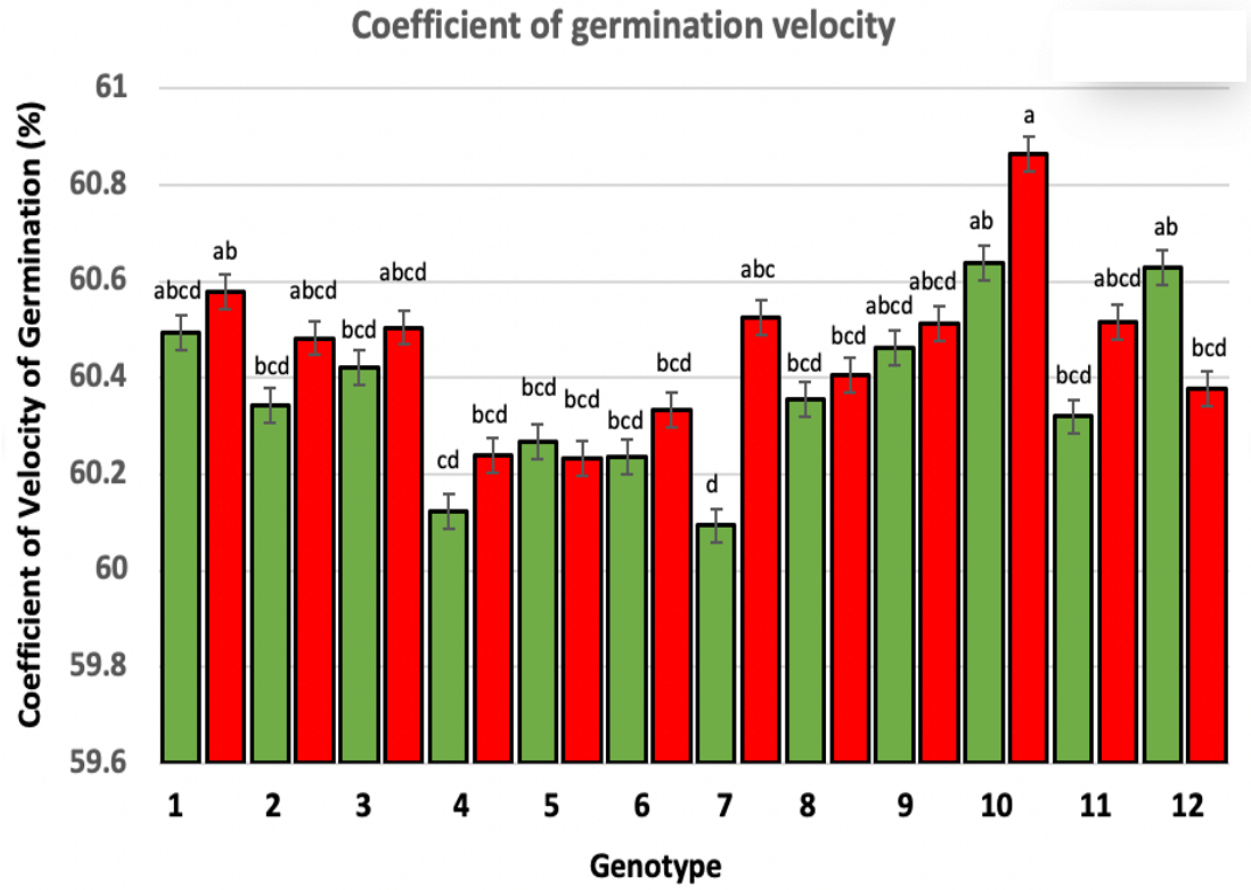


Figure 2.9. Genotype 1 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

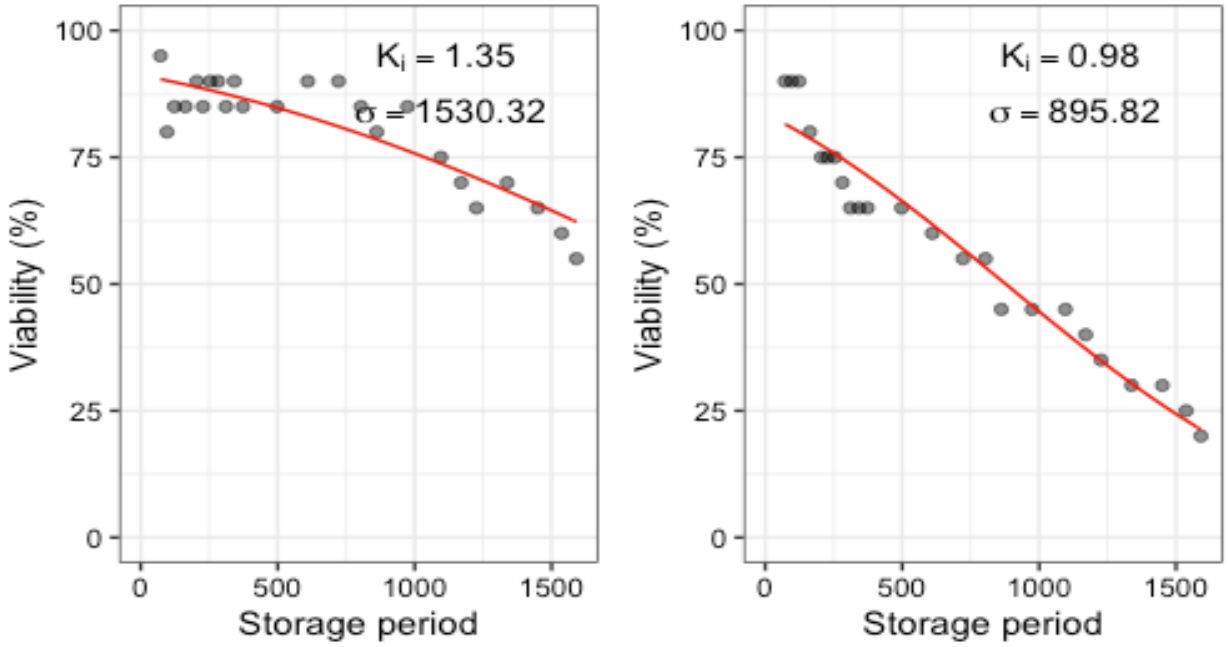


Figure 2.10. Genotype 2 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

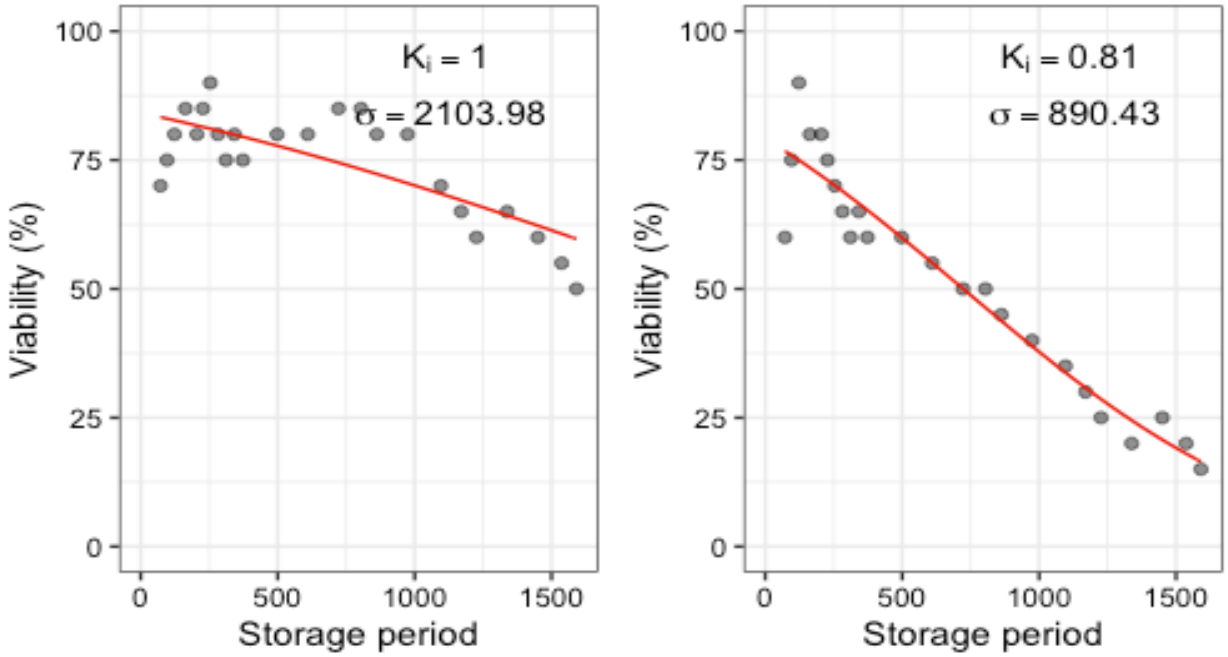


Figure 2.11. Genotype 3 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

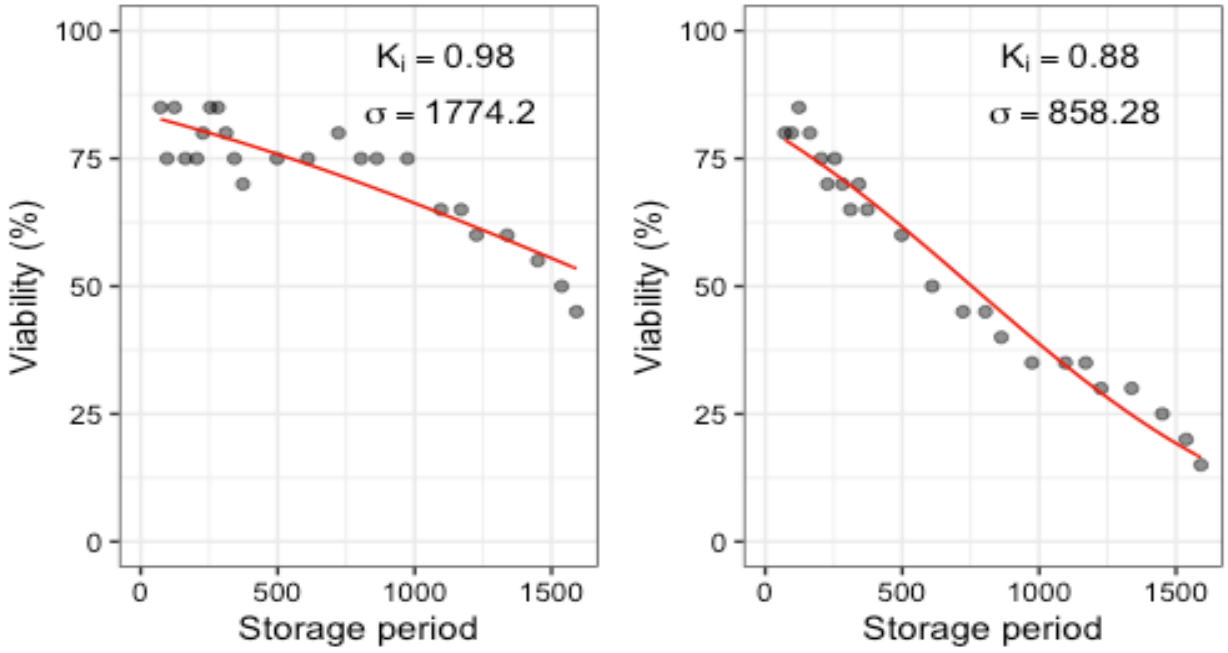


Figure 2.12. Genotype 4 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

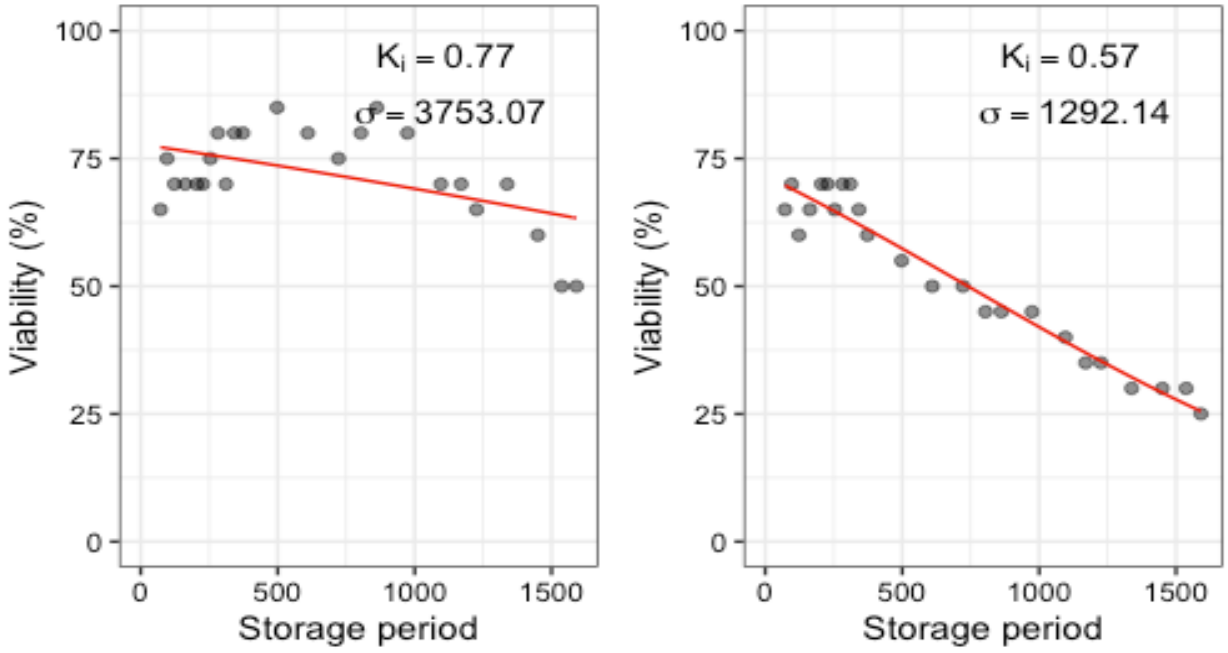


Figure 2.13. Genotype 5 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

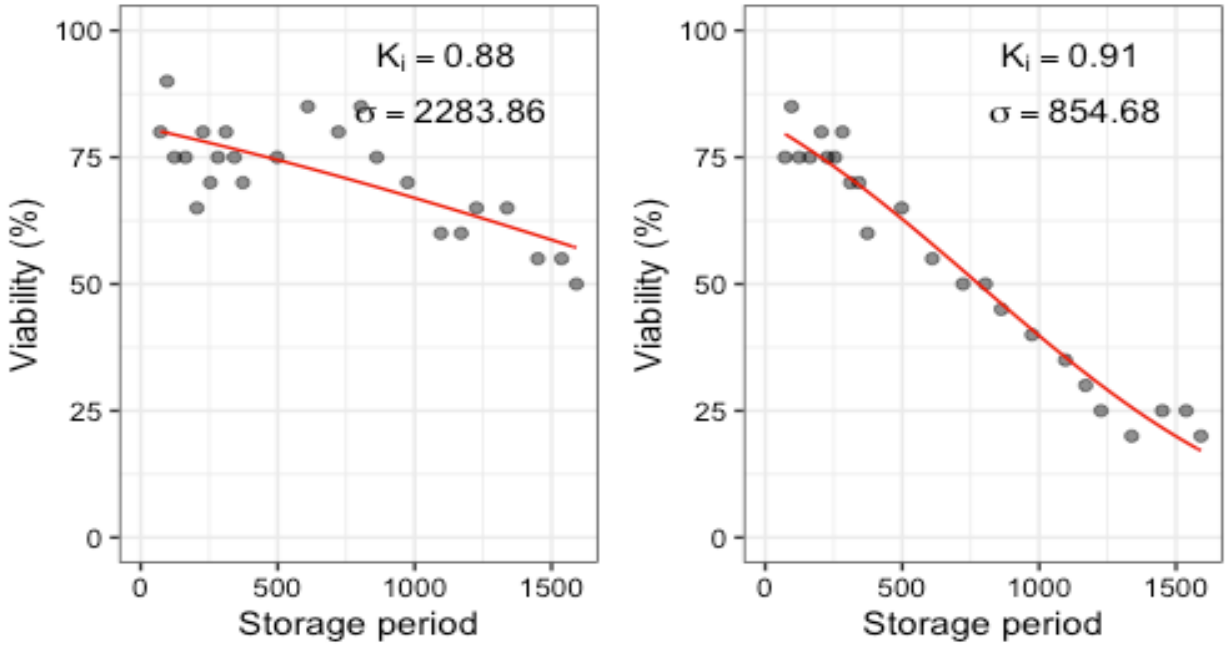


Figure 2.14. Genotype 6 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

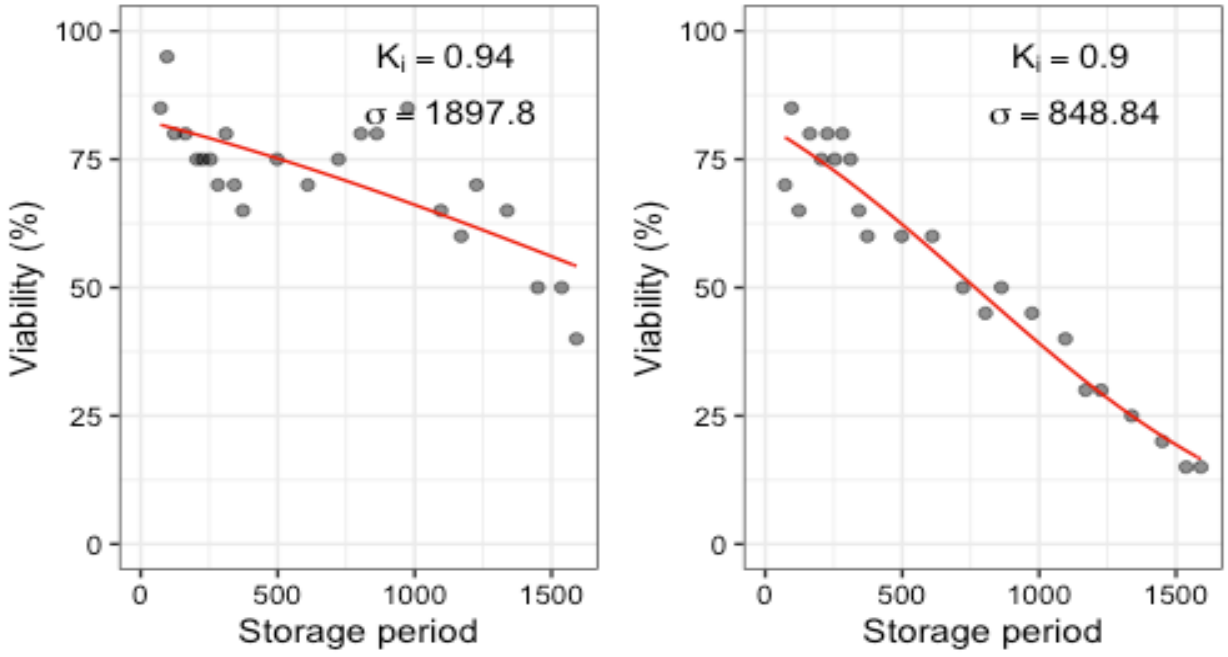


Figure 2.15. Genotype 7 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

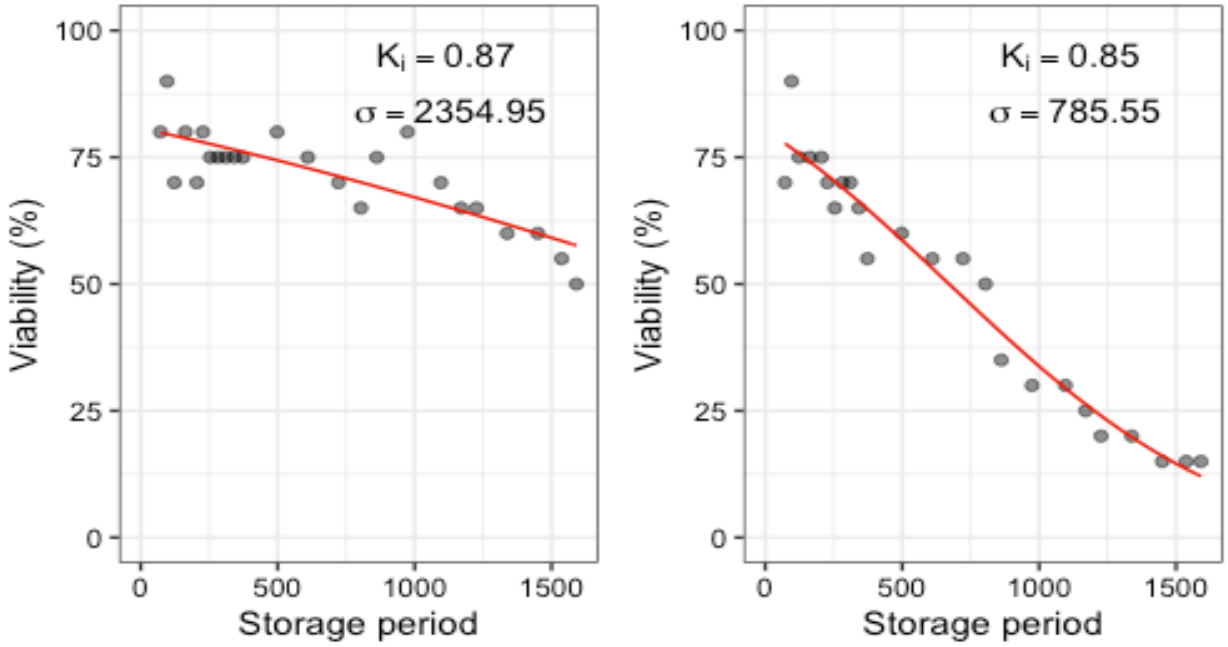


Figure 2.16. Genotype 8 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

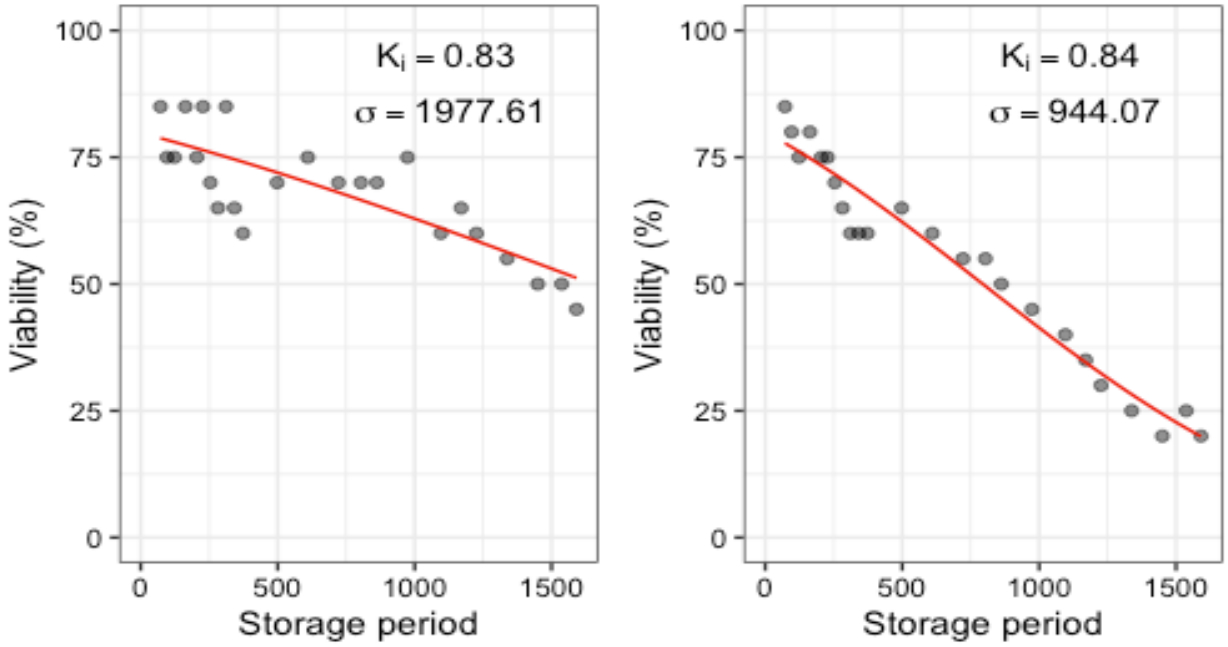


Figure 2.17. Genotype 9 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

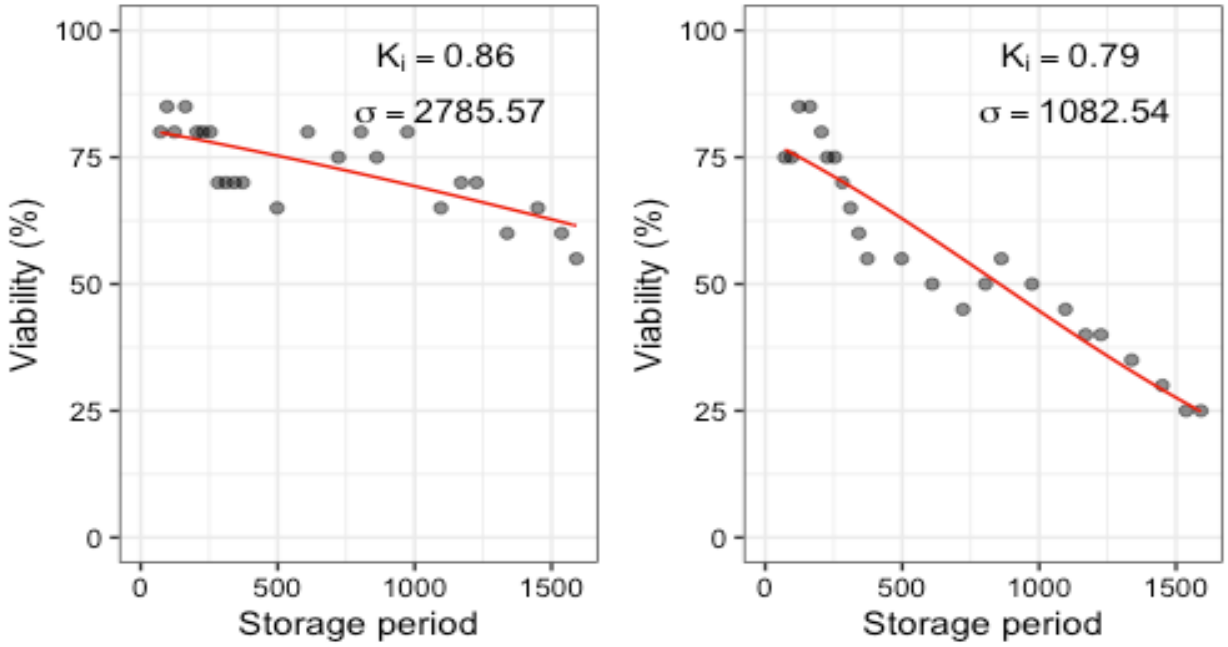


Figure 2.18. Genotype 10 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

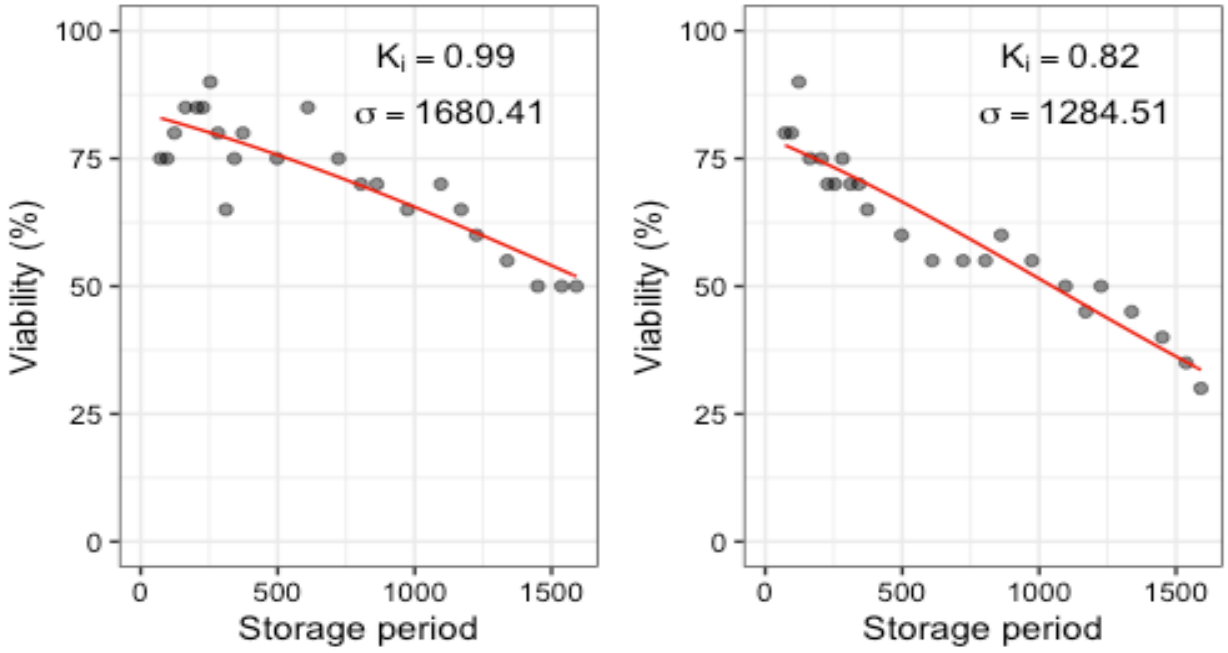


Figure 2.19. Genotype 11 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.

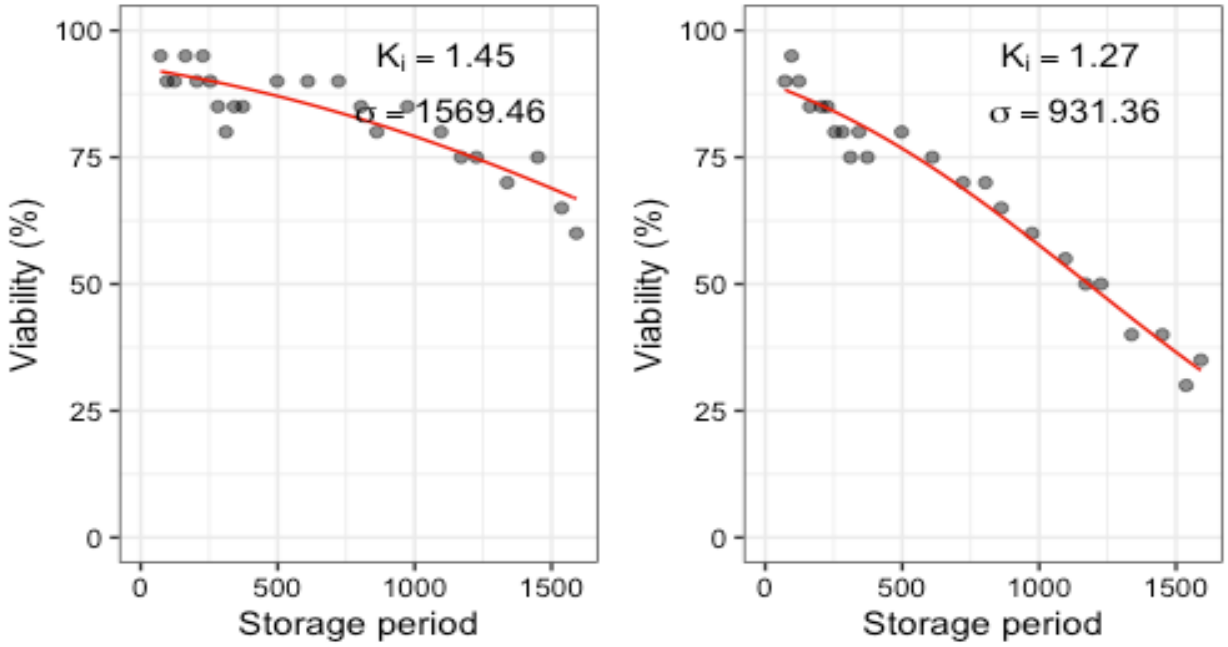
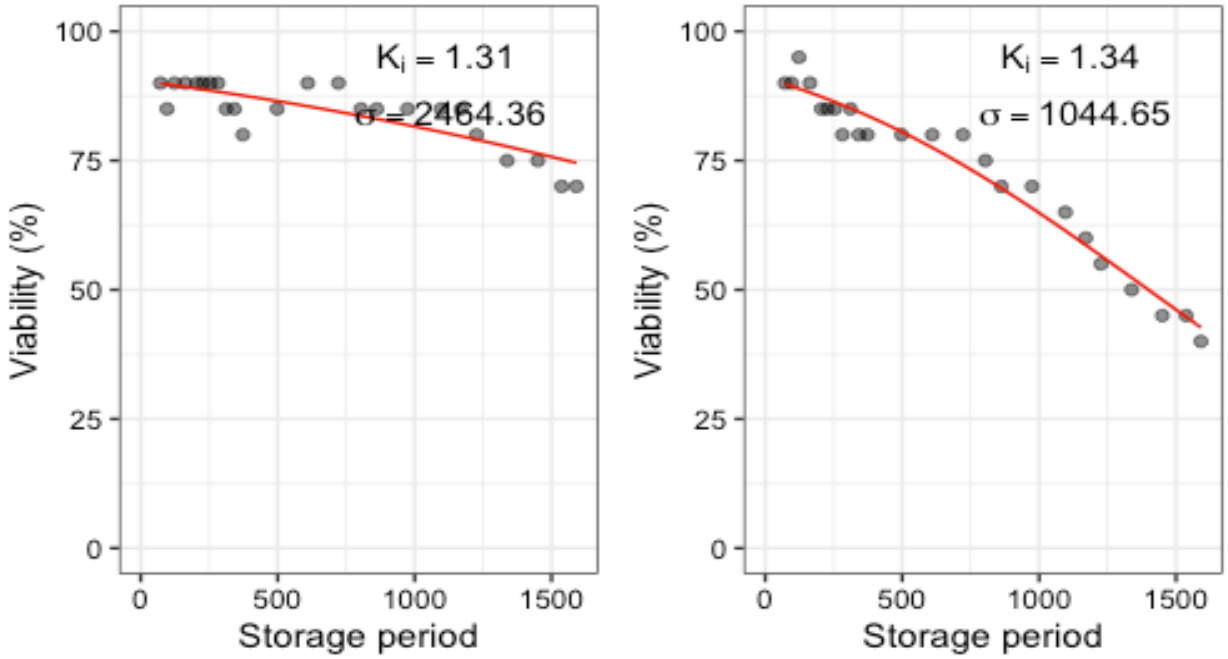


Figure 2.20. Genotype 12 seed survival curve. Cold room storage (left) and room temperature storage (right), initial seed viability, K_i, and days to lose one probit of seed viability, σ. Seed viability (%) listed on y axis and days in storage listed on x axis.



CHAPTER 3

Heritability for Agronomic and Steviol Glycoside Traits in Five Stevia Populations Estimated Using Parent-Offspring Regression

Abstract

Stevia (*Stevia rebaudiana* Bertoni) is becoming an economically valuable species for natural, plant-based sweeteners. In this research, we were interested in understanding the inheritance of traits for use in a breeding program. To do that, we used estimates of phenotypic and genotypic variance from data collected in the field and laboratory from several populations and their offspring (half-sib families). Our results showed that there was significant variation in phenological, agronomic, and steviol glycoside traits in parental and offspring genotypes. Significant differences in objective measurements and subjective ratings were observed in the parents and offspring ($p<0.05$). Furthermore, biomass yield (fresh and dry weight), as well as steviol glycoside concentrations, were significantly different among genotypes in parental and offspring generations ($p<0.05$). We also estimated the narrow-sense heritability by using parent-offspring regression analysis. In addition, to measure the phenological, agronomic, and steviol glycoside traits. open-pollinated populations of *S. rebaudiana* were used in 2015 and 2016 in two locations. Heritability estimates were pooled across open-pollinated populations. Subjective ratings for stem height, branch width, and leaf size had low heritability (0.06, 0.006, and 0.06, respectively). Objective measures of stem height, branch width, leaf length, length width, and leaf area also had low to moderate heritability estimates (0.30, 0.14, 0.22, 0.22, and 0.24, respectively). Dulcoside A, Reb-B, Reb-D, and Reb-E had low to moderate heritability (0.26, 0.08, 0.40, and 0.34, respectively). Steviolbioside and rubusoside also had low heritability estimates (0.06, and 0.24, respectively). Reb-A, Reb-C, stevioside, and total steviol glycosides

(TSG) had higher heritability estimates (0.56, 0.50, 0.22, 0.24, respectively). Pearson correlations comparing plant densities (6-plant and 30-plant plots), using data collected in 2015 and 2016, were high for subjective ratings taken early of stem height (0.72). A positive correlation for early subjective ratings of branch width in 6-plant and 30-plant plots was observed (0.62). Early season subjective branch width ratings in 6-plant plots were correlated with early season stem height ratings in 30-plant plots (0.66). Early season subjective stem height of 30-plant plots was correlated with branch width ratings of 6-plant plots (0.63). There was a low correlation of objective observations for (0.24) stem height, (0.06) branch width, (0.11) leaf length, (0.25) leaf width, and (0.20) leaf area between 6- and 30-plant plots. However, since most agronomic traits were not correlated for the two densities, data should be taken only at the high density.

Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a member of the Asteraceae family. It has become a valuable new crop in the market for natural, plant-based sweeteners (Ashwell, 2015; Tavarini et al., 2018). Since its approval in European markets by the Standing Committee of the European Commission in 2011, research on stevia has increased dramatically, along with worldwide demand for steviol glycosides (SG) (Calorie Control Council, 2011; Angelini et al., 2016). Stevia produces SGs that accumulate in its leaves (up to 30% concentration). The SGs are 250 to 300 times sweeter than sucrose, stable at high temperatures, and stable over a range of pH levels (3 to 9) (Kinghorn and Soejarto, 1985; Brandle et al., 1998; Prakash et al., 2014; Ashwell, 2015). The high amount of SG produced per hectare is more significant than that produced from sugarcane (sucrose) and other sweeteners, reducing the amount of land required (Ashwell, 2015).

Stevia plants (2n=22) proliferate and are adapted to tropical and sub-tropical environments; the plants have the ability to overwinter in cool-temperate regions (Ramesh et al., 2006; Chester et al., 2013). Stevia is an obligate short-day plant with critical day length of 13 hours for flower initiation (Ceunen et al., 2011; Zaidan et al., 1980). Stevia is an outcrossing species, requiring pollen transfer from one genotype to another to produce viable seeds (Yadav et al., 2011). Sporophytic self-incompatibility prevents inbreeding and leads to low seed yield as well as low seed germination in programs aimed at developing pure lines (Raina et al., 2013). There is a large environmental variance in stevia for traits such as flowering time, leaf yield, plant height, and SG content (de Oliveira et al, 2004).

Stevia breeders want high leaf yield, high leaf: stem ratio, improved plant vigor, regeneration capacity, high SG content, low stevioside: Reb-A content, adaptability to production regions, photoperiod insensitivity, self-fertility, and resistance to insects and diseases (Tavarini et al., 2018). Biomass production is a primary focus. Generally, yields of 1,600 to 2,000 kg/ha dried leaves can be produced commercially, with higher yields reported when using multiple harvests during a growing season and even higher yields reported from two-year or three-year production systems compared to annual production (Miyazaki et al., 1978). An important breeding objective for researchers is to develop cultivars that have high levels of a specific SG with stable expression in all plant leaves. Identifying germplasm capable of self-pollination might make genetic gains faster and provide a method to produce cultivars using seeds rather than cuttings (Tavarini et al., 2018). Photoperiod insensitivity is a desirable trait to delay flowering and lengthen the amount of time for SG accumulation in leaves, since SG content is highest just before flowering (Ramesh et al., 2006). Drought-tolerant cultivars are needed to expand cultivation to regions with little precipitation or unavailable irrigation. Drought

conditions reduce photosynthesis, resulting in lower plant yield (Tavarini et al., 2015). Resistance is needed for the few pests and diseases that attack stevia. The most important fungal pathogens are *Septoria steviae* and *Sclerotinia sclerotiorum,* which have been reported worldwide (Tavarini et al., 2018).

Plants with a large main stem with few secondary branches are less desirable due to low yield. Better would be a highly branched plant with erect branches, short internodes, and erect leaves. Selecting compact plant types with short internode spacing would improve leaf yield on a per-plant basis (Tavarini et al., 2018). Leaf : stem ratio is an important trait because stevia stems contain not much SG, and selecting plants with a higher proportion of leaves than stems would increase SG yields and leaf yields (Ceunen and Geuns, 2013). Stevia leaves have a diverse sizes and shapes; they are relatively small, subsessile, and ovate to lanceolate-shaped (Othman et al., 2015). An increase of leaf size might be valuable to maximize the site of SG accumulation to improve production. Reb-A content, large leaf area, rate of photosynthesis, chlorophyll content, and protein content were correlated (Weng et al., 1996). Plants with dark green leaves have higher chlorophyll content and a higher photosynthetic rate, which results in a higher growth rate and leaf biomass production (Tavarini et al., 2015). These results suggest plants with larger leaves and higher chlorophyll content photosynthesize more efficiently and produce more SG than small-leaf cultivars.

Stevia breeding research has been conducted in many countries, including China, Indonesia, Japan, Korea, Russia, and Taiwan, producing cultivars that have improved yield and stevioside content (Lee et al., 1978; Lee et al., 1982; Shu, 1989; Suhendi, 1989; Shyu et al., 1994; Shu, 1995; Kornienko and Parfenov, 1996; Weng et al., 1996). SG content has risen from 2 to 20 % of dry biomass after 30 years of selection (Yadav and Guleria, 2012). Stevia has

shown wide adaptation to different geographical locations, and some genotypes showed high cold tolerance under controlled growth room experiments, which might enable seasonality expansion of production (Kozik, et al., 2020; Ramesh et al., 2006; Tavarini et al., 2018). Cosson et al. (2019) reported high genotypic and phenotypic variance among stevia cultivars, landraces, and populations. However, only 90 cultivars are available on the market, and many farmers are using old cultivars such as 'Criolla', 'Eirete I', and 'Morita II' (Angelini et al., 2016; Cosson et al., 2019).

The success of a breeding program depends on selecting parents with beneficial traits from a population and the ability to estimate the genetic value of selected parents based on phenotypic value (Lorenzana and Bernardo, 2009). To select parents for a hybrid breeding program, a genetic divergence study was conducted using 15 clonal cultivars with high SG content and high Reb-A: stevioside ratio (Anami et al., 2010). Clonal cultivars exhibited high variability for many traits, including fresh and dry weight, plant height, branch number, SG content, and Reb-A: stevioside ratio. Therefore, they concluded that hybridization using four of the 15 cultivars possessing the highest divergence would be useful for developing segregating populations to select superior genotypes for crop improvement (Anami et al., 2010).

A two-year trial was conducted, at two locations in France, to assess overwintering ability of 96 genotypes derived from a population of 'Criolla' (Barbet-Massin et al., 2016). High variability of SG content was observed among the 96 genotypes, with some genotypes possessing a high Reb-A: stevioside ratio and others having high amounts of other specific SGs. Furthermore, SG composition remained stable at both locations, which suggested these traits are subject to a high genotypic predisposition for total SG content (Barbet-Massin et al., 2016). Gauray et al. (2008) reported that broad-sense heritability for leaf yield was 99 %, stevioside

content was 93 %, and plant height was 97 % for ten cultivars. Brandle and Rosa (1992) evaluated half-sib families derived from plants of a Chinese landrace and reported high heritability for yield, leaf: stem ratio, and stevioside content (62.1, 78.8, and 76.6 %, respectively), suggesting these traits can be improved through selection (Brandle and Rosa, 1992). Brandle et al. (1999) studied Reb-A and Reb-C inheritance in two crosses derived from divergent parents and reported a single dominant gene controlling Reb-A content in the first population and a single additive gene controlling SG content in the second population. Reb-A and Reb-C were linked in the first population, and an inverse relationship was observed in the second population with high Reb-C, low stevioside, and low Reb-A (Brandle, 1999). The type line 95-002A-336 was used to identify *Rce*, the single additive gene responsible for Reb-C content. No reciprocal differences were observed, indicating that the cross could be evaluated in either direction. Reb-A and Reb-C can be manipulated through selection simultaneously when the appropriate parents are selected for those traits (Brandle, 1999).

Growth traits and production traits can be compared using regression analysis to understand their relationship and to aid in selecting superior genotypes with enhanced growth parameters, yield, and SG content. For instance, leaf number and branch number are highly correlated with production traits such as single-plant leaf weight and dry leaf yield, while plant height has shown an inconsistent relationship with both of those growth traits and was inconsistently correlated with production traits (Buana and Goenadi, 1985; Buana, 1989; Shu and Wang, 1988). Total stevioside content was shown to be positively correlated with leaf : stem ratio; leaf thickness was positively correlated with stevioside: Reb-A ratio (Shyu et al., 1994; Tateo et al., 1998). Objective measurements of leaf length, leaf width, leaf area, and leaf thickness can improve the selection of higher leaf-yielding genotypes. A positive correlation of

dry leaf yield with plant height, number of branches, leaves per plant, and dry matter yield was observed by Chalapathi et al. (1998) with 96.88 % variation in dry leaf biomass explained by a linear function of all four traits. Nakamura and Tamura (1985) found Dulcoside A and Reb-C, and Reb-A and Reb-C to be positively correlated. A negative correlation was observed for stevioside and Reb-A, and Dulcoside A and Reb-C. Stevioside content is highly variable (4 % to 16 %) among individual plants even after undergoing continuous selection (Bian, 1981; Nakamura and Tamura, 1985).

Landraces and off-patent cultivars of stevia seeds were used to start a population to evaluate single plants' morphological, agronomic, and SG traits. In 2015, two experiments were run at two locations each, with half-sib families (offspring) from single plants (parents). Growth traits (stem height, branch width, leaf length, and leaf width) were measured objectively, as well as being rated subjectively on a scale of 0 to 9. We were interested in understanding the inheritance of traits for use in a breeding program. To do that, we used estimates of phenotypic and genotypic variance from data collected in the field and laboratory from the parent population in 2015 and the offspring in 2016. Analysis of variance, parent-offspring regression, and Pearson product-moment correlations were used to evaluate stevia populations developed in the stevia breeding project at North Carolina State University (NCSU). Results from this research will help in the optimization of cultivar improvement programs.

Materials and Methods

Populations

In 2015, seeds of landraces and off-patent cultivars were used to start the first breeding cycle of the stevia breeding program at NCSU. Seeds were planted in February 2015 using 72-

cell seedling trays (550 plants / m^2) in greenhouses at the Horticultural Field Laboratory. Fafard 4p soilless media (48% sphagnum moss, 30% bark, 11% perlite, 11% vermiculite, traces of dolomitic limestone, and gypsum as a wetting agent) was used for seedling establishment. Due to low germination rate, each cell contained four seeds to ensure the adequate seedling quantity. The overhead mist was used from seeding through true-leaf stage. Seedlings were thinned to one plant per cell, and a 100 ppm liquid fertilizer solution (20-10-20%) was applied weekly until plants were transplanted into the fields 12 weeks later. Greenhouse temperatures ranged from 28ºC during the day to 2ºC at night with a 12-hour photoperiod. Plants were acclimated to field conditions under 50% shade cloth for one week prior to transplanting (Huber and Wehner, 2021).

The parental generation was intercrossed in the field as part of a narrow-base (5 starting accessions) as well as a wide-base population (30 starting accessions). Beehives were placed next to the field sites to ensure adequate cross-pollination in late August to end of November 2015. Flowering started at the end of September and lasted until late October. Individual stevia plants used for SG analysis were harvested weekly for open-pollinated seeds in mid-October through November 2015 to generate offspring seeds for the 2016 field season. Seeds were collected once the seeds and seed heads turned brown and fell off the plant when shaken. Seed heads of individual plants were gathered and shaken into a gallon (4.5 L) Ziploc bags. Seeds were dried at 60ºC for 1 to 2 days until they reached 10% to 15% moisture content. Seeds were stored at 22°C for three months before sowing the following February 2016.

A total of 121 offspring were evaluated based on 2015 SG content and growth traits. Twenty-one offspring were selected to create a new elite population and were grown in separate polycross experiments, consisting of two replications of 6-plant plots at two field locations. One hundred of the 121 offspring were evaluated at two locations (the remainder had poor

germination). Agronomic yield trials consisted of two replications of 30-plant plots to evaluate biomass production. Populations were separated by border rows to reduce outcrossing with other experiments.

Locations

The parental genotypes were transplanted in May 2015, and the offspring families were transplanted in May 2016 (Huber and Wehner, 2021). Raised beds were covered in 1.25 mm black plastic. Beds were arranged on 1.5 m between-row spacing, and plots measured 3 m for 30 plants per plot for destructive biomass evaluations for whole plant yield and leaf weight. Adjacent 6-plant plots of each treatment combination (year-location-replication-genotype) were used for non-destructive objective measurements and subjective ratings of growth parameters, lodging, disease resistance, and SG analysis. A commercial plant density of 64,550 plants/ha was represented by 30-plant plots, while 6-plant plots represented a plant density of 12,910 plants/ha (Huber and Wehner, 2021). A 1.5 m alley separated plots. Drip irrigation was used to supply a minimum of 25 mm of water per week, including rainfall. A total of 50 mm of weekly rainfall could be expected during heavy rainfall events. Liquid fertilizer was injected through drip irrigation on a weekly basis. The broad-spectrum pesticide Seven® (carbaryl) was applied as needed to control insects early in the growing season. Plots were hand weeded throughout the season as needed (Huber and Wehner, 2021).

Weather data were collected during the growing season (May to September) at the Cunningham research station, Kinston, NC, and at the horticultural crops research station, Clinton, NC. The rainfall total at the Kinston location was 77 cm and 90 cm for 2015 and 2016, respectively. The rainfall total at the Clinton location was 56 cm and 73 cm for 2015 and 2016, respectively. Temperatures ranged from 30.3 ºC during the day to 19.4 ºC at night for both

coastal plain field locations during the 2015 and 2016 growing seasons. The average photoperiod was 13:42 hours of daylight, with a range of 13:38 hours in May, 14.30 hours during peak season, and 11:49 hours in September. Both locations had sandy soils (Huber and Wehner, 2021).

Growth Traits

Morphological characters were objectively measured and included stem height, plant branch width, leaf length, and leaf width using an mm ruler on 29 August 2015 and 30 August 2016 (Huber and Wehner, 2021). Leaf area was estimated using leaf length and width (0.5 x length x width). Plant size traits were measured on the third plant in the center row of 30-plant plots to represent performance at commercial density. In addition, we measured every plant in the 6-plant plots.

Subjective ratings were made early and late in the growing season on each plant in the 6-plant plots using a 1 to 9 scale, similar to the one developed by Jenkins and Wehner (1983) for stem height (0=short, 9=tall), branch width (0= no secondary branches, 9=many secondary branches), leaf size (0=small, 9= large), lodging (0=none, 9= stems on the ground), disease resistance (0=none, 9=dead), and flowering (0=none, 9=fully flowering). Transplant survival was recorded after the plants were growing for two weeks, and the final stand count was recorded at the last date of data collection.

Yield Traits

The 30-plant plots were harvested at the end of September by cutting the plants 5 cm above the soil (Huber and Wehner, 2021). Harvested plants from each plot were collected into burlap sacks. Fresh weights were collected on the day of harvest, followed by drying samples at

60 ºC in a tobacco barn. Dry weights were recorded after two days of drying. The yield was reported as $Mg \cdot ha^{-1}$.

Glycoside Traits

The SG content was measured using leaves harvested from five nodes nearest the top of the canopy (Ceunen and Geuns, 2013; Bondarev et al., 2003). Four plants from each 6-plant plot were selected to sample for SG analysis in mid-July before flower initiation to avoid SG decline at flowering (Yadav et al., 2011). Four replications were used in the experiments in 2015. Two replications of families from those experiments were evaluated in 2016. Leaves from each plant were kept separate in coin envelopes (5.72 cm x 8.90 cm) and dried at 60 ºC before grinding to a fine powder for analysis. SG samples were shipped to a third-party (PepsiCo) for ethanol-based extraction and quantification using LC-MS/MS methods similar to Shafii et al. (2012). The SG content for stevioside, steviolbioside, rubusoside, dulcoside A, Reb-A, Reb-B, Reb-C, Reb-D, and Reb-E were reported as mg/g (concentration of dry leaf weight). We also measured total steviol glycosides (TSG). Each SG's percentage of SG content was calculated based on the proportion of the TSG content x 100. Selected individual plants were harvested for clones to fix desired selections and seeds for the next breeding cycle.

Experiment Design

In 2015, the experiment was run as a randomized complete block design with 24 genotypes, two locations (Clinton and Kinston, NC), and four replications for both the 30-plant and 6-plant plots. In 2016, the experiment was run as a randomized complete block design with 21 genotypes, two locations (Clinton and Kinston, NC), and two replications for both the 30-plant and 6-plant plots.

Statistical Analysis

Analysis of variance was performed on phenotypic traits (objective measurements and subjective ratings) and SG content. Populations were evaluated by year separately using R code rgxe 1.1 (Dia et al., 2017). Genotypes were treated as fixed effects; location and replication were treated as random effects. Results were reported separately by year for parents and offspring.

Narrow-sense heritability (h^2) was estimated based on the slope from parent-offspring regression using the following formula:

$b_{O.P} = cov_{O.P} / var_P = 1/2\ var_A / var_P = 1/2\ h^2$; where $h^2 = 2\ b_{O.P}$

The slope ($b_{O.P}$) of the regression is equal to the covariance (cov) of the offspring-parent regression divided by the parental variance (var_P); which is equal to half of the additive variance (var_A) divided by the phenotypic variance var_P, and estimates half the h^2. The $b_{O.P}$ multiplied by two estimates h^2.

Pearson product-moment correlations were estimated for pairwise comparisons to determine trait associations from parent and offspring generations. Correlation analysis was performed separately for the 2015 populations and 2016 populations to assess trends in trait associations for each generation. Then the study was analyzed across both years to see if any traits were correlated between parent and offspring. Correlation analysis was also performed on 6-plant and 30-plant plots for plant size data to gauge the association of yield trial data with single-plant growth traits with a significant level was $p<0.05$.

Results

Analysis of Variance

There were significant differences in subjective stem height ratings for the early season among genotypes in 2015 ($p>0.05$) (Table 3.1). There were significant differences in stem height ratings between locations, and an interaction of genotype by location was observed in 2015 ($p>0.05$). Early season stem height ratings showed significant differences among genotypes in the 2016 field season ($p>0.05$). The location had a significant impact on subjective ratings for stem height, and there was a significant interaction of genotype by location in 2016 ($p>0.05$) (Table 3.1). In 2015, early season ratings of branch width had significant differences among genotypes, and there was a significant interaction of genotype by location ($p>0.05$) (Table 3.1). Branch width was significantly different among genotypes for early evaluations of the growth stage in 2016 ($p>0.05$) (Table 3.1). Location was significant for a subjective rating of branch width in 2016 as well ($p>0.05$) (Table 3.1). Subjective rating of leaf size in the early season was significant for genotype in 2015, and there were significant differences in ratings for leaf size in genotypes at different locations ($p>0.05$) (Table 3.1). In 2016, early season leaf size ratings were significantly different among genotypes, and there was an interaction of genotype by location ($p>0.05$) (Table 3.1).

Subjective ratings for stem height in the late season were significantly different among genotypes in 2015 ($p>0.05$). There was a significant interaction of location and genotype for stem height ratings late in the 2015 growing season ($p>0.05$) (Table 3.1). Late-season subjective ratings for stem height showed significant differences among genotypes in 2016 ($p>0.05$). Late season ratings for branch width depended on genotype in 2015 ($p>0.05$) (Table 3.1). Late season ratings for branch width were significantly different among genotypes and locations, and there

was a significant genotype by location interaction in 2016 ($p>0.05$) (Table 3.1). In 2015, ratings for leaf size were significant for genotype during late-season evaluations, and significant differences were observed for genotypes grown at different locations ($p>0.05$) (Table 3.1). In 2016, ratings for late-season leaf size were significantly different among genotypes and locations, and there was an interaction of genotype by location ($p>0.05$).

Stem height measured objectively was significantly different among genotypes in 2015 ($p>0.05$) (Table 3.1). There was an interaction of location and genotype for stem height ($p>0.05$). In 2016, stem height measurements showed significant differences among genotypes and locations, and there was a genotype-by-location interaction ($p>0.05$). In 2015, branch width was significantly different among genotypes and varied by location, with a significant interaction of genotype and location ($p>0.05$) (Table 3.1). In 2016, branch width measurements showed significant differences among genotypes and locations, and there was a significant genotype by location interaction ($p>0.05$). In 2015, leaf length was significantly different among genotypes ($p>0.05$) (Table 3.1). In 2016, leaf length was significantly different among genotypes and varied by location ($p>0.05$). In 2015, leaf width was significantly different among genotypes ($p>0.05$) (Table 3.1). In 2016, leaf width was significantly different among genotypes and varied by location ($p>0.05$). Canopy volume was significantly different among genotypes and varied by location in 2015 ($p>0.05$) (Table 3.1). Canopy volume was significantly different among genotypes and varied by location in 2016 ($p>0.05$). Leaf area was calculated based on leaf length and width and was significantly different among genotypes and locations in 2015 ($p>0.05$). Leaf area measurements were significantly different among genotypes and locations in 2016 ($p>0.05$).

The SG content varied by genotype and location in 2015. Significant differences in dulcoside A content were observed among genotypes ($p>0.05$) (Table 3.1). The location had an

impact on dulcoside A content ($p>0.05$). in 2016, dulcoside A content differed significantly among genotypes, location, and a genotype by location interaction ($p>0.05$) (Table 3.1). Reb-B varied significantly among genotypes in 2015 ($p>0.05$) (Table 3.1). Location had a significant impact on Reb-B content that year ($p>0.05$). In 2016, Reb-B content was significantly different among genotypes, and there was a genotype by location interaction ($p>0.05$) (Table 3.1). Location had a significant impact on Reb-D content in 2015 ($p>0.05$) (Table 3.1). The Reb-D concentration differed significantly among genotypes, and locations, and there was an interaction of genotype by location in 2016 ($p>0.05$). Reb-E was significantly different among genotypes in 2015 ($p>0.05$) (Table 3.1). Reb-E content was significantly different among genotypes and locations in 2016 ($p>0.05$).

Steviolbioside content was significantly different among genotypes and differed among locations in 2015 ($p>0.05$) (Table 3.1). In 2016, steviolbioside concentration varied by genotype and location, and there was a genotype by location interaction ($p>0.05$) (Table 3.1). Rubusoside content was not significantly different among genotypes and locations in 2015. In 2016, rubusoside content was significantly different among genotypes grown in different locations, and there was an interaction of genotype and location ($p>0.05$) (Table 3.1). Reb-A content varied significantly among genotypes and locations, and there was an interaction of location and genotype in 2015 and 2016 ($p>0.05$) (Table 3.1). Reb-C concentration was primarily affected by the location where the genotypes were grown in 2015 ($p>0.05$) (Table 3.1). Reb-C content was significantly different among genotypes and locations in 2016 ($p>0.05$). Stevioside content was significantly different among genotypes and locations in 2015 ($p>0.05$) (Table 3.1). Stevioside concentration varied by genotype and location in 2016, and there was a genotype by location interactions ($p>0.05$). The TSG content varied significantly by genotype and location in 2015

($p>0.05$) (Table 3.1). The TSG content was significantly different among genotypes and locations, and there was a significant interaction of genotype by location in 2016 ($p>0.05$).

Fresh and dry weight yield parameters were significantly different among genotypes and locations. In 2015, the location did not have a significant effect on yield traits, but in 2016, the location was significant for both fresh and dry weight ($p>0.05$) (Table 3.1). There was a significant interaction between location and genotype for fresh weight yield in both field seasons ($p>0.05$). Genotypes differed significantly in fresh weight yield for location in both years ($p>0.05$).

Trait Correlations

Pearson correlations of phenotypic traits were compared using trait data from 6-plant plots in 2015 and 2016. Stem height and branch width in the early season were correlated (0.70). Objective branch width and subjective branch width ratings in the late season were correlated (0.52). Canopy volume was correlated with objective stem height and branch width measurements (0.60 and 0.92, respectively). Leaf area was correlated with objectively measured leaf length and width measurements (0.88 and 0.92, respectively). Objective stem height and branch width measurements were correlated (0.46). Late-season branch width measurements were correlated with late-season subjective branch width ratings (0.57). Objective leaf length and leaf width measurements were correlated (0.66).

Pearson correlation of pairwise SG trait comparison showed stevioside was moderately correlated with steviolbioside (0.40). The TSG content was highly correlated with Reb-A (0.85) and moderately correlated with Reb-B (0.57). Reb-A was moderately correlated with Reb-B (0.52). Reb-A was moderately correlated with Reb-D (0.47). Reb-C and dulcoside A were correlated (0.49). Reb-B and Reb-D were correlated (0.48). Reb-E was correlated with

rubusoside (0.65). Stevioside was slightly correlated with steviolbioside (0.40) and rubusoside (0.43). A negative correlation was observed between Reb-A and stevioside (-0.18); and between Reb-E and Reb-B (-0.30).

Pearson correlations comparing traits measured at the two plant densities (6-plant and 30-plant plots), using data collected in 2015 and 2016, were as follows. Correlations were high for subjective ratings of stem height in the early season (0.73). They were also high for subjective ratings of branch width in the early season (0.62). However, most traits were not correlated when measured at the two densities. Subjective leaf size ratings in the early season had a low correlation between plant densities (0.20). Subjective leaf rating in late season had low correlation between plant densities (0.21). Subjective leaf size rating in late season of 6-plant plots had low correlation (0.26) with objectively measured leaf area in 30-plant plots. Subjective leaf size rating of 6-plant plots in the late season had a low correlation (0.26) with objectively measured leaf width in 30-plant plots. There was a low correlation of objectively measured stem height (0.24), leaf width (0.25), and leaf area (0.20) between 6-plant and 30-plant plots. Plants grown at higher densities were more affected by shading than plants at lower densities. It would be better to collect data from low-density plots for selections and then use standard density trials for yield and plant growth, as was done in this experiment.

Heritability

Narrow-sense heritability was estimated based on the slope of the parent-offspring regression analysis of 2015 parents and 2016 offspring. Heritability was generally low for the traits evaluated. Subjective growth ratings had low heritability estimates (0.08, 0.08, and 0.20, respectively) for early season stem height, branch width, and leaf size. Late-season ratings for stem height, branch width, and leaf size also had low heritability (0.06, 0.006, and 0.06,

respectively). Objective stem height, branch width, leaf length, length width, and leaf area also had low heritability (0.30, 0.14, 0.22, 0.22, and 0.24, respectively). Canopy volume had a low heritability value of 0.06. Dulcoside A, Reb-B, Reb-D, and Reb-E had low to moderate heritability (0.26, 0.08, 0.40, and 0.34, respectively). Steviolbioside and rubusoside also had low heritability (0.06 and 0.24, respectively). Reb-A, Reb-C, stevioside, and TSG had moderate to high heritability (0.56, 0.50, 0.22, and 0.24, respectively).

Parent-offspring regression analysis was conducted using data from 30-plant plots from 2015 and 2016 to determine the narrow-sense heritability of fresh and dry-weight biomass production. The heritability of fresh-weight biomass was 0.02, and dry weight was estimated to be 0.005. Therefore, yield should not be measured using single plants.

Discussion

Large genotypic and phenotypic variation has been reported in stevia (Cosson et al., 2019). Variation among and within genotypes was high for objectively measured traits, subjectively rated traits, yield, and SG traits. In this study, yields were higher in 2016 compared to 2015. Also, the content of Reb-A, Reb-C, Reb-D, and Reb-E were higher in 2016. The single-plant selection effectively improved SG traits in a single cycle of selection. The ratio of Reb-A: stevioside is a good indicator of the sweetness of a genotype. Reb-A is usually 30 to 40% of the SG profile, and stevioside makes up 60 to 70% of the profile. Stevioside is not as desirable as Reb-A because it has a lingering licorice aftertaste (Yadav et al., 2011). In this study, a negative correlation was observed between stevioside and Reb-A, which has been reported previously (Tavarini et al., 2018). Plant breeders are focused on increasing the Reb-A: stevioside ratio, and these findings indicate specific SGs can be improved rapidly. The TSG content was similar in

2015 and 2016 and showed high heritability. The high phenotypic variance was observed among progeny from the same parent, even at the same location. Thus, increased genetic variation is easily detected regardless of the environment. Improved cultivars are needed that have high levels of individual SG compounds that remain stable over plant age or canopy position.

The environment plays a critical role in the expression of phenotype in stevia. There is large variability in both phenotype and genotype response to the environment for traits such as flowering time, leaf yield, plant height, and SG content (Yadav et al., 2011; de Oliveira et al, 2004). In this study, location played a significant role in genotype performance. Kinston had a higher rain fall total in both years (77 cm and 90 cm, respectively) compared to Clinton (56 cm and 73 cm, respectively). In 2015, Kinston had higher yields compared to Clinton, and yields were similar in 2016 when both locations received similar rainfall totals. Soil type varied by location, with sandy soil found in Clinton and sandy loam soil in Kinston. The Kinston soils contain more organic matter, which increases soil moisture retention compared to the sandy soils at Clinton.

Genotype-by-location effects have been reported for growth, reproduction, and SG traits. The SG content varied significantly among genotypes, by plant age, and by the environment in previous studies (Gaurav et al., 2008; Luwańska et al., 2015; Tavarini et al., 2018). Genotype performance was significantly different at the two locations (Clinton and Kinston) and in two years of field trials (2015 and 2016). Subjective rating for stem height in the early season was lower in Kinston compared to Clinton in both years. The mean branch width ratings were higher in 2015 than in 2016 for both locations, with slightly higher values observed in Kinston. A higher subjective rating for stem height in the late season was observed in Clinton in both field seasons. Subjective ratings for branch width in the late season were higher in 2016 compared to

2015. Mean leaf size ratings were consistent across years in Clinton, and leaf size ratings were higher in Kinston in 2016 compared to 2015. In Kinston, stem height was similar both years; and in Clinton, the mean stem height was higher in 2016 compared to 2015. Leaf area in Clinton was greater in 2016 than in 2015, and in Kinston were lower in 2016 compared to 2015.

Dulcoside A content was similar in Clinton across the years and increased threefold in Kinston in 2016 compared to the 2015 field season. Reb-B content in Clinton was higher in 2015 than in 2016 and consistent across years in Kinston. Reb-D content was lower in Clinton in 2016 compared to 2015, whereas Reb-D content in Kinston was higher in 2016 than in 2015. Clinton Reb-E content was higher in 2015 than in 2016, and Reb-E content was lower in Kinston in 2015 compared to 2016. Clinton location had a higher mean steviolbioside content than Kinston in both years. The mean Reb-A concentration was higher in Clinton compared to Kinston in 2015, and the inverse was seen in 2016. In 2015, Clinton had a higher mean Reb-C content than Kinston; however, in 2016, the mean was higher in Kinston. Stevioside content was twice a high in 2015 compared to 2016. Clinton had a higher mean TSG compared to Kinston in 2015, and Kinston had a higher mean TSG content than Clinton in 2016. Fresh and dry biomass yield was significantly different among genotypes grown at various locations in 2015 and 2016. Differences in yield may be due to multiple factors, including genotype, environment, drying conditions, and survival rate.

Narrow-sense heritability is the additive genetic variance as a proportion of total (phenotypic) variance. We estimated it based on the slope of the parent-offspring regression of parents in 2015 and their offspring in 2016. Heritability estimates ranged from low to high across all traits evaluated. There was a high variance for biomass among offspring, replications, locations, and years.

Heritability estimates were lower than expected using parent-offspring regression analysis compared to a previous report by Huber (2017) using half-sib family means analysis on the same populations. Dry leaf yield was reported to be moderately heritable (0.41) using half-sib family analysis (Huber, 2017); and using parent-offspring regression, dry leaf yield had markedly lower heritability (0.005). The highest heritability estimates using half-sib family analysis were reported for objectively measured stem height (0.68), plant size (0.61), branch size (0.59), and leaf area (0.54) (Huber, 2017). In the current study, heritability for objectively measured stem height (0.31), branch width (0.14), and leaf area (0.25) were significantly lower. Huber (2017) reported moderately low heritability estimates for subjectively measured stem height, branch size, leaf area (0.30, 0.08, 0.13, respectively), which was higher than those reported in this study (0.06, 0.006, and 0.06, respectively).

The discrepancy in heritability estimates can be attributed to the different methods used. The use of variance components has been shown to overestimate heritability, while parent-offspring regression underestimates heritability and makes use of single-plant data in the parental generation. Half-sib family analysis provides more data points. In addition, we had a smaller sample size for the pairs of parents and offspring.

Knowledge of trait associations aids in the selection of elite genotypes with improved characteristics. For instance, leaf and branch numbers are highly correlated with production traits. However, plant height has demonstrated an inconsistent relationship with both of those characteristics and does not necessarily correlate to production traits (Buana and Goenadi, 1985; Buana, 1989; Shu and Wang, 1988). A positive correlation of total stevioside content with the leaf : stem ratio has been reported, and leaf thickness was shown to have a positive correlation with the stevioside: Reb-A ratio (Shyu et al., 1994; Tateo et al., 1998).

In this study, a positive correlation of TSG with Reb-A, Reb-D, and Reb-A was observed. Also, there was a negative correlation of Reb-A with stevioside, steviolbioside, and Reb-E. This information can be used to improve breeding methods for stevia. Interestingly, a high correlation between subjective and objective measures for stem height and branch width indicated that either could be used. The subjective rating was a faster method if conducted by a trained observer. However, subjective and objective measures for leaf size were not correlated. Leaf size varies depending on leaf age and canopy position. This trait may not have been accurately assessed depending on where in the canopy the observer measured the leaf. Objectively measuring leaf traits can improve the selection of higher leaf-yielding genotypes. Stevia leaves range in size and shape, and a larger leaf size helps maximize leaf area and increase SG yields (Othman et al., 2015). Selection of a higher leaf: stem ratio is important because stevia stems contain little SG; selection of plants with more leaves will improve leaf yield and SG yield as well (Ceunen and Geuns, 2013). Stem height and branch width subjective ratings were positively correlated for 6-plant and 30-plant plot densities, while objective rating showed low correlations between plant densities. Plant density played a role in observed phenotypic differences. Further research is needed to optimize the translatability of small plot research to commercial production scale.

Plant height is an interesting characteristic to evaluate because numerically high values can be an indication of high yield. However, a plant can have one long main stem with few or no branches, which results in a lower yield. Canopy spread rating, branch number, internode length, and nodes per branch are useful traits to couple with plant height to improve the picture of plant architecture. The ideotype is a compact plant with many upright, straight branches, short internode lengths, and large leaves (Tavarini et al., 2018). Breeders must evaluate as many growth traits as possible, and four characteristics (stem height, branch width, leaf length, and leaf

width) provide insight, along with 10 SG traits for researchers to expand on. The highest heritability estimates in this study were Reb-A, Reb-C, and Reb-D, indicating that progress can be made for these specific SG targets.

References

Anami, E., Poletine, J.P., Gonçalves-Vidigal, M.C., Filho, P.S., Lacanallo, G.F., Kvitschal, M.V., & Gonela, A. (2010). Characterization and genetic divergence in *Stevia rebaudiana* (Bert.) Bertoni clones based in agronomical and morphological characteristics. *Journal of Food Agriculture & Environment*, 8: 463-469.

Angelini, L. G., Martini, A., Passera, B., & Tavarini, S. (2016). Reference Series in Phytochemistry. *Sweeteners, Pharmacology, Biotechnology, and Applications* , ed. J.-M. Mérillon and K. G. Ramawat.

Ashwell, M. (2015). Stevia, Nature's Zero-Calorie Sustainable Sweetener: A New Player in the Fight Against Obesity. *Nutrition Today, 50*(3): 129–134.

Barbet-Massin, C., Giuliano, S., Alletto, L., Daydè, J., & Berger, M. (2016). Towards a Semi-Perennial Culture of *Stevia rebaudiana* (Bertoni) Bertoni Under Temperate Climate: Effects of Genotype, Environment and Plant Age on Steviol Glycoside Content and Composition. *Genet. Resour. Crop Evol*., 63: 685-694.

Bian, Y.M. (1981). Studies on *Stevia rebaudiana* a New Sweet- tasting Plant: Refining Stevioside and Determination of its Concentration. *Plant Physiol. Commun*., 3: 15-17.

Bondarev, N.I., Sukhanova, M.A., Reshetnyak, O.V., & Nosov, A.M. (2003). Steviol Glycoside Content in Different Organs of *Stevia rebaudiana* and Its Dynamics During Ontogeny. *Biologia Plantarum*, 47: 261-264.

Brandle, J.E., & Rosa, N. (1992). Heritability for Yield, Leaf : Stem Ratio and Stevioside Content Estimated from Landrace Cultivar of *Stevia rebaudiana. Can. J. Plant Sci*., 72:1263-1266.

Brandle, J.E., Starratt, A.N., & Gijzen, M. (1998). *Stevia rebaudiana*: Its Agricultural, Biological, and Chemical Properties. *Can. J. Plant Sci.,* 78: 527-536.

Brandle, J. (1999). Genetic Control of Rebaudioside A and C Concentration in Leaves of the Sweet Herb, *Stevia rebaudiana*. *Can. J. Plant Sci*., 79: 85–92.

Buana, L. (1989). Determination of the Required Growth Variables in an Agronomic Experiment with Stevia. *Menara Perkebunan*, 57(2): 29-31.

Buana, L., & Goenadi, D.H. (1985) A Study on the Correlation Between Growth and Yield in Stevia. *Menara Perkebunan*, 53(3): 68-71.

Calorie Control Council. (2011). Stevia Gaining Approval in Europe. Accessed Sept. 30, 2021. https://caloriecontrol.org/stevia-gaining-approval-in-europe/

Ceunen, S., Werbrouck, S., & Geuns, J. M. C. (2011). Accumulation Pattern of Steviol Glycosides in *Stevia rebaudiana* Bertoni and its Stimulation by Red-Light. EUSTAS Stevia Symposium "Stevia: Break-through in Europe", 5.

Ceunen, S., & Geuns, J. M. C. (2013). Spatio-temporal Variation of the Diterpene Steviol in *Stevia rebaudiana* Grown Under Different Photoperiods. *Phytochemistry,* 89: 32-38.

Chalapathi, M.V., Thimmegowda, S., & Sridhara, S. (1998). Correlation Studies in Stevia. *Ind. Agric*., 42: 137-138.

Chester, K., Tamboli, E.T., Parveen, R., & Ahmad, S. (2013). Genetic and Metabolic Diversity in *Stevia rebaudiana* using RADP and HPLC Analysis. Pharmaceutical Biology, 51(6): 771-777.

Cosson, P. Hastory, C., Errazzu, L.E., Budeguer, C.J., Boutié, P., Rolin, D., & Schurdi-Levraud, V. (2019). Genetic Diversity and Population Structure of Sweet Leaf Herb, *Stevia*

rebaudiana B., Cultivated and Landraces Germplasm Assessed by EST-SSRs Genotyping and Steviol Glycoside Phenotyping. *BMC Plant Biology*, 19: 436.

Dia, M., Wehner, T. C., & Arellano, C. (2017). RGxE: An R program for genotype x environment interaction analysis. *Amer. J. Plant Sci*., 8: 1672-1698.

Gaurav, S. S., Singh, Y. P., & Sirohi. S. P. S. (2008). Genetic Variability for Yield and Quality Traits in *Stevia rebaudiana* (Bertoni). *Progressive Res.,* 3: 95-96.

Huber, B. M. (2017). Studies on Stevia (*Stevia rebaudiana*). *Master of Science Thesis*, North Carolina State University, Raleigh, NC.

Huber, B.M., & Wehner, T.C. (2021). Performance of 16 *Stevia rebaudiana* seed cultigens for glycosides and yield in North Carolina. *Scientia Horticulturae, 277*: 109803.

Jenkins Jr., S.F., & Wehner, T.C. (1983). A system for the measurement of foliar diseases of cucumber. *Cucurbit Genetics Cooperative Report,* 6: 10–12 (article 5).

Kinghorn, A.D., & Soejarto, D.D. (1985). Current status of stevioside as a sweetening agent for human use. In: Economic and medical plant research. Academic Press, London, UK. H. Wagner, H. Hikino, and N.R. Farnsworth, eds.

Kornienko, A.V., & Parfenov, A.M. (1996). Some Results of Work at the All-Russian Sugarbeet and Sugar Institute. *Sakharnaya Svekla*, (5): 6-7.

Kozik, E.U., Yücesan, B., Saravitz, C., & Wehner, T.C. (2020). Cold tolerance of diverse stevia cultigens under controlled environment conditions. *Agrosystems, Geosciences & Envi*r. 3(1): https://doi.org/10.1002/agg2.20120

Lee, J.I., Kang, K.H., & Park, H.W. (1982). New High Reb-audioside-A Stevia Variety 'Suweon 11'. *Research Reports*, *Office of Rural Development* (*Suweon Korea*), 24(C):186-188.

Lee, J.I., Kang, K.H., & Ham, Y.S. (1978). The High Yielding Stevia Variety 'Suweon 2'. *Annual research report, Office of Rural Development (Suweon Korea),* 21: 21.

Lorenzana, R.E., & Bernardo, R. (2009). Accuracy of genotypic value predictions for marker-based selection in biparental plant populations. *Theor Appl Genet,* 120: 151-161.

Luwańska, A, Perz, A., Mańkowska, & Wielgus, K. (2015). Application of *in vitro* Stevia (*Stevia rebaudiana* Bertoni) Cultures in Obtaining Steviol Glycoside Rich Material. *Kerba Rolonica*, 61(1): 50-63.

Miyazaki, Y., Watanabe, H., & Watanabe, T. (1978). Studies on the Cultivation of *Stevia rebaudiana* Bertoni. III Yield and Stevioside Content of 2-year-old Plants. *Eisi Shikenjo hokoku*., 96: 86-89.

Nakamura, S., & Tamura, Y. (1985). Variation in the Main Glycosides of Stevia. *Jpn. J. Trop. Agric.,* 29: 109-115.

de Oliveira, V.M., Forni-Martins, E.R., Magalhães, P.M., & Alves, M.N. (2004). Chromosomal and morphological studies of diploid and polyploid cytotypes pf *Stevia rebaudiana* (Bertoni) Bertoni (Eupatorieae, Asteraceae). *Genetics and Molecular Biology*, 27(2): 215-222.

Othman, H.S., Osman, M., & Zainuddin, Z. (2015). Morphological Assessment of Stevia rebaudiana Bertoni Accessions in the IIUM's Germplasm as Initial Material for Stevia Breeding. Austrialian J of Basic and Applied Sci., 9(25): 1-9.

Prakash, I., Markosyan, A., & Bunders, C. (2014). Development of Next Generation Stevia Sweetener: Rebaudioside M. *Foods*; 3(2): 162-175.

Ramesh, K., Singh, V., & Megeji, N.W. (2006). Cultivation of *Stevia* [*Stevia rebaudiana* (Bert.) Bertoni]: a comprehensive review. *Adv Argo*., 89(5): 137-177.

Raina, R., Bhandari, S. K., Chand, R., & Sharma, Y. (2013). Strategies to improve poor seed germination in *Stevia rebaudiana*, a low-calorie sweetener. *J. Medicinal Plants Res*., 7: 1793-1799.

Shafii, B., Vismen, R., Beaudry, R., Warner, R., & Jones, A.S. (2012). Large-scale Profiling of Diterpenoid Glycosides from *Stevia rebaudiana* Using Ultrahigh Performance Liquid Chromatography/Tandem Mass Spectrometry. *Anal. Biol. Chem*., 403: 2683-2690.

Shu, S.Z., & Wang, W.Z. (1988). Variation in Quantitative Characters in Stevia. *Acta Agronomica Sinica*, 14(2): 167-173.

Shu, S.Z. (1995). A Study on Good Variety Selection in *Stevia rebaudiana*. *Scientia Agricultura Sinica*, 28(2): 37-42.

Shu, S. (1989). *Stevia rebaudiana* Variety Trials. *Zuowu Pinzhong Ziyuan*, (1): 17-18.

Shyu, Y. T., Liu, S. Y., Lu, H. Y., Wu, W. K., & Su, C. G. (1994). Effects of Harvesting Dates on the Characteristics, Yield, and Sweet Components of Stevia (*Stevia rebaudiana* Bertoni) Lines. *Journal of Agricultural Research China,* 43:29–39.

Suhendi, D. (1989) Mass Selection of *Stevia rebaudiana* Bertoni. *Menara Perkebunan*, 56(4): 93-95.

Tateo, F., Mariotti, M., Bonini, M., Lubian, E., Martello, S., & Cornara, L. (1998). Stevioside Content and Morphological Variability in a Population of *Stevia rebaudiana* (Bertoni) Bertoni From Paraguay. *Italian Journal of Food Science*, 10(3): 261-267.

Tavarini, S., Passera, Guidi, L., & Angelini, L.G. (2015). Impact of Nitrogen Supply on Growth, Steviol Glycosides and Photosynthesis in *Stevia rebaudiana* Bertoni. *Plant Biosystems*, 150(5): DOI: 10.1080/11263504.2014.993743

Tavarini, S., Passera, & Angelini, L.G. (2018). Crop and Steviol Glycoside Improvement. In: Stevia by Breeding, in *Steviol Glycosides: Cultivation, Processing, Analysis and Applications in Food,* 1-31.

Weng, X.Y., Sun, J.Y., & Zang, R.C. (1996). Study on the Growth and Physiological Characteristics of *Stevia rebaudiana* SM4. *Journal of Zhejiang Agricultural University*, 22(5): 538-540.

Yadav, A.K., Singh, S., Dhyani, D., & Ahuja, P.S. (2011). A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)] *Can. J. Plant Sci*., 91: 1-27.

Yadav, S.K., & Guleria, P. (2012). Steviol Glycosides from Stevia: Biosynthesis Pathway Review and Their Application in Foods and Medicine. *Crit Rev Food Sci Nutr,* 52: 988-998.

Zaidan, L.B.P., Dietrich, S.M.C., & Felippe, G.M. (1980). Effect of Photoperiod on Flowering and Stevioside Content in Plants of Stevia rebaudiana Bertoni. Japanese J of Crop Sci., 49(4): 569-574.

Table 3.1. Analysis of variance for parent genotypes in 2 locations (Clinton and Kinston, NC) in 2015. Degrees of freedom (df), sums of squares (SS), mean square error (MSE), F ratio (F), and probability (P) with a significance level of p>0.05. Results for early and late season subjective ratings for stem height, branch width, and leaf size, objectively measured stem height, branch width, leaf length, and leaf width (mm), and calculated canopy volume (mm^3) and leaf area (mm^2) ; Rebaudioside (Reb-)A, Reb-B, Reb-C, Reb-D, Reb-E, dulcoside-A, rubusoside, steviolbioside, stevioside, total steviol glycoside content (mg/g), and biomass reported as fresh and dry weight (Mg/ha).

Trait	Source of Variation	df	SS	MSE	F	P
Early Season Subjective Plant Height Rating	Location	3	67.7	33.83	24.18	6.00x10-11
	Genotype	23	488.4	21.24	15.18	< 2x10-16
	Location x Genotype	23	88.1	3.83	2.739	2.27x10-5
	Residuals	881	1232.8	1.4		
Early Season Subjective Branch Width Rating	Location	3	293.1	146.57	106.278	< 2x10-16
	Genotype	23	261.6	11.37	8.248	< 2x10-16
	Location x Genotype	23	46.7	2.03	1.472	0.071
	Residuals	881	1215	1.38		
Early Season Subjective Leaf Size Rating	Location	3	40.2.6	201.32	245.093	< 2x10-16
	Genotype	23	200.1	8.7	10.594	< 2x10-16
	Location x Genotype	23	29.6	1.29	1.568	0.044
	Residuals	881	723.7	0.82		
Late Season Subjective Plant Height Rating	Location	3	92.2	46.1	33.08	1.40x10-14
	Genotype	23	279.3	12.14	8.714	< 2x10-16
	Location x Genotype	23	61	2.65	1.902	0.007
	Residuals	881	1227.9	1.39		
Late Season Subjective Branch Width Rating	Location	3	47.7	23.832	16.52	9.05x10-8
	Genotype	23	110.8	4.819	3.34	2.38x10-7
	Location x Genotype	23	38.6	1.678	1.163	0.27
	Residuals	881	1270.9	1.443		
Late Season Subjective Leaf Size Rating	Location	3	13	6.502	5.701	0.003
	Genotype	23	94.2	4.097	3.592	3.29x10-8
	Location x Genotype	23	40.9	1.776	1.557	0.046
	Residuals	881	1004.8	1.14		

Table 3.1. (Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Objective Stem Height	Location	3	8637149	4318574	337.75	< 2x10-16
	Genotype	23	2565600	111548	8.724	< 2x10-16
	Location x Genotype	23	851414	37018	2.895	7.13x10-6
	Residuals	881	11264802	12786		
Objective Branch Width	Location	3	5845162	2922581	170.46	< 2x10-16
	Genotype	23	4119737	179119	10.45	< 2x10-16
	Location x Genotype	23	964811	41948	2.45	1.83x10-4
Objective Leaf Length	Location	3	496	248.2	2.46	0.086
	Genotype	23	7910	343.9	3.41	1.39x10-7
	Location x Genotype	23	2870	124.8	1.24	0.203
	Residuals	881	88875	100.9		
Objective Leaf Width	Location	3	403	201.41	8.156	3.09x10-4
	Genotype	23	2706	117.64	4.764	2.35x10-12
	Location x Genotype	23	816	35.49	1.437	0.084
	Residuals	881	21757	24.7		
Canopy Volume	Location	3	4.66x1018	2.33x1018	246.07	< 2x10-16
	Genotype	23	2.07x1018	9.01x1016	9.515	< 2x10-16
	Location x Genotype	23	6.34x1017	2.76x1016	2.91	6.33x10-6
	Residuals	881	8.34x1018	9.47x1015		
Leaf Area	Location	3	194041	97021	2.363	0.095
	Genotype	23	4327383	188147	4.583	1.05x10-11
	Location x Genotype	23	1424288	61926	1.508	0.06
	Residuals	881	36168339	41054		

Table 3.1. (Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Dulcoside A Content	Location	3	11.7	5.83	9.78	6.33x10-5
	Genotype	23	33.8	1.471	2.47	1.60x10-4
	Location x Genotype	23	14.4	0.625	1.05	0.402
	Residuals	881	525.5	0.596		
Reb-B Content	Location	3	33.19	16.594	64.45	< 2x10-16
	Genotype	23	14.45	0.628	2.441	1.91x10-4
	Location x Genotype	23	4.95	0.215	0.836	0.687
	Residuals	881	226.79	0.257		
Reb-D Content	Location	3	188.6	94.28	33.66	8.82x10-15
	Genotype	23	90	3.91	1.397	0.101
	Location x Genotype	23	62.1	2.7	0.963	0.512
	Residuals	881	2467.5	2.8		
Reb-E Content	Location	3	2.8	1.401	1.74	0.177
	Genotype	23	58.9	2.56	3.17	8.80x10-7
	Location x Genotype	23	25.3	1.102	1.37	0.118
	Residuals	881	711.5	0.808		
Steviolbioside Content	Location	3	23.72	11.86	106.63	< 2x10-16
	Genotype	23	4.8	0.209	1.88	0.008
	Location x Genotype	23	2.28	0.099	0.89	0.61
	Residuals	881	97.98	0.11		
Rubusoside Content	Location	3	0.068	0.034	1.24	0.29
	Genotype	23	0.53	0.023	0.84	0.682
	Location x Genotype	23	0.501	0.022	0.79	0.741
	Residuals	881	24.18	0.03		
Reb-A Content	Location	3	45170	22585	41.312	< 2x10-16
	Genotype	23	26870	1168	2.137	1.50x10-3
	Location x Genotype	23	33052	1437	2.63	5.04x10-5
	Residuals	881	481639	547		

Table 3.1. (Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Reb-C Content	Location	3	738	368.8	5	0.007
	Genotype	23	2480	107.8	1.46	0.076
	Location x Genotype	23	551	24	0.324	1
	Residuals	881	65111	73.9		
Stevioside Content	Location	3	12248	6124	11.023	1.87x10-5
	Genotype	23	48204	2096	3.772	7.84x10-9
	Location x Genotype	23	18185	791	1.423	0.899
	Residuals	881	489495	556		
Total Steviol Glycoside Content	Location	3	67328	33664	38.751	< 2x10-16
	Genotype	23	25600	1113	1.281	0.17
	Location x Genotype	23	23566	1025	1.179	0.254
	Residuals	881	765335	869		
Fresh Weight (Mg/ha)	Location	3	57	57.49	2.126	0.148
	Genotype	22	4663	211.96	7.839	4.32x10-14
	Location x Genotype	21	1659	79.01	2.922	1.49x10-4
	Residuals	110	2974	27.04		
Dry Weight (Mg/ha)	Location	3	1.1	1.07	0.095	0.759
	Genotype	22	1074.5	48.84	4.32	1.37x10-7
	Location x Genotype	21	295.8	14.09	1.246	0.229
	Residuals	110	1243.8	11.31		

Table 3.2. Analysis of variance for pooled offspring populations in 2 locations (Clinton and Kinston, NC) in 2016. Degrees of freedom (df), sums of squares (SS), mean square error (MSE), F ratio (F), and probability (P) with a significance level of p>0.05. Results for early and late season subjective ratings for stem height, branch width, and leaf size, objectively measured stem height, branch width, leaf length, and leaf width (mm), and calculated canopy volume (mm^3) and leaf area (mm^2); Rebaudioside (Reb-)A, Reb-B, Reb-C, Reb-D, Reb-E, dulcoside-A, rubusoside, steviolbioside, stevioside, and total steviol glycoside content (mg/g) and biomass reported as fresh and dry weight (Mg/ha).

Trait	Source of Variation	df	SS	MSE	F	P
Early Season Subjective Plant Height Rating	Location	3	184	61.33	153.99	< 2x10-16
	Genotype	99	137.7	1.39	3.492	< 2x10-16
	Location x Genotype	127	87.9	0.69	1.738	3.27x10-6
	Residuals	1069	425.8	0.4		
Early Season Subjective Branch Width Rating	Location	3	435.5	145.15	506.4	< 2x10-16
	Genotype	99	86.1	0.87	3.033	< 2x10-16
	Location x Genotype	127	102	0.8	2.801	< 2x10-16
	Residuals	1069	306.4	0.29		
Early Season Subjective Leaf Size Rating	Location	3	285.5	95.18	97.33	< 2x10-16
	Genotype	99	390	3.94	4.029	< 2x10-16
	Location x Genotype	127	161.2	1.27	1.298	0.0194
	Residuals	1069	1045.4	0.98		
Late Season Subjective Plant Height Rating	Location	3	168.8	56.27	68.64	< 2x10-16
	Genotype	99	208.2	2.1	2.565	1.90x10-13
	Location x Genotype	127	162.6	1.28	1.561	1.65x10-4
	Residuals	1069	876.4	0.82		
Late Season Subjective Branch Width Rating	Location	3	87.4	29.12	40.331	< 2x10-16
	Genotype	99	150.5	1.52	2.106	1.10x10-8
	Location x Genotype	127	151.6	1.194	1.653	2.27x10-5
	Residuals	1069	771.8	0.722		

Table 3.2.(Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Late Season Subjective Leaf Size Rating	Location	3	27.7	9.238	24.473	2.55x10-15
	Genotype	99	100.4	1.014	2.686	8.98x10-15
	Location x Genotype	127	76	0.599	1.586	9.77x10-05
	Residuals	1069	403.5	0.377		
Objective Stem Height	Location	3	1334303	444768	33.01	< 2x10-16
	Genotype	99	3572563	36086	2.678	1.10x10-14
	Location x Genotype	127	2904865	22873	1.698	8.38x10-6
	Residuals	1069	14403907	13474		
Objective Branch Width	Location	3	827904	275968	20.714	4.76x10-13
	Genotype	99	3179868	32120	2.411	8.49x10-12
	Location x Genotype	127	3144175	24757	1.858	1.77x10-7
	Residuals	1069	14242371	13323		
Objective Leaf Length	Location	3	82812	27604	158.339	< 2x10-16
	Genotype	99	23435	237	1.358	0.0143
	Location x Genotype	127	24093	190	1.088	0.2488
	Residuals	1069	186363	174		
Objective Leaf Width	Location	3	3667	1221.9	45.972	< 2x10-16
	Genotype	99	5282	53.4	2.007	9.69x10-8
	Location x Genotype	127	4055	31.9	1.201	0.0737
	Residuals	1069	28413	26.6		
Canopy Volume	Location	3	1.09x1017	3.62x1016	5.172	1.50x10-3
	Genotype	99	1.84x1018	1.85x1016	2.649	2.31x10-14
	Location x Genotype	127	1.76x1018	1.39x1016	1.98	7.75x10-9
	Residuals	1069	7.48x1018	7.00x1015		

Table 3.2.(Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Leaf Area	Location	3	21258420	7086140	102.527	< 2x10-16
	Genotype	99	10078249	101800	1.473	2.64x10-3
	Location x Genotype	127	10223873	80503	1.165	0.114
	Residuals	1069	73884071	69115		
Dulcoside A Content	Location	3	283.1	94.37	56.026	< 2x10-16
	Genotype	99	1531.4	15.47	9.184	< 2x10-16
	Location x Genotype	127	785.6	6.19	3.672	< 2x10-16
	Residuals	1069	1800.6	1.68		
Reb-B Content	Location	3	0.48	0.16	1.742	0.157
	Genotype	99	20.7	0.209	2.272	2.35x10-10
	Location x Genotype	127	35.7	0.281	3.054	< 2x10-16
	Residuals	1069	98.38	0.092		
Reb-D Content	Location	3	408	136.1	26.169	2.45x10-16
	Genotype	99	1784	18.02	3.465	< 2x10-16
	Location x Genotype	127	851	6.7	1.289	0.022
	Residuals	1069	5560	5.2		
Reb-E Content	Location	3	80.4	26.785	16.846	1.07x10-10
	Genotype	99	451.9	4.565	2.871	< 2x10-16
	Location x Genotype	127	149.2	1.175	0.739	0.984
	Residuals	1069	1699.7	1.59		
Steviolbioside Content	Location	3	5.46	1.8184	24.415	2.77x10-15
	Genotype	99	17.54	0.1772	2.379	1.85x10-11
	Location x Genotype	127	12.06	0.0949	1.275	0.0273
	Residuals	1069	79.62	0.075		

Table 3.2.(Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Rubusoside	Location	3	1.646	0.549	25.397	7.11x10-16
Content	Genotype	99	8.082	0.0816	3.779	< 2x10-16
	Location x Genotype	127	5.416	0.0426	1.974	9.04x10-9
	Residuals	1069	23.093	0.0216		
Reb-A	Location	3	98019	32673	52.144	< 2x10-16
Content	Genotype	99	263599	2663	4.249	< 2x10-16
	Location x Genotype	127	104236	821	1.31	0.0162
	Residuals	1069	669822	627		
Reb-C	Location	3	32422	10807	73.113	< 2x10-16
Content	Genotype	99	77950	787	5.327	< 2x10-16
	Location x Genotype	127	25643	202	1.366	6.56x10-3
	Residuals	1069	158018	148		
Stevioside	Location	3	6923	2307.6	16.206	2.63x10-10
Content	Genotype	99	35194	355.5	2.497	1.04x10-12
	Location x Genotype	127	21345	168.1	1.18	0.095
	Residuals	1069	152215	142.4		
Total Steviol	Location	3	122825	40942	48.235	< 2x10-16
Glycoside Content	Genotype	99	252535	2551	3.005	< 2x10-16
	Location x Genotype	127	152868	1204	1.418	2.65x10-3
	Residuals	1069	907368	849		

Table 3.2.(Continued).

Trait	Source of Variation	df	SS	MSE	F	P
Fresh Weight (Mg/ha)	Location	3	2863	954.3	24.972	1.15x10-14
	Genotype	121	22440	185.5	4.853	< 2x10-16
	Location x Genotype	144	7927	55	1.44	0.004
	Residuals	351	13414	38.2		
Dry Weight (Mg/ha)	Location	3	326.2	108.72	32.416	< 2x10-16
	Genotype	121	1206.4	9.97	2.973	2.12x10-15
	Location x Genotype	144	593.7	4.12	1.229	0.065
	Residuals	351	1177.2	3.35		

Table 3.3. Parent-offspring regression in 2 locations (Clinton and Kinston, NC) and 2 years (2015, 2016). The coefficients of regression are listed for early and late season subjective ratings for stem height, branch width, and leaf size, objectively measured stem height, branch width, leaf length, and leaf width (mm), and calculated canopy volume (mm^3) and leaf area (mm^2); Rebaudioside (Reb-)A, Reb-B, Reb-C, Reb-D, Reb-E, dulcoside-A, rubusoside, steviolbioside, stevioside, and total steviol glycoside content (mg/g) and biomass reported as fresh and dry weight (Mg/ha). Significance level of $p<0.05$.

Trait	Intercept	Slope	h2	Adjusted R2	F	P
Early Season Subjective Plant Height Rating	1.877	0.038	0.076	0.011	2	0.161
Early Season Subjective Branch Width Rating	2.186	0.034	0.069	0.006	1.543	0.217
Early Season Subjective Leaf Size Rating	4.51	0.093	0.185	0.033	3.993	0.049
Late Season Subjective Plant Height Rating	5.934	0.031	0.061	-0.006	0.459	0.5
Late Season Subjective Branch Width Rating	5.72	0.003	0.006	-0.011	0.005	0.944
Late Season Subjective Leaf Size Rating	5.175	0.029	0.058	-0.002	0.829	0.365
Objective Stem Height	545.234	0.154	0.308	0.09	9.81	0.002
Objective Branch Width	433.068	0.069	0.138	0.011	2.003	0.161
Objective Leaf Length	49.144	0.113	0.226	0.028	3.536	0.063
Objective Leaf Width	15.916	0.162	0.325	0.166	18.76	3.91x10-5
Canopy Volume	7.88x107	3.15x10-2	6.30x10-2	-0.006	0.45	0.504
Leaf Area	488.022	0.122	0.245	0.059	6.538	0.01227

Table 3.3. (Continued).

Trait	Intercept	Slope	h2	Adjusted R2	F	P
Dulcoside A Content	2.101	0.126	0.253	0.035	4.27	0.042
Reb-B Content	0.511	0.036	0.071	0.033	4.012	0.048
Reb-D Content	-0.193	0.202	0.404	0.335	45.89	1.36x10-9
Reb-E Content	0.725	0.168	0.335	0.206	24.07	4.22x10-6
Steviolbioside Content	0.511	0.028	0.055	-0.006	0.472	0.494
Rubusoside Content	0.179	0.117	0.234	0.018	2.653	0.107
Reb-A Content	37.391	0.279	0.557	0.266	33.33	1.15x10-7
Reb-C Content	10.426	0.247	0.494	0.278	35.33	5.49x10-8
Stevioside Content	25.37	0.109	0.219	0.207	24.28	3.88x10-6
Total Steviol Glycoside Content	95.341	0.102	0.204	0.051	5.772	0.018
Fresh Weight (Mg/ha)	20.756	0.011	0.022	0.02	0.03	0.867
Dry Weight (Mg/ha)	5.283	0.003	0.005	0.174	0.011	0.918

Table 3.4. Trait Correlation of Parent and Offspring Generations. Pooled populations for agronomic traits: early (E) and late (L) season subjective (Sub.) ratings for stem height (SH), branch width (BW), leaf size (LS), and objective (Obj.) measurements for stem heigh (SH), branch width (BW), leaf length (LL), leaf width (LW), and calculated canopy volume (CV) and leaf area (LA); and steviol glycoside content (mg/g) of dulcoside-A (Dulc-A), rebaudioside (reb)-B, reb-D, reb-E, steviobioside (Bios.), rubusoside (Rub.), reb-A, reb-C, stevioside (Stev.), and total steviol glycoside content (TSG).

										2015														
				Sub. E	Sub. E	Sub. E	Sub. L	Sub. L	Obj.	Obj.	Obj.													
2016	SH	BW	LS	SH	BW	LS	SH	BW	LL	LW	CV	LA	Dulc-A	Reb-B	Reb-D	Reb-E	Bios.	Rub.	Reb-A	Reb-C	Stev.	TSG
Sub. E SH	1.00	0.70	0.08	0.13	0.06	0.09	0.25	0.07	0.17	0.19	0.12	0.20	0.09	-0.03	-0.14	0.00	0.08	0.07	-0.22	-0.06	0.10	-0.21
Sub. E BW		1.00	0.14	0.11	0.02	0.06	0.27	0.02	0.27	0.22	0.10	0.27	0.15	-0.06	-0.20	0.01	0.15	0.14	-0.26	-0.08	0.11	-0.26
Sub. E LS			1.00	0.30	0.23	0.34	0.20	0.16	0.04	0.21	0.15	0.13	-0.01	-0.11	-0.11	0.05	0.05	0.05	-0.08	-0.02	0.14	-0.05
Sub. L SH				1.00	0.52	0.40	0.71	0.49	0.11	0.10	0.51	0.10	-0.04	-0.06	0.02	0.08	0.07	0.09	0.02	-0.14	0.18	0.01
Sub. L BW					1.00	0.40	0.39	0.57	-0.01	0.05	0.51	0.01	0.03	-0.10	-0.08	0.00	0.03	-0.02	-0.03	0.02	0.12	0.01
Sub. L LS						1.00	0.28	0.25	0.19	0.32	0.23	0.27	0.02	-0.09	-0.08	-0.01	0.01	-0.03	-0.03	-0.02	0.10	-0.02
Obj. SH							1.00	0.46	0.25	0.18	0.60	0.22	0.04	-0.07	-0.06	0.08	0.04	0.09	-0.11	-0.09	0.14	-0.10
Obj. BW								1.00	0.03	0.06	0.92	0.05	0.10	-0.09	-0.06	-0.02	-0.04	-0.07	-0.03	0.08	0.07	0.03
Obj. LL									1.00	0.66	0.08	0.88	0.01	-0.02	-0.08	-0.04	0.08	0.02	-0.08	-0.04	0.01	-0.11
Obj. LW										1.00	0.09	0.92	0.02	-0.08	-0.18	0.07	0.07	-0.03	-0.20	0.06	0.03	-0.17
CV											1.00	0.09	0.11	-0.09	-0.07	0.01	-0.02	-0.03	-0.07	0.05	0.09	-0.02
LA												1.00	0.03	-0.05	-0.15	-0.07	0.08	0.00	-0.15	0.01	0.03	-0.15
Dulc-A													1.00	0.06	-0.21	-0.17	0.01	-0.09	-0.10	0.49	0.02	0.17
Reb-B														1.00	0.48	-0.30	-0.13	0.05	0.52	0.10	-0.14	0.57
Reb-D															1.00	0.07	-0.29	0.00	0.47	-0.24	-0.19	0.43
Reb-E																1.00	0.17	0.65	-0.28	-0.27	0.16	-0.28
Bios.																	1.00	0.38	-0.24	-0.13	0.40	-0.17
Rub.																		1.00	-0.18	-0.23	0.43	-0.08
Reb-A																			1.00	-0.12	-0.18	0.85
Reb-C																				1.00	-0.26	0.26
Stev.																					1.00	0.06
TSG																						1.00

Table 3.5. Trait Correlation of 6-plant (6-plant) Plot and 30-plant (30-plant) Plot Densities. Agronomic traits: subjective (Sub.) ratings for early (E) and late (L) season stem height (SH), branch width (BW), leaf size (LS), and objective measurements of stem height (SH), branch width (BW), leaf length (LL), and leaf width (LW), and calculated canopy volume (CV) and leaf area (LA).

		6-plant plot											
30-plant plot		Sub. E SH	Sub. E BW	Sub. E LS	Sub. L SH	Sub. L BW	Sub. L LS	Obj. SH	Obj. BW	Obj. LL	Obj. LW	CV	LA
Sub. E	SH	0.73	0.66	0.15	-0.10	-0.13	0.15	0.04	0.01	0.02	0.19	0.06	0.12
Sub. E	BW	0.63	0.62	0.19	-0.07	-0.10	0.10	0.03	-0.02	0.07	0.17	0.02	0.13
Sub. E	LS	0.07	0.11	0.20	0.11	0.10	0.20	0.02	0.01	0.07	0.20	0.00	0.14
Sub. L	SH	0.08	0.12	0.11	0.10	0.08	0.16	0.12	0.05	0.16	0.19	0.07	0.18
Sub. L	BW	0.12	0.10	0.03	0.04	-0.05	-0.02	0.05	-0.01	0.12	0.08	0.01	0.11
Sub. L	LS	-0.08	-0.05	0.12	0.11	0.14	0.21	0.10	0.01	0.12	0.23	0.02	0.18
Obj.	SH	0.18	0.23	0.13	0.06	0.01	0.10	0.24	0.04	0.23	0.19	0.13	0.22
Obj.	BW	0.19	0.15	-0.01	-0.04	-0.03	0.04	0.13	0.07	0.04	0.05	0.12	0.04
Obj.	LL	-0.15	-0.16	0.02	0.15	0.16	0.20	0.10	0.02	0.11	0.16	0.02	0.14
Obj.	LW	0.03	0.00	0.05	0.05	0.09	0.26	0.06	-0.03	0.09	0.25	-0.02	0.19
CV		0.22	0.19	0.05	-0.01	-0.03	0.06	0.18	0.08	0.08	0.08	0.14	0.08
LA		-0.03	-0.06	0.04	0.08	0.10	0.26	0.09	-0.02	0.10	0.24	-0.01	0.20

APPENDIX

Appendix A

Stevia Plant Diseases

Seedling diseases observed from germination experiments and transplant production:

Rhizoctonia sp.

Pythium sp.

Botrytis cinerea

Alternaria sp.

Septoria steviae

Saprophyte:

Slim mold sp.

Diseases observed during field research:

Alternaria sp.

Athelia rolfsii

Fusarium oxysporum

Septoria steviea

Sclerotinia sclerotiorum

Macrophomina phaseolina

Tomato Spotted Wilt Virus