ABSTRACT

HUBER, BRANDON MICHAEL. Studies on Stevia (*Stevia rebaudiana*). (Under the Direction of Dr. Todd Wehner.)

Stevia rebaudiana (Bertoni) is a valuable natural sweetener, 300 times sweeter than sucrose but containing zero calories (Yao et al., 1999). Stevia, a herbaceous perennial of the Asteraceae family and native to Paraguay, has potential as a crop for North Carolina and many other subtropical areas of the world. Leaves of stevia contain diterpene steviol glycosides that are non-nutritive and non-toxic with high potency when compared to sucrose, and can potentially replace artificial sweeteners. However, the highly desirable steviol glycosides rarely occur at a high concentration, but breeding can improve these steviol glycosides.

Field trials were conducted using 25 different cultigens of *S. rebaudiana* (Bertoni) to measure agronomic traits including plant size, leaf size, yield, disease and lodging resistance, and glycoside profiles in 2015 and 2016. Cultigens differed across many of these traits among trials. Year and location were significant for some traits, for example stem height, indicating that differences in soil type and rainfall affected growth of the various stevia cultigens.

High yielding cultigens were identified, including ‘Katupyry’, NC-1009 & NC-1028 (Jung), ‘Eirete I’, and ‘Eirete II’. NC-1022 (Seed Savers), and NC-1010 (R.H. Shumway) had the highest rebaudioside A level and the highest ratio of rebaudioside A to stevioside, but only a moderate yield. Cultigens also differed in resistance to lodging and several diseases present in the trial locations. NC-1024 (Park Seed) had the lowest ratings for lodging among cultigens and NC-1023 (Johnny's) and NC-1021 (Territorial) had the lowest occurrence of foliar disease caused by *Septoria steviae* Ishiba. Cultigens differed in height (tallest being Swallowtail NC-1032) and plant width (‘Eirete-II’ was most branched). Some cultigens also produced exceptionally large leaves (Everstevia, NC-1011).

Pearson correlations on agronomic and glycoside traits were generally low, however there was a high correlation between fresh and dry yield. A strong negative correlation was observed between percent rebaudioside A and percent stevioside (-0.93).

Heritability of agronomic traits and glycoside yield were measured in four elite *S. rebaudiana* (Bertoni) breeding populations from elite selections from the cultigens grown in 2015. Heritability estimates across the four populations ranged from low to high for agronomic traits. Estimates of heritability for glycosides were high when heritability was measured in both percent of total steviol glycosides and in mg/g of dry weight, including rebaudioside A, rebaudioside C, rebaudioside D, stevioside, and total steviol glycosides. Yield, in dry weight, had moderate heritability (0.41) with a gain resulting in 1.19 Mg/ha per breeding cycle at 20% selection.

Finally, gain from selection (20%) was estimated for agronomic and glycoside characteristics. Gain for subjective and objective measurements 50 days after planting were moderate to high for plant size, stem height, and plant width, but low for leaf size. Late-season ratings at 100 days after planting showed lower heritability and gain than when estimated in June. Gain in yield (dry weight) was at 1.19 Mg/ha per breeding cycle; however, gain per breeding cycle for percent survival, and lodging resistance were low. Gains were moderate to high for the glycoside compounds rebaudioside A, rebaudioside C, rebaudioside D, stevioside, and total steviol glycosides at 20% selection.

Overall, agronomic traits in stevia have variable heritabilities and gain of selection, but glycoside traits have moderate to high heritability and gain from selection. As a result, much gain in these desirable traits could result per breeding cycle.

Studies on Stevia (*Stevia rebaudiana*)

by
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BIOGRAPHY

Brandon Huber was born in Philadelphia, Pennsylvania on the 18th of April 1989 to Ronald and Judith Huber. At an early age, his parents and grandparents had an influence on my passion of horticulture. Despite growing up in a large city, plants fascinated him and he surrounded himself with them by growing many plants in containers in a small city yard. He also started collecting and growing many rare plants including giant pumpkins. By the time he was a teen he began exhibiting award-winning plants in the Philadelphia Flower show and was noted for the blooming of bizarre plants such as *Amorphophallus* species. After many years of culture, he bloomed the world's largest flowering species *Amorphophallus titanum* (Beccari) currently housed at NC State. As an undergraduate he focused his studies on ornamental horticulture which eventually led to my interest in plant breeding and admittance to the Horticulture program at North Carolina State University under the direction of Dr. Todd Wehner. After he finishes his M.S. in plant breeding his desire is to continue his education and better my horticultural skills via a Ph.D. program at North Carolina State University. He looks forward to using my skills, knowledge, and the connections made during his M.S. degree to his benefit as he pursues a Ph.D.

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CHAPTER 1: A REVIEW OF STEVIA BREEDING AND PRODUCTION

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Introduction

Stevia rebaudiana (Bertoni) is of value as a natural sweetener because it contains zero calories and is 300 times sweeter than sucrose (Yao, Ban & Brandle, 1999). The genus *Stevia* consists of 230 species of herbaceous plants, but *S. rebaudiana* (Bertoni), a native of Paraguay, is of most interest as a potential sweetener (Soejarto et al. 1983). Leaves of stevia contain diterpene steviol glycosides, which include steviosides and rebaudiosides. These diterpenes are produced naturally in the plant and may be a defense mechanism or repellent to predators (Smith and Van-Stadin, 1992, Nanayakkara, 1987); however, their function(s) is still largely unknown (Yadav et al, 2010). Steviol glycosides are non-nutritive and non-toxic, and have high potency so they can replace the use of artificial sweeteners with a naturally-occurring one (Yadav et al, 2010; Hutapea, 1997) that does not metabolize in the human body (Strauss, 1955). Stevia first became useful as a sweetener in Japan when it was commercialized in the 1970s (Kinghorn and Soejarto, 1985). One downside, however, is the bitterness or off-taste that some people experience when used in products. Ingredient suppliers that utilize sweeteners would like to increase desirable steviol glycosides without a bitter aftertaste, unlike stevioside (Yadav, 2010), to maximize the useful properties of stevia in products. Rebaudioside A is the most studied rebaudioside and is the main compound of this class that is extracted and processed for commercial use. Stevia is now available for sale on the market as either powdered extracts or in whole leaf form for usage in food, candies, and beverages.

Currently, desirable steviol glycoside ratios, such as high rebaudioside A to stevioside, rarely occur in leaves but breeding can improve levels of the desirable glycoside compounds. Since commercialization, stevia usage has increased. In addition, previous limitations affecting

commercialization involved bans from the Food and Drug Administration (Bespalhok-Filho and Hattori, 1997). Plant breeding of stevia is beginning to occur worldwide, and programs are operating in China, Japan, India, Brazil, Canada and in the United States of America. Oregon State University and Fort Valley State University in Georgia are also involved in breeding stevia. Sweet Green Fields Co. was at onetime involved with the production of stevia in North Carolina.

Improved cultivars of stevia are available for production around the world. However, there is limited research in trait discovery. There is also limited research on methods for effective breeding, determining qualities of interest, and estimating heritability for key traits such as glycoside content. A breeding method for stevia could be adopted from other cross-pollinated crops such as sunflower and chrysanthemum which also are members of the *Asteraceae* family.

Stevia production is projected to increase dramatically over the next few years around the world. Stevia has much potential as a crop for North Carolina and many other sub-tropical climates of the world. Stevia is a perennial or small shrub of the *Asteraceae* family and can be grown and harvested for many years as a perennial in climates where annual low temperatures do not fall below -7 Celcius (Yadav, et al., 2010). Stevia plants regenerate from basal shoots annually, either naturally in the spring or after it has been cut after harvest.

Seeds typically display a low rate of germination (Shock, 1982; Duke, 1993; Carneiro et al., 1997; Lester, 1999), which prevent direct-sowing and require plants to be grown as transplants. Sourcing quality seed is a challenge because stevia seed has a short shelf life (Brandle et al. 1998a). Stevia seeds have small endosperms and are wind dispersed in nature. A contributor to the poor seed germination may be that seed is harvested before maturity (Colombus, 1997), but also timing of flowering and pollination play a role as well (Melis and Sainati 1991; Strauss, 1995). Seeds are either black or tan in color. The black seeds had 59-86% germination rate and tan had 0% germination in a study by Raina et al. in 2013. Studies on seed production and fertility show that selection for high germination is possible (Carneiro and Guedes 1992). Seed yield of up to 8.1 kg/ha have been recorded and one ha of plants will plant approximately 200 ha for leaf production (Lester, 1999).

LED lighting was reported to influence the germination rate of stevia seeds when controlling the light spectrum (Simlat, 2016). Blue LED wavelength (430-485nm) was shown to confer enhanced seed germination in comparison to red light (620-660nm). Blue LED light was also found to improve growth of leaves and roots of young stevia plants. However, a red LED wavelength (620-660nm) increased stem length and roots but had the least effect on synthesis of chlorophyll and carotenoids. Blue and red light were found to have an opposite effect on the activity of antioxidant enzymes. The highest fresh weight among the experiment was found where a combination of red and white LED lighting was used. Addition of CO_2 at 700 ppm can optimize production of glycosides and yield by 25% in northern climates where greenhouse production is used (Weber, 2016).

Taxonomy

Stevia is found in the tribe *Eupatorium* among the large family of *Asteraceae* containing 950 genera (Soejarto, 1983; Lester, 1999). The genus has approximately 230 species of herbaceous, or shrub like plants (Gentry, 1996

The taxonomic classification of *S. rebaudiana* (Bartoli) is as follows:

Kingdom	Plantae
Subkingdom	*Tracheobionta*
Superphylum	*Spermatophyta*
Phylum	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Asteridae*
Group	*Monochlamydae*
Order	*Asterales*
Family	*Asteraceae* (Compositae)
Subfamily	*Asteroideae*
Tribe	*Eupatorieae*
Genus	*Stevia*
Species	*rebaudiana*

S. rebaudiana offers the most sweetening properties of the genus, whereas other species are noted for containing interesting biochemicals (Frederico, 1996). Some other species include *Stevia plummerae* (A. Gray), *Stevia micrantha* (Lag)., *Stevia serrata* (Cav.), *Stevia lemmonii* (A. Gray), *Stevia ovata*

(Willd.), *Stevia anisostemma* (Turcz.), *Stevia salicifolia* (Cav.), *Stevia bertholdii* (B. L. Rob.), *Stevia crenata* (Benth), *and Stevia origanoides* (H.B. & K.).

Cytogenetics

In Mexico 244 stevia accessions were collected and classified into 73 species with a monoploid chromosol number of 11 or 12 with ploidy levels of 2x, 3x, 4x, 5x, and 6x (Watanabe et al., 2001). Eleven chromosome pairs were found in a cytogenetic study, with lengths of 1.70 to 3.76 µm (Cimpeanu et al., 2005).

Polyploidy can be useful in plant breeding as a way of doubling the production of genes in a plant and can be a valuable tool in genetic improvement (Thao et al. 2003). A study conducted on colchicine-induced stevia polyploids resulted in significant differences in traits such as leaf thickness, fresh, and dry leaf weight and leaf area (Hedge et al. 2015). In addition, delayed flowering would be a valuable characteristic in stevia, allowing for later and potentially larger yields. Leaf area and yield was greater with the polyploid than in the diploid control.

Breeding of polyploids could be valuable as they have larger leaves, and potential for higher glycoside content than the standard diploid (Sanyo, 1990; Shuichi et al. 2001). Breeding of triploids was conducted by crossing a tetraploid with high rebaudioside A content with a diploid of high rebaudioside A, resulting in triploid progeny that had higher rebaudioside A content than either parent. Eight triploid cultivars were obtained and categorized by leaf shape based on needle-like or wide needle-like leaves. There was a correlation with rebaudioside A and stevioside content based on the leaf shape of triploids (Hata, et al. 2001). Cultivars with wide needle-like foliage resulted in high stevioside content, whereas needle-like foliage had high content of rebaudioside A across various plants. Therefore, phenotypic screening for glycosides like stevioside and rebaudioside A is possible (Hata, et al. 2001). Triploids are additionally valuable to a plant breeder due to sterility, making it difficult for others to use their lines in breeding (Allard, 1960). Therefore, polyploidy could be an additional tool used in developing elite lines with higher yield and or higher glycoside content.

Qualitative and Quantitative Genetics

Genotype, environment, cultural practices and propagation play a role in the content of steviol glycosides and leaf yield in stevia (Tavarini et al., 2010). Broad-sense heritabilities were measured for yield and quality across ten cultivars at one location and three replications. Heritability for leaf yield was 99%, 93% for stevioside content, and 97% for plant height (Gaurav et al., 2008). In a previous study, heritability was 76.6% for stevioside, 62.1% for leaf yield and 78.8% for leaf/stem ratio (Brandle, 1999). This study provides an estimate of heritability and suggests that high heritabilities such as these should allow for substantial gains. (Brandle and Rosa, 1992).

A study conducted by Othman, et al. (2015) with 14 stevia accessions from Malaysia and Paraguay found that most of the evaluated accessions were morphologically different, which may suggest genetic divergence among accessions. In that study evaluations included plant height, number of branches, and leaf yield. Leaf yield is a valuable trait in stevia, because the desired glycosides are derived in highest quantity from the leaves. The authors also found that a tall plant or multi-branched plant does not necessarily yield more leaves. Sufficient genetic variation exists among stevia accessions that can be used for future breeding to develop elite lines (Sakaguchi and Kan, 1992). A similar cultivar trial was conducted on stevia yield and glycosides in the western United States using the same cultivars across many diverse regions. Leaf yield for cultivars were found to have significant differences across different locations. Rebaudioside A differences were found as well among cultivar and location by cultivar interaction (Parris, C. Shock, C. Qian, M., 2016).

Two crosses using different parents with similar glycoside profiles were evaluated for genetics of steviol glycosides and significant reciprocal differences were not found (Brandle et al. 1998). Rebaudioside A content was controlled by a single dominant gene in the first cross, and another single gene in the second cross. In the first cross rebaudioside A and rebaudioside C were highly correlated. However, in the second cross rebaudioside C was highly correlated when stevioside and rebaudioside A had low correlation. Despite no reciprocal differences found here, additional crosses and additional steviol glycoside compounds should be evaluated for genetic effects.

Correlations of traits can aid in selection and suggest likelihood of genetic gain in a crop (Yadav et al, 2010). Plant height and leaf yield were shown to have a positive correlation with overall biomass (Buana and Goenadi, 1985). However, in another study, plant height had no significant correlation with yield (Buana, 1989). Stevioside was found to be uncorrelated with yield, (Brandle and Rosa, 1992), whereas rebaudioside A was correlated with yield (Shyu, 1994). Total stevioside content also was correlated with a high leaf to stem ratio (Tateo et al. 1998). Dry leaf yield was correlated with branch number of a given plant, leaf count, plant height and dry matter accumulation (Chalapathi et al. 1998). Leaf dry weight showed to have the most influence on yield (Shu and Wang, 1988). Leaf area was observed to have a correlation with stevioside content (Truong et al. 1999). High rebaudioside A content was correlated with large leaves (Weng et al. 1996). Rebaudioside A and rebaudioside C were positively correlated with each other, and dulcoside A and stevioside were positively correlated (Nakamura and Tamura, 1985). Additionally, they found stevioside was not correlated with rebaudioside A, and dulcoside A was not correlated with rebaudioside C (Nakamura and Tamura, 1985). Correlations such as high rebaudioside A and yield offer breeders promising results when breeding for one or the other trait.

Molecular Genetics

A linkage map of stevia was created with 183 Random Amplified Polymorphic DNA (RAPD) markers with 21 linkage groups covering 1389 cM (Yao et al.,1999). However, as much as 1250 cM has yet to be mapped. The authors found 35.5% polymorphism and 62.5% 1:1 segregation for the markers. Currently simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers have replaced RAPDs, so this RAPD map has minimal use at this point because new advanced technologies are more efficient and provide more information. RAPD markers are also difficult to replicate and therefore are not often accepted in journal articles anymore. However, the previous linkage map suggests high level of polymorphism in the genome of stevia despite its limited coverage of the genome.

Marker-assisted selection has been deployed in most major crops and would help aid in more efficient breeding progress in stevia (Staub et al. 1996). New advanced technologies such as next generation sequencing and transcriptome can be more efficient in characterizing the genomics of stevia. Chen et al. (2014) using RNA-Seq found 143 UDP-glucosyltransferase unigenes, including those likely involved in steviol glycoside biosynthesis. This indicates that new technologies such as RNA-Seq can be useful in species that have not been explored.

Steviol Glycoside Pathway

Considerable work has been done to elucidate the biosynthetic pathway of glycosides in stevia, where roughly half of the steps of the steviol glycoside pathway (Brandle et al., 2002). At least eight different steviol glycosides were found to accumulate within the leaves of stevia and their production branches of the pathway responsible for gibberellic acid biosynthesis. Kaurene synthase (KS) and copalyl diphosphate synthase (CPS) are intermediate steps shared by both pathways, with the gene that produces KS being duplicated in stevia (Richman et al., 2005). The synthesis in the mesophyll cells occurs via the same pathway as gibberellic acid until the step of Kaurenoic acid synthesis. The conversion of kaurenoic acid to steviol by the enzyme kaurenoic acid hydroxylase commits the flow of metabolites toward steviol glycoside production. Steviol is further metabolized through a series glycosylation reactions (and to a lesser degree rhamnosylation and xylosylation) to produce the array of sweet compounds classified as steviol glycosides (Yadav et al.,2010). The specific quantity and arrangement of the sugar groups influences the taste perception; thus, a knowledge of the enzymes involved in the synthesis can ultimately be used to help control flavor (DuBois and Stephenson, 1984). UDP-glycosyltransferases (UGTs) are a group of enzymes responsible for transferring a sugar residue from activated donors to an acceptor molecule. Identification of UGTs in plant species such as Arabidopsis, has allowed for rapid progress towards understanding functional characteristics within that species. Further understanding of glycosyltransferase function in other species such as stevia is important, because many sugar-

containing secondary metabolites are produced in a host of plant species that are not found in model systems like Arabidopsis. The UGTs responsible for the formation of steviol glycosides are unique in comparison to other species that do not produce these sweet compounds.

Expressed sequence tags (ESTs) are effective tools in gene discovery (Brandle et al., 2002; Ohlrogge and Benning, 2000), and have helped elucidate key genes in novel metabolic pathways for other crops such as the identification genes responsible for isoprenoid biosynthesis in peppermint (Lange and Croteau, 1999; Lange et al. 1998, 2000). By characterizing ESTs in stevia that showed homology to UGTs from other species, three different classes of genes that code for enzyme activities toward the glycosylation of steviol were identified, and designated UGT74G1, UGT76GI, and UGT85C2. The specific placement of these genes within the steviol glycoside pathway is shown in the figure below from the manuscript of Richmond et al. (2005). Further application of the powerful tools of ESTs, or more current transcriptome-based methodologies such as RNA-seq, can be applied to determining genes responsible for additional glucosylation reactions in stevia.

The chemical structure of steviol contains two hydroxyl groups, one attached to the C-19 of the C-4 carboxyl and the other attached to the C-13. Both can be further glycosylated to yield 19- and 13- monosides and a 13, 19 bioside, rubusoside. Additional glycosylation of the 13-O-glucoside at the C-2’ and C-3’ produces a mixture of mono-, di-, tri-, and tetra-glycosides that can be found in the leaves of stevia (Brandle et al., 1998; Starrat et al., 2002). However, most steviol glycosides found in the leaves of stevia include the tetra-glycoside rebaudioside A and the tri-glycoside stevioside.

The pathway for Steviol glycoside synthesis begins with steviol. Glucosylation occurs at the C-13 alcohol, yielding steviolmonoside then a glucose is added at the C-2’ position of the 13-O-glucoside which forms steviolbioside (Shibata et al., 1991). It is thought that stevioside is then produced by glycosylation to position C-19 on the C-4 carboxyl. Glucosylation at this step appears to be critical for glycoside transportation to the vacuole, as accumulation of glycosides occurs only following this step. The pathway is thought to continue with the C-3’ position of the 13-O-glucose of stevioside to form rebaudioside A (Shibata et al., 1991). It is also thought that rebaudioside A can be

produced via rebaudioside B, with steviolbioside as an intermediate step. Other minor glycosides can be formed by the addition of either a rhamnose or xylose moiety to steviolmonoside or rubusoside (Brandle et al., 1998: Starratt et al., 2002). Most of the steviol glycosides are formed by four glucosylation reactions that start with steviol and end with rebaudioside A where other minor glycosides can be formed by the addition of a rhamnose or xylose moiety (Richman et al., 2005).

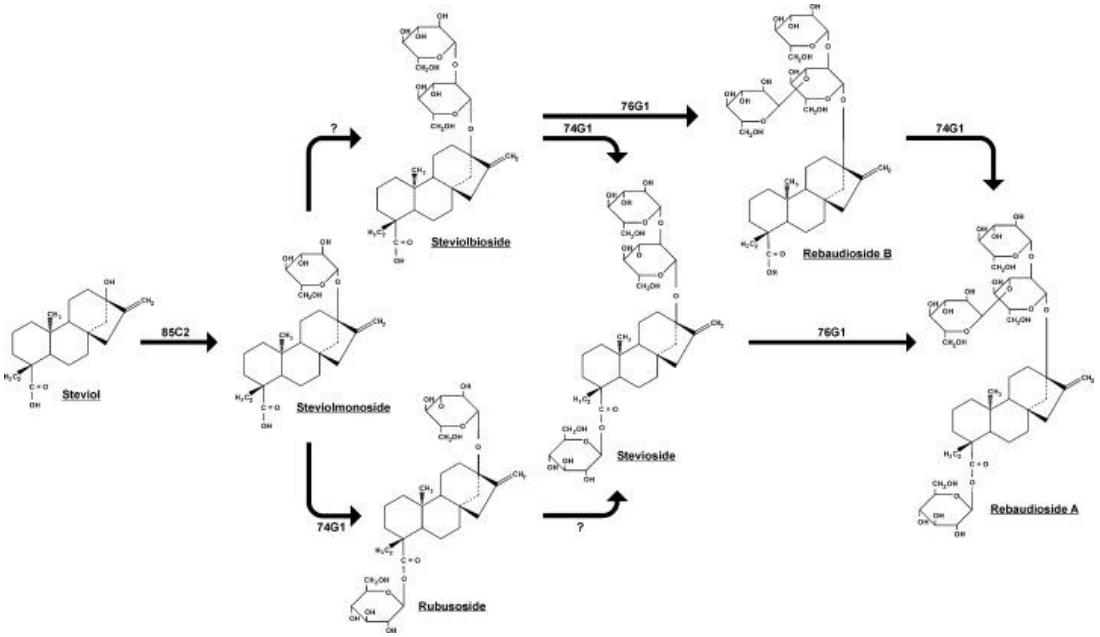

Figure 1.1. "Proposed enzyme and biosynthetic pathway for synthesis of rebaudioside A from the aglycone steviol" (Richman et al. 2005).

Taste of Various Steviol Glycosides

Stevia has high potential as a crop across the world. In stevia, many different glycosides compounds are of interest. There are many other steviol glycosides in stevia in addition to rebaudioside A that are sweet and are being studied, including rebaudioside B, rebaudioside C, and rebaudioside D, but rebaudioside A and stevioside are the major glycosides in the steviol glycoside profile. Previous research has suggested that rebaudioside B may be a byproduct during the extraction process for glycosides (Brandle, 1998).

The various glycoside compounds in stevia have different sweetening potency when compared to sucrose. For example, stevioside is noted to be 250 to 300 times sweeter than sucrose. Rebaudioside A is estimated to sweeten 350 to 450 times sucrose, rebaudioside B 300 to 350 times, and rebaudioside C 50 to 120 times. Rebaudioside D has 200 to 300 times, rebaudioside E 300-520 times, dulcoside A 50 to 120 times, and steviolbioside 100 to 125 times the sweetness of sucrose (Crammer and Ikan, 1986).

Stevioside has been reported to range naturally in the plant between 4 and 20% in dry weight based on differences among cultivars and environmental conditions (Kennely 2002; Starrat et al. 2002). These rebaudioside molecules are distributed all throughout the plant. However, they are at highest concentrations in leaves, particularly young ones; in the same leaves veins contain bitter compounds (Maiti and Purohit, 2008).

Despite the availability of these other minor steviol glycosides, the major glycosides including stevioside and rebaudioside A make up most commercial stevia sweeteners, likely due to their availability in the plant. Minor glycosides are noted as less potent than the major glycosides rebaudioside A, and stevioside (Crammer and Ikan 1986; Brandle 1999; Oddone 1999). A typical stevia sweetener will either contain more than 80% stevioside or more than 90% rebaudioside A (Gardana et al., 2010). Truvia® Natural Sweetener, produced by Cargill utilizes stevia in the product with 84% of rebaudioside A with no trace of stevioside, however other stevia extracts contained mostly stevioside (Gardana et al., 2010). Stevioside is noted for having a “licorice” taste, with a lingering aftertaste undesirable for many consumers. (Yadav et al., 2010). Other compounds found in stevia include flavonoid glycosides, coumarins, phenylpropanoids, cinnamic acids, and some essential oils (Yadav et al; 2010). There are variable concentrations of steviol glycosides found across all plant parts, however stevioside is not accumulated in the roots (Sekaran et al. 2007).

Human taste tests have shown various steviol glycosides to be unique in sweetness and bitterness. Despite rebaudioside A being most readily used, it was found to be more bitter than rebaudioside D (Allen, 2013). This difference in bitterness makes rebaudioside D a potential target

for stevia breeding but as a minor glycoside it is produced in small quantities in the plant (Hellfritsch et al., 2012). Recent studies have indicated the potential for another minor glycoside, rebaudioside M, is also sweeter and less bitter than rebaudioside A (Prakesh, 2014) with an overall better taste than rebaudioside A. However, rebaudioside D and M, represent very small portions of the profile. Therefore, to extract large quantities of these minor compounds in stevia where the major glycosides occupy most of, much of the profile would require many plants. Breeding for higher accumulation of these minor compounds can make them more efficient to extract. Synthesis may be the next viable option. Companies like Cargill have already began investigating the synthesis of these minor compounds via fermentation rather than producing them in plants (Cargill, 2015). Eversweet™ from Cargill takes advantage of the sweetener qualities of rebaudioside D, and rebaudioside M produced by fermentation. Current breeding programs have shown success in reducing stevioside content and increasing rebaudioside fractions, so selection for the right compounds through breeding to make a quality sweetener for the market shows potential.

Production Methods

Stevia seed is first sown in seedling flats prior to planting due to poor germination and slow development of seedlings (Colombus, 1997; Brandle et al., 1998). Stevia is a photoblastic species (Brandle et al. 1998) requiring light to germinate, therefore seeds will not germinate if they are covered by soil. Although stevia is quite tolerant of various temperatures, the optimal growing temperature is 15 to 30 degrees Celsius (Sakaguchi and Kan, 1982). Seeds will begin to germinate between seven and ten days on average upon imbibing water (Yadav et al. 2010). Seedlings are ready for transplant at about 45 to 60 days after sowing (Colombus, 1997; Brandle et al., 1998).

The soil pH recommendation is between 5.5 and 6.5 (Oddone, 1999). Stevia leaves are harvested toward the end of the season at flower initiation and they have reached their maximum biomass (Sumida 1980; Xiang 1983; Shuping and Shizhen, 1995). At this point in the season the leaves have their highest concentration of steviol glycosides but a steady decline as much as 35-50%

of steviol glycosides occur as flowering progresses (Bian 1981; Hoyle 1992; Singh and Rao 2005). Plants are harvested by cutting the plants above the soil level to allow sufficient nodes for re-sprouting for a future harvest (Donalisio et al., 1982).

Once established, stevia can yield multiple times each growing season. In a study in Mississippi leaf yield was measured and it was found that the highest yield came from a once-over harvest in a 180-day season rather than two harvests taken every 90 days or three harvests every 60 days (Moraes et al., 2013). In Northern climates where a 180-day growing season is not possible, stevia can still be grown and harvested once before frost. The crop can last for seven to eight years, with increases of yield the first four years, but a decline the following years (Megeji, N.M., et al. 2005). Stevia is quite tolerant of various temperatures where in its natural habitat at altitudes between 200 and 500 m will experience temperatures ranging from -6 to 43 degrees Celsius (Yadav et al, 2010).

Improvements in propagation techniques will be beneficial to the stevia industry and plant breeders. Seed germination is generally low, however one report showed a stevia selection did not produce viable seed at all, and therefore vegetative propagation was required to reproduce this line (Shock, 1982). However, vegetative propagation is labor intensive. Unlike crops like sweetpotato, direct planting of stem cuttings into the field has little success (Chalapathi et al. 1999a). Propagation by cuttings produces successful results with 98-100% rooting efficiency (Gvasaliya et al., 1990). Despite the high success rate of this propagation technique, cuttings taken in late winter were observed to have the best rooting success than when taken at other times of the year (Carvalho and Zaidan, 1995). Propagation is done using stem cuttings with four internodes per cutting for best results (Tirtoboma, 1988). Growth regulators can also help stimulate rooting (Zubenko et al., 1991b; Carvalho and Zaidan 1995; Kornienko and Parfenov 1996; Kornilova and Kalashnikova 1996; Bondarev et al. 1998). Better rooting and sprouting was found by dipping cuttings in 50 ppm Indole-3-butyric acid (IBA) (Zubenko et al. 1991b). Paclobutrazol was also found to increase rooting success and induce sprouts, resulting in more vigorous cuttings than IBA treatments (Chalapathi et al. 2001).

Tissue culture techniques are also successful methods of stevia propagation, where various plant parts including leaves, auxiliary shoots, shoot primordia, and intermodal explants can be used (Yadav et al., 2010). Cell-suspension cultures were developed for the mass propagation of stevia for field production (Ferreira and Handro, 1988). Recent advancement such as an improved 500 l bioreactor was developed to mass-propagate shoots of stevia, producing 64,600 g of shoots from 460 g of propagules (Akita et al., 1994). Once a desired cultivar is developed, this mass-propagation technology is beneficial to accelerate breeding and commercial production. This would help lower the cost of clonal propagation and make it cost-effective for growers to plant elite cultivars rather than growing from seed. Researchers could also use this technology to produce large experiments of clonal material that was previously difficult due to limitations in labor.

Breeding Objectives

A key breeding objective for stevia is to develop the plant for improved steviol glycosides and yield. There is interest to develop and select a plant with a higher rebaudioside A content and a higher ratio of rebaudioside A to stevioside (Yadav et al., 2010) to make stevia more suitable as a natural sweetener with minimal bitterness. However recent patents and articles show that rebaudioside C, rebaudioside D and rebaudioside M are most desirable. Rebaudioside D was found to be less bitter than rebaudioside A (Allen, 2013). Additionally, rebaudioside M was also reported as a sweeter and less bitter compound than rebaudioside A (Prakesh, 2014). Rebaudioside C is another desirable glycoside that GLG filed a patent for a plant with high content in 2014. Breeding a more prolific plant with a higher dry leaf weight would lead to more usable yield per plant for extraction and purification of target rebaudioside fractions. In my opinion, having a higher leaf to stem ratios is another desirable characteristic, where a plant produces more leaves per plant and less stevioside considering the negative correlation with yield. A branching habit that resists lodging will allow for a higher volume of leaf production is also desirable given that the high levels of desirable rebaudiosides are present.

Currently, stevia is less domesticated than most agricultural crops due to limited breeding efforts. Breeding will help improve the seed germination rates by natural selection for lines that have good germination. Potentially one could select for lines that are self-compatible to develop an improved line that comes true from seed that doesn't need clonal propagation to reproduce.

Breeding Methods

To begin breeding stevia you would need to acquire germplasm with wide genetic diversity to begin screening for traits. Stevia can be allowed to intercross naturally in the field, where you could also make selections throughout the season. In the greenhouse, one can accelerate breeding, by utilizing the short-day nature of stevia to increase breeding cycles per year. Selections can be cross bred in the greenhouse by hand or paired up into cages with pollinators. Stevia can be induced to flower by exposing the crop to a short-day length, which naturally occurs through the fall and winter months outdoors, typically 54 to 104 days after transplanting depending on the cultivar. Photoperiods shorter than 14 hours will encourage development of flowers with current cultivars (Valio and Rocha, 1977; Zaidan et al., 1980; Chalapathi, 1997). Flowering can be induced at the 4-leaf stage using two short-day cycles (Valio and Rocha, 1977). Therefore, early flowering can be induced by this treatment so that plants flower after planting to pollinate early. Small white flowers (15-17mm) with a light purple corolla are arranged in indeterminate heads (Marsolais et al., 1998; Dwivedi, 1999). Flowers are perfect or hermaphroditic (Goettemoeller and Ching, 1999). Once flowering is initiated in the field, stevia can take over a month to reach full flowering (Taiariol 2004; Ramesh et al. 2006). Once seeds are matured and selections are made, seeds from superior lines can be saved to replant the following year.

Selection and estimation of glycosides must be done at plant maturity, right before flowering has exceeded 10% as steviol glycosides have reached their maximum at this stage (Yadav et al., 2010). The consequence of sampling too early is that you would not capture the full potential of the

glycosides in the plant, and high-performance liquid chromatography (HPLC) is a very expensive test to waste (Brandle and Rosa, 1992).

Advancements have been made in quantifying glycosides using HPLC and tandem mass spectrometry, measuring a sample every nine minutes. (Shafii et al., 2012). The group worked with the cultivars ‘Eirete’, ‘Criolla’, and a population of stevia developed at Michigan State University. These were grown in fields and greenhouse. Rebaudioside A content ranged from 0.25% to 16.4% in the plants measured. A need exists for simpler and more cost-effective methods. This is necessary as there will be a need to measure large numbers of samples for a stevia breeding program. Breeding programs can enable variations and selections to improve the desirable rebaudioside fractions in stevia.

Population improvement methods such as recurrent selection could be an effective breeding method in increasing yield and total glycoside content selection in a cross-pollinated species. HPLC can be used to determine glycoside ratios to select plants with desirable compounds. Development of composites and synthetic cultivars were noted as the most effective and practical breeding method for stevia breeding (Yadav et al., 2010). Utilizing isolation cages and clonal propagation can help develop elite lines, which was utilized to develop cultivar AC Blackbird (Brandle, 2001).

Cultivars

A wild stevia population was noted in having two to ten percent of total glycoside concentration, but after three decades of selection and breeding this concentration has increased to as high as 20% (Huang et al., 1995). Current cultivar releases indicate that there is sufficient genetic variability to make the desired genetic gains for traits such as leaf yield, rebaudioside A, and the ratio of rebaudioside A and stevioside (Lee et al. 1982; Morita 1987; Brandle and Rosa 1992; Shizhen 1995). In addition, the constant outcrossing nature of stevia may help to maintain a high level of natural variability to make gains possible through recombination and allelic reassortment. (Shu 1989; Huang et al. 1995).

Plant breeders from various countries around the world have been releasing cultivars of stevia for many years now. A stevia germplasm collection was developed from accessions in Paraguay for the Institute of Himalayan Bioresource Technology in India. Several elite cultivars from their large breeding program have been developed such as cultivars ‘Modhuguna’ and ‘Modhuguni’ which are noted for high yield and stevioside. The cultivars ‘Yunri’, ‘Yunbing’, and ‘Zongping’ that feature high levels of rebaudioside A and stevioside were developed by researchers in China, in addition to ‘SM4’ with high yield and a high rebaudioside A to stevioside ratio. Researchers at Balai Penelitian Perkebunan in Indonesia (The Plantation Research Institute) released a cultivar known as ‘BPP72’, with features unknown. Morita from Japan released the cultivars ‘Morita-I’, ‘Morita-II’ and ‘Morita-III’, where a ratio of 9:1, rebaudioside A to stevioside was reported (Morita 1987; Shizhen, 1995). Due to self-incompatibility, however, the cultivar cannot be reproduced by seed using sexual reproduction (Yadav et al., 2010). ‘Suweon 11’ was released by breeders in Korea that features high rebaudioside A. Researchers at the FSBSI “Primorsky SRIA” in Russia released a cultivar called ‘Ramonskaya Slastena’, noted for high yield and lodging resistance (Romashova et al., 2017). Plant breeders in Taiwan released ‘K1’, ‘K2’, and ‘K3’ which are high-yielding lines with a high ratio of rebaudioside A to stevioside. Researchers at The State University of Maringá in Brazil released the cultivar UEM-320 featuring high rebaudioside A (Carvalho et al., 2013).

Cultivars such as the ‘RSIT’ breeding lines have demonstrated that rapid progress could be made for high or low rebaudioside A, and high and low stevioside content. RSIT was developed starting with a Chinese landrace where 1000 plants were tested and 15 were selected. The selected plants were then transplanted to a greenhouse and intercrossed and the half-sib families were tested in trials. Based on trial data, the breeders selected the two best plants and clonally propagated them for further research. ‘RSIT 94-1306’ had 0% rebaudioside A, and 17.25% stevioside whereas ‘RSIT 94-751’ had 11.82% rebaudioside A and 4.88% stevioside (Sys et al. 1998; Marsolais et al. 1998). Large gains were made in the ‘RSIT’ lines using only two breeding cycles of selection in one population. A

1.5% selection intensity was followed by the crossing of the 15 HS families and by selecting two selections.

The synthetic cultivar ‘AC Blackbird’ developed by Brandle in 2001 also demonstrated that progress could be made for a high ratio of rebaudioside A to stevioside. Synthetic cultivars are developed by crossing inbreds that have good combining ability. To develop this line, a field trial used 12 families of 60 plants each where twenty plants per family were tested using HPLC. Selections were made on the best four and they were used to intercross in the greenhouse. In addition, clones were propagated to place the best four in the field to intercross. This led to the release of the synthetic cultivar in generation one, known as ‘syn1’. ‘AC Black Bird’ has 14% total glycosides, with 9:1 ratio of rebaudioside A to stevioside, 12.49% rebaudioside A, and 1.27% stevioside in percentage of dry matter. As a synthetic cultivar, new versions can be released each year with improved glycoside profiles.

In 2011 a patent was filed for a new and distinct cultivar called ‘AKH L1’, noted for its late harvesting ability, light green leaves, with more branching on the main stem (US PP23728 P3, 2013.) ‘AKH L1’ is also noted for high rebaudioside A with a high yield of dry leaves and disease resistance. The yields of the dried leaves were 4,500 kg/ha. This cultivar was a result of selection from a controlled breeding program in San Pedro, Paraguay. ‘AKH L1’ was bred by a controlled pollination of the female parent of ‘Eirete’ crossed with the male pollen parent of ‘AKH/EM1’, an unpatented cultivar. This new cultivar ‘AKH L1’ was a selection within a population of seedlings from this cross. The selection criteria were for high rebaudioside A with a content more than 50% of the total steviol glycoside with high yield of leaves and resistance to leaf spot diseases (US PP23728 P3, 2013).

In 2015 a patent was filed for a new release of stevia by Purecircle SDN BHD, Malaysia (US 20160057966 A1, 2016). This cultivar name is ‘814011’ and is noted to have a higher rebaudioside M and Rebaudioside D percentage than other cultivars. When compared to ‘PC Star’, which is a similar but unpatented cultivar, cultivar ‘814011’ had a rebaudioside-M content of 1.12%, while ‘PC Star’ has only 0.21%. Cultivar ‘814011’ had a rebaudioside D content of 1.11% compared to ‘PC Star’

with 0.40%. The stevioside content, which is often non-desirable is lower in cultivar ‘814011’ than ‘PC Star’ also making it a more desirable and unique cultivar release. The cultivar was bred using two-step crossbreeding method. ‘Eirete’, and ‘PC Star 2’ were used as the female, and the male pollen parent was ‘AKH L1’. In the next generation, a male parent called ‘YF001’ was selected from the progeny with desirable agronomic traits and used in the cross to the initial parental lines. Cultivar ‘814011’ was selected in the progeny (US 20160057966 A1, 2016).

The S&W Seed Company filed for a patent for a unique new cultivar of stevia named ‘SW 129’ (U.S. Plant Patent PP27815, 2017). This cultivar is noted to have sweet leaves, low bitterness and after-taste, winter hardiness, high levels of steviol glycosides, and is late flowering. In field trials, ‘SW 129’ had leaf yields of over 2200 Kg per acre. ‘SW 129’ was also noted to have 98% more rebaudioside A content, with an increase of 475% in the rebaudioside A to stevioside ratio when compared to standard cultivars. This was the third patented unique cultivar released by S&W Seed. ‘SW 201’ was released as an improved cultivar with a better leaf taste profile suited for fresh and dry leaf production. ‘SW 107’ was a cultivar released for commercial processing for the mass market (U.S. Plant Patent PP27815, 2017).

Current breeding work with stevia has resulted in increased glycoside concentration by 20%. There has also been progress in the ratio of rebaudioside A to stevioside which was initially 0.36:1, and now is 0.96:1 in some cultivars (Brandle, 2001). It appears evident that the glycoside focus based on recent cultivar releases include improvements of rebaudioside A, rebaudioside D, and rebaudioside M. Rebaudioside A has been well utilized as a good choice for sweeteners such as Truvia®, with a focus on lowering stevioside. However, other sweeteners seem to utilize stevioside as a primary sweetener. Rebaudioside D and rebaudioside M are more of a recent focus in new cultivars of stevia based on recent releases although there is limited research on these compounds. Yield is also an important factor that is considered with all cultivar releases.

Disease Resistance

Diseases of stevia can occur and affect production. (Yadav et al. 2010). Septoria (*Septoria steviae* Ishiba, C. Tani T.) appears to be the most common fungal disease of Stevia. Symptoms generally appear as sunken, angular, olive-gray lesions that can rapidly spread. Leaves quickly become necrotic and drop (Lovering et al., 1996). A screening method was developed to test for resistance and found that stevia clone 598-1 was resistant in both field and greenhouse tests (Reeled, 1999).

Other oomycete and fungal diseases of stevia include *Pythium* spp., (Koehler et al., 2017) Rhizoctonia (*Rhizoctonia solani* Kuehn.), Alternaria leaf spot (*Alternaria alternata* Keissl.), stem rot (*Sclerotium delhinii* Welch.), root rot (*Sclerotium rolfsii* Curzi), *Schlerotinia sclerotiorum* de Bary., and powdery mildew. (*Erysiphe cichoracearum* DC) are noted in stevia culture in some cases (Ishiba et al. 1982; Lovering and Reedleeder 1996; Chang et al., 1997; Thomas 2000; Megeji et al. 2005; Kamalakannan et al. 2007). Viruses can also be a concern for stevia. Tomato spotted wilt virus was discovered in a greenhouse in North Carolina on plants that were selected from the field and brought to the greenhouse for breeding (Kohler et al., 2016). TSWV can be spread to stevia in the field or greenhouse by insect vectors such as western flower thrips (*Frankliniella occidentalis* Pergande*)*. Symptoms show as distortion, mottling, and chlorosis of leaves which progresses to necrosis of leaves stems and eventual plant death. Chlorotic ring spots can also be observed with TSWV. (Koehler et al., 2016). Currently there are no pesticides labeled for stevia, so control of insects, weeds, and diseases can be challenging by chemical means, which can be a limitation in commercial production. Breeding of disease resistant lines is valuable to growers and is another important trait on which to focus breeding efforts.

Conclusions

Stevia has the potential to become an important crop in the future as a natural sweetener due to valuable sweetening compounds such as rebaudioside A, C, D and M. according to recent findings.

Research progress over the past decade or so have been productive, but many research topics still need to be addressed. It is still unknown as to why *S. rebaudiana* produces these diterpenes in such a high amount, despite theories of a defense mechanism. With a better understanding of the process, it could be possible to induce stevia to produce higher levels of these compounds. Rebaudioside A has been well explored as a better sweetener than stevioside and therefore is used readily as a primary component in today's stevia products. However, recent findings show that rebaudioside C, D, and M are sweeter with a closer taste to sucrose than rebaudioside A, which could improve the quality of stevia sweeteners in the market. In addition, there are many cultivars that feature high levels of rebaudioside A, which is now less desirable. Research conducted on rebaudioside C, D, and M is limited and many variety trials do not include these compounds. Therefore, variety trials should include recent glycosides of interest, which would be beneficial to plant breeders. Also, recent releases of these cultivars have enabled trials to be conducted. Taste tests have been run on some of the glycoside compounds to identify sweet and bitter compounds, however more compounds have since been discovered that should be tested.

Self-incompatibility is known in stevia, but poor seed germination continues to be an issue with commercial production, where additional research is needed to explore the flowering physiology. Elite clonal varieties exist, but come with a higher cost as a plant than the cost of seed. But if clonal lines offer higher quality and yield than a seed cultivar would, than clonal lines may be cost-effective. Studies are needed to compare costs of clonal lines and yield in comparison to seed varieties. Another issue with commercial production is control of weeds, and diseases where chemicals are not labeled as safe for stevia. Growers will be hesitant to plant a crop that requires hand-weeding which is labor-intensive. Disease resistant lines have been developed and offer some control, but multiple more stevia diseases have been reported recently to affect the crop.

Additional work on the biosynthetic pathway of stevia is needed, as not all the steps have been identified. The pathway is helpful in understanding compounds that are linked which can help direct breeding progress. Modern techniques can also be explored such as molecular markers, HPLC,

and mass spectrometry to assist in quicker breeding for higher glycoside and yield traits. Glycoside evaluation by HPLC is effective, but expensive which limits the ability of large populations to be analyzed. Phenotypic screening methods would be valuable to help identify the best plants in a breeding population without the need to test them all via HPLC.

Mutation breeding can also be explored for cultivar development in stevia, as it has been successful with other crops. Polyploidy has been reported to have benefits in stevia breeding, but research is limited as to the effect on glycoside production. Polyploidy can potentially further improve cultivars that are currently available. Genome editing through new techniques in biotechnology such as CRISPR could prove effective in developing varieties and eliminating bitter compounds.

Current estimates of genetic variance components and heritability have indicated great potential in breeding for glycosides and yield. Additional heritability estimate studies should be conducted using large populations, as it can be a beneficial tool for breeding stevia and can help increase breeding efficiency. In addition, further identifying trait correlations can help breeders in effective selection.

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CHAPTER 2: PERFORMANCE OF 25 STEVIA (*STEVIA REBAUDIANA*) CULTIGENS FOR YIELD, GLYCOSIDES, AND OTHER PLANT TRAITS IN NORTH CAROLINA

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Abstract

A field trial was conducted using 25 seed sources of stevia (*Stevia rebaudiana* Bertoni), to measure yield, glycoside profile and concentration, plant size, and other agronomic traits. There are many sources of commercial stevia seed available. However, the original sources of these populations are often unknown. The cultigens evaluated were obtained from garden seed companies as well as commercial sources. The trials were conducted for two years at two locations. Multiple objective measurements and subjective ratings were collected during each growing season to determine stem height, plant width, and leaf size. Phenotypes were assessed both subjectively (e.g., plants rated on a scale of 1-9) and objectively (e.g., individual plants measured using meter stick). Other data on traits such as lodging and disease also were collected. At the end of the season, plants were harvested for measuring total fresh and dry weight as well as leaf and stem weight since leaves are the primary source of sweetener. Pearson correlations were generated for yield and glycoside traits. Data were collected on a single-plant basis to evaluate homogeneity within seed source. Cultigens were significantly different from each other for most traits measured, indicating a diverse genetic base available to growers in the world. High-yielding cultigens were identified with year, location, and block significant for many of the yield and glycoside traits. Cultigens with highest leaf dry weight were ‘Katupyry’, ‘Eirete-I’, and ‘Eirete-II’ The most desirable cultigen for glycoside level was Seed-Savers (NC-1022).

Introduction

Stevia rebaudiana (Bertoni) is a new crop that can be used to produce non-caloric, plant-derived sweetener replacements (Yao, Ban & Brandle, 1999). The leaves contain diterpene steviol glycosides. These diterpenes are produced naturally in the plant most likely as a defense mechanism (Nanayakkara et al., 1987), however has not been confirmed. Rebaudioside A is one of the most explored of the steviol glycosides and is the main rebaudioside extracted and processed for market currently, although recent research suggests that other compounds are more sweet. Stevia has a bitter taste, making it less desirable to consumers. Ingredient suppliers of sweeteners would like to develop cultivars with different glycoside concentrations in order to improve stevia for use in products. Stevia has been studied and grown in Southeast Asia and South America. It is now being grown and sold in the US. In Japan, the market value of stevia is reported to be 2-3 billion yen per year (Megeji et al., 2005).

Phenotypic differences have been reported in stevia among the available sources. Unfortunately, seeds are often sold generically as stevia without cultivar information. Seeds purchased by growers range from local seed savers to wholesale sources. A study conducted by Othman et al. (2015) evaluated 14 stevia accessions from Malaysia and Paraguay. The study showed that the accessions were morphologically different from each other for traits such as plant height, number of branches, and leaf yield. They reported that tall or wide growing plants did not necessarily yield more leaves. However, leaf size may affect leaf weight and sweetener concentration. Another study demonstrated large variation in production of steviol glycosides and yield for stevia germplasm (Sakaguchi and Kan, 1992).

Stevia breeding is underway worldwide. We identified breeding programs in China, Japan, India, Brazil, France, and Canada. Research in the United States is increasing as well, including Oregon State and Fort Valley State University in Georgia. Cultivars are needed with high yield and improved taste, along with traits such as overwintering tolerance for northern climates.

There are improved cultivars of stevia available around the world, with improved levels of desirable rebaudiosides. Cultivar trials have identified improved leaf yield, rebaudioside A content, as well significant genotype by environment interaction leading to limited stability of performance (Parris et al., 2016). Rebaudioside D was recently found to be less bitter than rebaudioside A. The preferred taste over rebaudioside A makes rebaudioside D a potential product for sweetener, but in current cultivars it is produced in too small of a concentration to be cost effective (Hellfritsch et al., 2012). Rebaudioside M, also with less bitterness than rebaudioside A, has been recently explored as well, however again as a minor glycoside faces similar problems for large scale production as does rebaudioside D (Prakesh et al., 2014).

Stevia production continues to gain interest over the years (Megeji et al., 2005). Mechanized planting and harvesting systems being developed will further encourage farmers to grow stevia. In United States Department of Agriculture Plant Hardiness Zone nine and warmer, or elsewhere as long as temperatures remain above -6 Celsius, stevia can be grown for seven to eight years as a perennial, providing greater return on establishment costs.

Poor seed germination makes direct seeding not practical for commercial stevia production, so plants grown in protected environments before being transplanted into the field (Shock (1982), Duke (1993), and Carneiro (1997)). Stevia seed has a short storage life, making high quality seed more difficult to obtain. This is a problem when evaluating cultivars from diverse sources because some cultivars fail to germinate. Thus, much work remains to be done in improving seed production, storage, and germination for this crop.

Stevia diseases include a leaf spot caused by *Septoria steviae* (Ishiba) that appears to be the most common fungal pathogen. Symptoms generally appear as sunken, angular, olive-gray lesions that can spread rapidly. Leaves become necrotic and drop from the plant, causing reduced yield (Lovering et al., 1996). Disease screening has identified clone 598-1 which was resistant in both field and greenhouse tests (Reeled, 1999). Other diseases are caused by Rhizoctonia (*Rhizoctonia solani* Kuehn.), Alternaria leaf spot (*Alternaria alternata* Keissl.), stem rot (*Sclerotium delhinii* Welch.),

root rot (*Sclerotium rolfsii* Curzi), *Schlerotinia sclerotiorum* de Bary., and powdery mildew. (*Erysiphe cichoracearum* DC). Currently, there are no fungicides labeled for stevia, limiting disease control. Genetic resistance through breeding may offer some level of control for many of these diseases. Thus, much work remains to be done improving this crop.

The objective of this study was to evaluate the available seed sources of stevia to identify if differences were found among these cultigens by genotype and environmental effects for traits such as yield, high glycoside content, and large plant size. Environmental effects in this study include year, location, and replicate. If differences were found, cultigens that have superior traits such as high glycoside, yield, and disease resistance can be valuable to include in elite breeding populations. With limited germplasm available for stevia, assembling a collection for the USDA could also prove useful. Traits that affect yield such as plant height, plant width, leaf size, plant weight, lodging, and disease resistance were evaluated at different times in the season to see if significant correlations exist. Additionally, cultigens were evaluated via subjective ratings and objective measurements to test effectiveness of these rating procedures to evaluate stevia. This study was intended to help growers and researchers identify sources for traits of interest.

Materials and Methods

Stevia seeds were obtained from seed companies and seed catalogs to make a total of 25 cultigens (Table 2.1). They were tested in 2015 and 2016 in Clinton and Kinston, NC. Seeds from each cultigen were planted in early February into 72-cell seedling flats in a greenhouse. Flats were filled with Fafard 4p, a multipurpose potting soil, comprised of sphagnum peat moss, perlite, vermiculite, dolomitic limestone, wetting agent and gypsum. Four seeds were planted into each cell to ensure sufficient seedlings per flat, due to the low germination rate expected. Each cultigen was planted in its own flat to prevent mixing of seed sources. Soil media was kept moist by watering twice daily with an overhead mist system. A liquid soluble fertilizer (20-10-20) at 100ppm was applied

starting at true-leaf stage and continuing weekly. The seedlings were thinned at the true-leaf stage to 72 per flat. Greenhouse temperatures were maintained at 29°C day and 21°C night.

Seedlings were grown for 8 weeks in the greenhouse before moving them to a cold frame outdoors with a 50% shade cloth. The plants were acclimated for one week prior to transplanting in the field.

Plants were transplanted into raised beds one meter wide covered with black plastic (early May in 2015 and 2016). Rows were on 1.5 m center-to-center. In each year and at each location plots consisted of either six plants or 30 plants. In 2015 and 2016, each cultigen was planted into a 6-plant per plot density and 30-plant per plot density, with plant data taken on the 6-plant plot (glycosides, lodging, disease), and yield data (yield, stem, leaf fresh and dry weight) taken on the 30-plant plot. The 30-plant plot represented a commercial density of 64,550 plants/ha. The 6-plant plot occupied the same plot size as the 30-plant plot with 0.6 m between plants at a density of 12,910 plants/ha. Plots were 3 m long with a 1.5 m alley at each end to separate them. Plants were irrigated after transplanting and received 25 mm per week of irrigation if no rainfall. Fertilizer was injected in the irrigation. Sevin® (carbaryl) was used as a broad-spectrum to control insects in the early season. Plots were hand-weeded since there are not any labeled herbicides for stevia.

The stand-count was taken two weeks after transplanting and again at the end of the season to calculate percentage survival through the summer.

Experiment Design

The experiment was a split-plot treatment in a randomized complete block design of four replications, two years (2015, 2016), two locations (Clinton and Kinston NC), and, 25 cultigens. Whole plots were years and locations, and subplots were cultigens. Cultigens were replanted each year and not left in the field to overwinter.

Data Collection - Plant Size

Traits measured were plant size, stem height, plant width, and leaf area. In 2015, the plants were given three subjective ratings over the growing season and one objective measurement (the

fourth rating of the season). Subjective ratings were taken in addition to objective measurements as an attempt to simplify data collection by a quick visual rating. In 2015, subjective rating one was taken June 16, subjective rating two July 21, and subjective rating three August 22. The objective measurement was taken August 29. In 2016, the plants were rated by two subjective and two objective measurements over the growing season. In 2016, subjective rating one was taken June 14 and objective measurement one June 21. Subjective rating two was August 23, and objective measurement two August 30.

The subjective scale was on a 1 to 9 continuous visual rating scale for stem height, plant width, and leaf size (1-3=small, 4-6=medium, and 7-9=large). Stevia can grow to about 1 m in stem height (Shock, 1982), so a 9 rating is based from that height. For plant width, ratings would be based from the widest plant reaching 0.6 m. For leaf size, the ratings would be based on a leaf size maximum reaching 10 cm.

The objective measures were similar to the subjective ratings, except for using a meter stick. Leaf length and width were used to calculate leaf area: 0.5 x leaf length x leaf width. Both the 30-plant plot and 6-plant plot were used for the subjective and objective plant size traits. For the 6-plant plot, each plant was rated individually for both subjective and objective measurements. For the 30-plant plot, we measured the third plant in the center row.

Data Collection - Yield

Yield (fresh weight, dry weight), stem dry weight, and leaf dry weight were measured on the 30-plant plots after harvest. Dry weights were taken after drying for two days in a dryer set at 60°C. Lodging and disease damage were rated subjectively on a 0 to 9 scale (0=none, 9=dead).

The 30-plant plots were harvested at the end of September in 2015 and 2016 by cutting plants at roughly five cm above the soil line. Live plants were counted to estimate survival during the summer, since plants were lost to wind and disease, including southern blight (*Sclerotium rolfsii* Sacc.). We calculated survival using spring vs. fall counts. Plants from each plot were bagged and the fresh weight taken.

All weights were measured in pounds and then converted to Mg/ha. The conversion is calculated by first converting to kg by multiplying by 0.4536. It is then multiplied by 0.001 to convert to mg. We then multiply by 0.02 to convert our plot size to square foot. Multiply by 43,560 to convert to an acre. Finally, we multiply by 2.47 to get measurements for a hectare. The calculation becomes (Mg/ha = lb/plot x 0.97609).

Data collection - Glycosides

Glycosides were measured using leaves taken 5 nodes from the top of the plant, where glycosides accumulate in highest quantity (Ceunen, 2013). Leaf tissue was dried and ground using a tissue grinder prior to analysis. Glycosides (stevioside, and rebaudiosides A, B, C, and D) were reported in mg/g as well as percentage of total steviol glycosides in the sample. Glycoside measurements were taken by taking mid-canopy leaves from four plants per plot in mid-July before flower initiation as steviol glycosides have reached their maximum at this stage (Yadav et al., 2010). Leaves from each plant were kept separate. They were then dried at 60°C for two days and then ground to a fine powder for analysis. Glycosides were extracted and then measured by high performance liquid chromatography and mass spectrometry, a common method used to quantify glycosides by others (Gardana et al., 2010).

After drying, leaves were separated from the stems by hand and weighed. Leaf and stem weights were taken for one replication in two locations and two years. One replication was used due to the time consumption of separating leaves from stems by hand. Yield (fresh and dry weight) was calculated per ha and per plant. Percentage of dry leaf weight to dry weight was measured to see if any differences occurred across cultigens.

Lodging resistance was evaluated in 2016 using a representative plant in the 6-plant plot. Lodging was a subjective scale from 0 to 9 in a continuous scale with approximate 10% intervals.

Severity of Septoria leaf spot was rated 0 to 9 in a continuous scale with approximate 10% intervals of leaf damage. Disease ratings were made using a representative plant in the 6-plant plot.

Data Analysis

Data were analyzed using SAS v9.4 (SAS Institute, Cary, N.C.). Analysis of variance was run after data were checked for normality and errors using Proc ANOVA. Least squares means were calculated to account for missing data. Data were analyzed using both years except for new traits added in 2016 (lodging, disease).

Pearson product-moment correlations were estimated for all pairs of traits. Also, since plant size was measured on both 6- and 30-plant plots, correlations were run to determine ability to predict one from the other.

Results and Discussion

Cultigens Tested

Since cultigens were obtained as generic stevia from most seed companies, they were assigned an accession number. (Table 2.1).

In 2015, 25 cultigens were obtained. Those with insufficient germination to run the test were eliminated (NC-1006 and NC-1019). NC-1016, NC-1017, and NC-1018 had low germination and therefore they were evaluated using fewer replications. The procedure was repeated in 2016. NC-1038, NC-10235, NC-1036, and NC-1037 had low germination. Fewer replications were used for 'Native', NC-1030 (6 of 8 plots), and NC-1022 and 'Eirete II' (7 of 8 plots). Because of insufficient germination, some cultigens were only present in one of the two-year trial. Some cultigens sources, despite being the same source for 2015 versus 2016, had varying germination one year to the next, despite the same methods used in sowing. It is possible that stevia seed lots used by garden seed companies change with supply.

Analysis of Variance

Plots at the 30-plant density were harvested for yield and leaf weight in September. Cultigen effect on yield (total fresh or total dry weight) was highly significant in both years (Table 2.2). Location and location by cultigen interaction was also significant for total fresh weight, indicating that these cultigens performed differently at each location. Year was significant for total dry weight

but not total fresh weight. Year by cultigen had a highly significant interaction for total fresh and total dry weight, indicating that some cultigens in this study varied in performance by the year they were grown in. Plant breeders would want to select cultivars that perform consistently well year to year.

There were no significant effects of year, location, or cultigen for stem dry weight (Table 2.2). Only year was significant for leaf dry weight and percent of dry leaf to dry weight. Thus, selection for high percentage leaves may not be as effective as selection for high yield, either dry or fresh. Researchers found significance for location for dry leaf yield (Parris et al. 2016), while we were unable to detect it. It is possible that with the use of more diverse locations, significance could be detected. The two locations we used have similar cultural environments towards the coast, but soil type and moisture can vary even within one field location

Percentage survival in 30-plant plots was significant for location, year, and cultigen. The interaction of year and cultigen was also significant. Significance for percent survival indicates that cultigens vary by their ability to become established after planting due to various factors, which we cannot identify based on our limited dataset. Perhaps some endure more stress after they are transplanted and become established.

Significant effects were observed for cultigen, year, and year by cultigen for rebaudioside A (Table 2.3). Similarly, researchers found significance for cultigen similar to our findings but also detected significance for location by cultigen (Parris et al. 2016), while we detected no significance. Rebaudioside B did not have any cultigen effect but it was affected by replication and location. Rebaudioside C had no significant effects. Rebaudioside D was affected by cultigen, year, and year by location. Stevioside was affected by cultigen and year.

Glycosides were also measured as percentage of total steviol glycosides (Table 2.4). Percentage of rebaudioside A was significant for cultigen and year by cultigen. Percentage of rebaudioside B was significant for cultigen and location. Percentage of rebaudioside C was significant for cultigen and year. Percentage of rebaudioside D was significant for cultigen, year, year by location, and year by cultigen. Stevioside was significant for cultigen, year by cultigen and location

by cultigen. Total steviol glycosides was significant for year, replicate, cultigen, and year by cultigen. Total steviol glycoside level was highly affected by environment effects such as year, rep, and the interaction of year x location and the average across all the cultigens in 2015 was much higher than in 2016 despite similar measurement times of samples collected in 2015 and 2016. It may be that differences between years were weather related as there was not any observed significance for location in this case, but both locations will vary in environment based on soil type and moisture levels on a year to year basis. Genotype and environment were observed to play a role in the content of rebaudioside A and stevioside (Tavarini et al., 2010). We could conclude a similar finding where genotype was significant for stevioside and rebaudioside A, and environment was significant as year but not as location. All glycosides that were measured had significance for cultigen effects which indicates a strong genetic component to the accumulation of these compounds despite some environmental factors that can affect the quantities measured. This suggests that selection for these glycosides are possible although some variation will be observed in the total of these compounds based on the environment. However, further studies like those conducted by Parris et al. (2016) could be conducted utilizing clonal lines with unique glycoside profiles to test how if any variation is observed it the total for these compounds as well as others such as rebaudioside B and rebaudioside D.

Stem height and plant width (subjective rating) measured in June was significant for year, location, year by location, cultigen, year by cultigen, and year by location by cultigen. Leaf area (subjective rating) measured in June was significant for year by location, cultigen, year by cultigen, location by cultigen, and year by location by cultigen. These results suggest that the traits plant size, stem height, plant width, and leaf size (subjective and objective) may be useful measures of growth (Table 2.5).

Plant size (subjective rating) assessed in August in 2015 and 2016 was significant for location, year by location, cultigen, year by cultigen, and year by location by cultigen (Table 2.5). Stem height (subjective rating) assessed in August was significant for year by location, cultigen, and

year by cultigen. Branching width (subjective rating) assessed in August was significant for year, year by location, cultigen, year by cultigen, and year by location by cultigen. Leaf area (subjective rating) assessed in August was significant for year, location, year by location, replicate, cultigen, and year by cultigen.

Plant size (objective measure) was significant for year, location, year by location, cultigen, and year by cultigen (Table 2.6). Stem height (objective measure) was significant for year, location, year by location, cultigen, and year by cultigen. Branching width (objective measure) was significant for year, location, year by location, cultigen, and year by cultigen. Leaf area (objective measure) was significant for year, location, year by location, cultigen, and year by cultigen.

Overall, estimates either subjective or objective gave similar F-values in estimating yield components such as plant size, stem height, plant width, and leaf size. Subjective and objective measures are effective measures of these traits, however subjective ratings are less time consuming but require a trained evaluator, and are potentially more error prone. Objective measurements are more precise, but require measurements by ruler taken and recorded, rather than a visual scale. Leaf area when measured as an objective measurement, requires length and width to be measured to calculate the leaf area while subjective rating only requires one visual rating. Advanced technologies could be utilized in future studies to measure plants using digital imaging offering more precise measurements.

Disease damage (subjective rating) in 2016 was significant for location, cultigen, and location by cultigen (Table 2.6). Lodging (subjective rating) was significant for cultigen, and location by cultigen. This study showed that there are significant differences between cultigens across agronomic traits including yield and glycosides profiles across the 25 cultigens that were evaluated. Other researchers (Othman et al. 2015) found similar results using improved cultivars. Despite the use of garden seed cultigens in our study with unknown origins beyond where we purchased them, sufficient genetic variation exists among the cultigens tested that could be used for breeding. Although we do not have enough data to determine these cultigens origin in this study, there is high genetic diversity

among these stevia, which is commonly observed by others, contributed by the out-crossing nature of stevia (Handro et al., 1993).

Best Cultigens

Plant size traits (subjective rating) measured in June 2015 and 2016 are in Tables 2.7 and 2.8. NC-1008 (Botanical Interest) had largest plant size in 2015, and 'Eirete II' and 'Katupyry' were the largest for 2016. NC-1007 (Hirt's seed) was the tallest in 2015 and 'Katupyry' was the tallest in 2016. For plant width, NC-1008 (Botanical Interest) had the widest plants in 2015, and 'Eirete II' the was widest in 2016. For leaf size, NC-1011 (Everstevia) had the largest in 2015 and NC-1032 (Swallowtail) had the largest in 2016.

Pearson correlations were calculated for traits measured in 6-plant and 30-plant plots (Table 2.8). Values were mostly around 0.50 and therefore too low to justify using the more easily collected data from 6-plant plots. This suggests that in the future data for these traits should be collected from 30-plant plots.

Plant size traits (subjective rating) measured in August 2015 and 2016 is in Tables 2.9 and 2.10. NC-1001 had the largest plant size in 2015 and NC-1020 had the largest plant size in 2016, both from Baker Creek. NC-1011 (Everstevia) had the largest stem height in 2015 and NC-1032 (Swallowtail) had the largest in 2016. 'Katupyry' had the largest plant width in 2015 and 'Eirete II' had the largest in 2016. NC-1011 (Everstevia) had the largest leaf area in 2015 and NC-1020 (Baker Creek) had the largest in 2016.

Plant size traits (objective measures) were measured in August in 2015 and 2016 (Tables 2.11 and 2.12). NC-1011 (Everstevia) had the largest plant size in 2015 and NC-1032 (Swallowtail) in 2016. 'Native' had the tallest stem height in 2015 and NC-1032 (Swallowtail) in 2016. 'Eirete I' had the widest plant width in 2015 and 'Eirete II' in 2016. NC-1011 (Everstevia) had the largest leaves in 2015 and NC-1020 (Baker Creek) in 2016.

Disease severity for foliar symptoms was rated subjectively in 2016 and 'Native' was the most resistant (Table 2.12). Screening for disease resistance seems likely as it was for the screening method

to find disease resistance for both field and greenhouse tests in clone 598-1 (Reeled, 1999). Breeding for disease resistance could be an important trait in stevia especially in production areas where high disease pressure causes defoliation of leaves, the primary product of the crop Breeders can incorporate any of the genes found in these cultigens into elite lines for improving lines and cultivar development. Future studies could include the inheritance of resistance for specific diseases and identifying sources of resistance. Screening for disease resistance should be conducted in areas of variable disease pressure and not just areas with high disease occurrence. Lodging tolerance was also measured in 2016 and 'Native' was the most tolerant.

'Eirete II' was the highest yielding (fresh wt.) cultigen in 2015 and 'Katupyry' was the highest yielding in 2016 (Tables 2.13 and 2.14). Yield (dry weight) was similar to fresh weight., with 'Eirete II' and 'Katupyry' the best in 2015 and 2016, respectively. Dry stem weight. was greatest for 'Native' in 2015 and 'Katupyry' in 2016. Dry leaf weights were the highest for 'Eirete II' in 2015, and 'Eirete II' and NC-1023 (Johnny's) in 2016. Yield of dry leaf across the cultigens tested averaged 1.7 Mg/ha in 2015 and 1.5 Mg/ha in 2016, with the highest being ‘Eirete-II’ at 2.7 Mg/ha. These averages are similar to those reported in Paraguay of 1.5 to 2.5 Mg/ha^{-1}per year (Ramesh et al. 2006). Although our yields appear low when compared to Parris et al., (2016), our cultigen trial was conducted using undeveloped lines from garden seed companies, rather than improved varieties used in typical commercial stevia trials. This information gives us directional knowledge of the variation observed in garden variety stevia and indicates sources of unique traits and characteristics that could be incorporated into elite stevia varieties. However, it would be interesting to have grown commercial lines as a control among our cultigens to see if yields varied drastically based in North Carolina. We could also get a good comparison of how these cultigens compare to improved cultivars.

Rebaudioside A (mg/g dry leaf tissue) was highest for NC-1010 (R.H. Shumway) in 2015 and NC-1022 (Seed savers) in 2016 (Tables 2.15 and 2.16). Rebaudioside B was highest in 'Eirete I' in 2015 and NC-1022 (Seed savers) in 2016. Rebaudioside C was highest in NC-1014 (Richters) in 2015 and in NC-1029 (R.H. Shumway) in 2016. Rebaudioside D was highest in NC-1003 (Seed Savers) in

2015 and in NC-1022 (Seed Savers) in 2016. Stevioside is generally selected to be low, and 'Eirete I' was the lowest in 2015 and 'Katupyry' in 2016. A high ratio of Rebaudioside A to stevioside content is considered desirable, and ‘Eirete I’ was the highest in 2015 and NC-1022 (Seed Savers) in 2016.

Glycosides were also evaluated for percentage of total steviol glycosides present in the leaves (Tables 2.17 and 2.18). In 2015 'Eirete I' had the highest percentage of Rebaudioside A and in 2016 NC-1022 (Seed Savers) had the highest. Percentage of Rebaudioside B was highest in 'Eirete I' in 2015 and NC-1022 (Seed Savers) in 2016. Percentage of Rebaudioside C was highest in NC-1014 (Richters) in 2015 and highest in NC-1029 (R.H. Shumway) in 2016. Percentage of Rebaudioside D was highest in NC-1004 (Johnny's) in 2015 and highest in NC-1022 (Seed Savers) in 2016. Stevioside percentage was lowest in 'Eirete I' in 2015 and lowest in NC-1029 (R.H. Shumway) in 2016. Glycoside concentration found across plants in cultigen trial had the highest accumulation of stevioside, followed by rebaudioside A, rebaudioside C, rebaudioside D, and rebaudioside B. Other researchers have also reported stevioside to be the main compound found, similar to our finding. (Behera et al., 2013; Moraes et al., 2013; Pal et al., 2015; Serfaty et al., 2013; Vasilakoglou et al., 2016). However, Parris et al. (2016) found that rebaudioside A was found in the highest concentration, followed by stevioside across a variety trial conducted in the western U.S. This is likely due to the improved cultivars that were evaluated in their study, where selection was for a higher ratio of rebaudioside A over stevioside. Despite the base population containing stevioside as the predominant glycoside, it appears evident that with breeding we can increase rebaudioside A to levels where it accumulates at higher concentrations than stevioside: for example, the cultivar Morita had a 9:1 ratio of rebaudioside A to stevioside (Morita, 1987). Through breeding it could be possible to breed other major glycoside compounds like rebaudioside C to these high levels, if they were desirable as a sweetening product. However, many other minor glycosides of interest such as rebaudioside D and M would be much more challenging to increase to these levels, considering the low percentage at which these occur naturally, whereas rebaudioside A naturally occurs as a large portion of a typical glycoside profile. The compounds of interest would depend on whatever

compound makes the most cost effective and feasible sweetener product for the non-caloric sweetener market and this is still not fully understood or agreed among end-users.

There is interest for developing stevia products that are sweet and less bitter. Research suggests that rebaudioside D and M are preferred over rebaudioside A (Allen, 2013; Prakash, 2014) with stevioside having the lowest quality among this group. However, meeting the preference for rebaudioside D and M could become challenging due to their minor accumulation in stevia plants. We observed rebaudioside D to accumulate 2% of the total steviol glycosides on average across cultigens evaluated (Table 2.18). To extract such minimal levels from a crop would not be cost effective until breeding was to improve the profile to acceptable levels of these compounds. Large accumulation of other compounds beside the target compound masks the taste of these minor compounds and adds to the difficulty of extracting them in pure form. For this reason, Cargill is using a fermentation process to produce rebaudioside D and M using yeast in a biofermentation plant to produce their sweetener product Eversweet™ (Cargill, 2014). This and similar methods of producing glycosides synthetically have been explored by others as well (Rumelhard, 2016). It may be that additional compounds become of interest either due to cost or feasibility, but further exploration and research on all compounds is necessary.

Correlations Among Traits

Pearson correlations were estimated for all pairs of traits (Table 2.19). Most correlations were low, although significant, but we were interested in those that were 0.7 or greater, which indicates a high correlation (as well as -0.7 or less).

Overall subjective plant size measured early in the season was correlated to subjective stem height at 0.75, plant width subjective at 0.71, and leaf size subjective at 0.68. Plant size (objective) as an index was highly correlated with stem height objective at 0.67 and leaf size objective at 0.89. However, plant size (objective) as an index had low correlation objective plant width, in other words the plant width had less contribution to the plant size than stem height and leaf area. Plant size (subjective) vs. correlation with plant size (objective) was only moderately correlated (0.40),

suggesting that subjective and objective measurements are unique estimates. We would assume that correlations between the subjective and objective measurements taken at the same time of year, would have correlations above 0.70. However, correlations for stem height measured in August (0.47), plant width (0.36), and leaf area (0.50) were moderate. These correlations, although significant, indicate that our subjective and objective measurements are aligned. Branch width had the lowest correlation of the plant size measurements likely due the difficultly to gauge plant width in stevia. An estimate that involved counting branches might be more effective to capture the branching ability because plant width and branch number might be two different traits. Objective measurements are more accurate because subjective ratings could vary by the person who rates them, however the key point is to find a balance between accuracy and cost (time and labor).

Yield as fresh weight. and yield as dry weight. were highly correlated (0.73). Yield (dry weight) and dry stem weight were highly correlated (0.89). Leaf dry weight and stem dry weight were uncorrelated (-0.01). Subjective or objective measurement correlations with yield could offer valuable information on determining the phenotype that may correspond to a high yielding line. Branch number subjective ratings and objective measurements in August had the highest correlation to yield (fresh weight) (0.21, 0.39), followed by stem height (0.22, 0.33). However, for yield as dry weight, stem height had the highest correlation for subjective ratings (0.11, 0.36) and plant width had the highest correlation for objective measurements in August (0.29). However, the correlation between yield as fresh weight and yield as dry weight was high (0.86). Our findings indicate that plant width, followed by stem height, corresponded to a high yielding phenotype in our population, whereas leaf area had very low correlation to yield. Estimates in early season had less correlation than late season estimates, indicating that early season estimates do not correlate well to yield. Specific phenotypes and cultural recommendations such as plant spacing might be desired for a mechanized harvesting system. Despite yield being important, the crop must fit into a harvesting system that can be mechanized. It would also be useful to find a phenotype that correlates to glycoside content as well.

In this cultigen trial, we harvested plots by hand, as limited mechanized harvesting systems exist for smaller plots. Hand harvests may be more precise but in a mechanized system there might be an amount of loss in yield depending on the phenotype. Selection in breeding for yield would occur if using a mechanized harvesting system, where plants that do not meet the harvest system would not yield high despite the potential of variation to occur when harvested by hand. In addition, growing on covered plastic as we did for this study is an added factor, where commercial growers would not likely grow on plastic unless there is added value. We utilized plastic to attempt to control for error, and for weed control which permits selection in a breeding program.

Correlations between percent glycoside of total steviol glycosides (TSG) and concentration mg/g was high across all glycosides measured. Rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, and stevioside total content with concentration were highly correlated (0.84) (0.93) (0.88) (0.92) (0.77). Stevioside was negatively correlated with rebaudioside A, rebaudioside B, rebaudioside C, and rebaudioside D (-0.16 to -0.31). Percent stevioside had the highest negative correlation with percent rebaudioside A (-0.93), but also negatively correlated with percent rebaudioside B, rebaudioside C, and rebaudioside D. A negative correlation of total Rebaudioside A was noted for concentration of stevioside (-0.74). All other traits measured did not have high correlation (0.7). Although correlations were moderate, a significant correlation between the percent of rebaudioside A, rebaudioside B, and rebaudioside D was found.

Glycoside correlations with yield offer valuable information for developing phenotypic markers for screening stevia with desirable glycosides with less need for expensive testing which can be a limitation. In our study, we were limited in sampling due to expensive analysis procedures, so rather than test the entire population we had to randomly select within cultigens. In our population, we detected a low, but significant correlation for rebaudioside A, rebaudioside C, and rebaudioside D with yield measured fresh or dry. Glycoside compounds that also correlate to yield, enable easier breeding progress to be made for both traits simultaneously, given the population we studied.

However, these correlations are not overwhelmingly large and could vary based on the population and environment.

With many crops, there tends to be a boundary of high yield versus quality of a crop product, in this case being glycosides. Although further data is needed for all compounds, breeding for a specific glycoside such as stevioside may have its limitations where yield or biomass is sacrificed at levels of high glycoside accumulation. Depending on which glycoside is the breeding focus, will affect yield potential. However, further research and data should be collected to investigate this further. If selecting for yield and glycoside is difficult, a potential solution may be to select an intermediate yielding plant that offers a medium-high quality glycoside level, rather than selecting the highest glycoside lines specifically, but rather a combination of both together. Regardless of how high yielding a line might be, if it has minimal glycoside accumulation of interest, then growers would not plant it. The same being true for a high glycoside plant that performs terribly.

In this study plant height and leaf dry yield were found to have a positive correlation with fresh weight and dry weight, which confirms the findings of Buana and Goenadi (1985). Stevioside concentration was found to have a negative correlation with yield (Brandle and Rosa, 1992), similarly to our results (-0.25). We found that rebaudioside A concentration was correlated with yield, like the findings of Shyu (1994). We could confirm the findings that rebaudioside A and rebaudioside C concentration were positively correlated as found by Nakamura and Tamura (1985). Brandle et al (1998) also found rebaudioside A and rebaudioside C concentration to be completely linked. Weng et al. (1996) found that plants with high rebaudioside A concentration correlated with large leaves while we detected no significant correlation in our study. Dry leaf yield was correlated with plant number and plant height similar to the finding of Chalapathi et al. (1998). Leaf area was found to have no correlation with stevioside content, unlike the findings of Truong et al. (1999).

Disease resistance has a low but significant correlation with yield as fresh and dry weight and rebaudioside B, but a negative correlation with rebaudioside D concentration. This correlation between yield and disease resistance maybe due to plants that have resistance can yield more due to

less defoliation. It would also be interesting to study disease resistance further to see if there is a phenotype prone to disease pressure such as a well branched plant where a dense canopy prevents airflow. Lodging tolerance is also an important trait for a crop and for a mechanized harvesting system because plants that lodge will be missed by a harvester. Lodging tolerance had near zero or a negative correlation with yield. There may be a challenge to select for yield with lodging resistance. It would be of interest to see if a particular phenotype contributes to lodging. For example, a tall plant may be more lodging prone at a specific height, or a more branched plant.

With the selection for an individual glycoside for example rebaudioside D, correlations and heritabilities may change and offer further insight about these correlations, where additional breeding and research is needed. Although we lack extensive data currently, these findings allow us to suggest ideas for further research.

Correlations of Traits Across Two Planting Densities

Correlations were calculated to compare how predictive two planting densities would be for the same traits at both the density of 64,550 plants/ha. and 12,910 plants/ha. If correlation is high between planting densities for the same trait, we may not need to rate the plants for the same trait in two different densities which can save time and resources.

Correlations for most traits were low to moderate and we are mostly interested in traits correlated above 0.70. However, none of the traits were correlated at this level. Majority of the correlations were around 0.50. Plant size subjective June rating for 6-plant plot had high correlation (0.54). Stem height subjective June rating for the 6-plant plot had high correlation with the 30-plant plot (0.50). Branching width subjective June rating for the 6-plant plot was correlated with subjective plant width at the 30-plant plot (0.58). Leaf size for subjective June rating at the 6-plant plot was highly correlated (0.52). Stem height subjective August rating at the 6-plant plot was correlated at the 30-plant plot at (0.51). Plant size objective August rating between the 6-plant plot and the 30-plant plot was highly correlated (0.59). Stem height objective June rating between the 6-plant plot and the 30-plant plot was highly correlated (0.52). Leaf size objective measurement between the 6-plant plot

and the 30-plant plot was highly correlated (0.55). Plant size subjective average, and subjective August rating at the 6-plant plot had no correlation to any trait in the 30-plant plot. A slight negative correlation was noted for leaf size subjective August rating, suggesting that leaf size varies greatly by density.

Overall correlations between planting densities for all given traits were found to be low to moderate. This concludes that traits cannot accurately estimated by measuring at one density, because none of them had an r value above 0.70. A correlation of 0.7 or higher usually indicates you can predict roughly half from the other estimate. Leaf area is a unique and significant measurement and had small to slightly negative correlation to stem height and plant width late in the season at rating two. Yield was not measured at the smaller plot density in this experiment and would be an interesting component to add to future studies. Additionally, this and other studies could help determine optimal plot density for commercial stevia production.

Conclusions

Significant differences were identified, where cultigen was significant for majority of the traits estimated. The various seed purchased from garden seed companies is in fact unique for many traits, although the initial source of these lines are unknown. Additional studies to include cultivars as a control would be interesting to compare to the standard garden seed varieties. The presented tables show the least significant difference (LSD) which can be used to see how cultigens are placed in similar or unique groups via Fisher's test, but overall these cultigens cover a wide spread from high to low for any trait. Breeders could therefore assemble elite populations using garden seed cultigens with preferred traits. Sourcing germplasm as a breeder is one of the first steps in beginning a breeding program, however there are limitations when trying to source germplasm of stevia, because no accessions are available at the USDA. Despite initial variation in cultigens found, a wider germplasm base would be preferred for long term breeding gains, where smaller populations will likely offer short term breeding progress due to limited genetic material.

Location had a significant effect for traits such as stem height and yield (fresh weight), where varying soil types and moisture can easily occur across locations. Similarly, an effect of year was noted on yield, agronomic traits, and glycosides when measured in mg/g. Block effect (replicate) was generally insignificant for most traits except for significance for yield (fresh), and yield (dry). Despite significance of location, additional locations covering a wider range of environments should be used for future studies, as it would be interesting to detect if more variation would occur. Additionally, less replications are needed within a location due to insignificant findings as an attempt to save resources.

Significant environmental effects and interactions of cultigen were identified for location and or year suggest varieties that prefer specific regions for example yield, drought tolerance, or disease tolerance. NC-1022 (Seed Savers) was 5th in yield at Kinston, but 19th at Clinton, and therefore preferred the environment at Kinston. Although varieties that show a genotype x environment are unstable across various environments, they can be quite useful if they excel in a specific region which could be identified.

Although two densities were used in this study, we gathered agronomic data on the 30-plant plot and glycoside data on the six-plant plot, therefore we cannot determine the effect on density for traits such as glycosides and yield, but they could be useful to explore in future studies. There are various densities reported for stevia experiments, however studies are needed to determine optimal plant density for commercial stevia production. Glycoside amounts shown in the table are from a low planting density, which glycosides could decrease when grown in a more compact commercial density, due to plant stress. Future studies, may want to sample glycosides directly in the commercial density, however the findings even at a smaller density should still indicate the best glycoside lines.

The only high correlation for glycoside content was a negative correlation was found for rebaudioside A and stevioside. No other high glycoside correlations were noted. Correlations across agronomic traits were generally low otherwise. Further studies on correlations should be conducted to

identify if any strong correlations exist for yield and glycoside concentration, as both traits are most valuable in the crop.

Subjective and objective measurements were both effective in estimating stevia traits, however had low correlation between each other when measured at the same time. Subjective measures are commonly used in breeding programs, and can offer quick estimation, however were too variable for estimating stevia yield traits. Therefore, objective measurements although time consuming were more reliable. Digital imaging and advanced technology may be useful to help automate or speed up data collection for future studies.

Agronomic measurements in the 6-plant plot density had low to moderate correlation when compared to the 30-plant plot. This indicates that most traits cannot be estimated for one density based on another density. Leaf size was the most variable across two densities. Future variety trials should use the 30-plant plot as a representation of a commercial density.

The highest yielding lines were 'Katupyry' in 2016, and 'Eirete I' in 2015, where lines that yield highest in fresh weight, were consistent with dry weight yield with high correlation between fresh and dry weight. Therefore, harvest of fresh weight is an effective method alone to measure yield, and collecting dry weights is secondary. Additionally, percent dry leaf weight was only significant for year as a measured trait, with similar amounts of dry leaf regardless of the cultigen. However, the cultigen NC-1021 (Territorial) in 2016 ranked in the middle of all the cultigens with a lower harvest weight, had an equal leaf dry weight amount than cultivars ranked at the top. This suggests that selection for cultigens that produce a higher dry leaf to stem ratio has potential.

The largest leaves were found in NC-1001 (Baker Creek) and NC-1013 (Swallowtail), which were high yielding lines. NC-1011 (Everstevia) and NC-1013 (Swallowtail) were the tallest growing cultigens, and high yielding lines but not the best. Plant width was largest in 'Katupyry' and 'Eirete I' which also were the highest yielding lines for both years. Similarly, correlations between yield and plant width were the highest, followed by stem height for yield components. Therefore, phenotypic

screening for yield is most effective by selecting plants with wide architecture. Leaf size generally had the lowest correlation to yield.

Screening for *Septoria steviae*, indicated that 'Native', NC-1021 (Territorial), and NC-1023 (Johnny's) had the best resistance among cultigens. Disease incidence was higher in Clinton than in Kinston in 2016, possibly due to higher rainfall and standing water after Hurricane Matthew. Lodging resistance is a desirable trait and was greatest in NC-1024 (Park seed). Lodging could be a valuable trait to breed for harvesting systems.

Growers and breeders will be interested in the high yielding cultigens 'Katupyry', NC-1009, 'Eirete I', and 'Eirete II' and high glycoside lines such as 'Eirete I', NC-1022 (Seed Savers), and NC-1029 (R.H. Shumway). From these lines, field polycrosses can be assembled to screen for elite lines, where the best lines are replanted for continued improvement each year following. Although these cultigens are readily available in the United States, they likely represent a small portion of the available cultigens available in the world.

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Table 2.1. Cultigens planted in 2015 and 2016 with sources listed.

Cultigen	2015 Cultigen	2015 Source	2016 Cultigen	2016 Source
1	Eirete I	Stevia Store	Eirete I	Stevia Store
2	Eirete II	Stevia Store	Eirete II	Stevia Store
3	Katupyry	Stevia Store	Katupyry	Stevia Store
4	Native	Stevia Store	Native	Stevia Store
5	NC-1001	Baker Creek	NC-1020	Baker Creek
6	NC-1002	Territorial	NC-1021	Territorial
7	NC-1003	Seed Savers	NC-1022	Seed Savers
8	NC-1004	Johnny's	NC-1023	Johnny's
9	NC-1005	Park Seed	NC-1024	Park Seed
10	NC-1006	Local Harvest	NC-1025	Seedway
11	NC-1007	Hirt’s Seed	NC-1026	Stokes
12	NC-1008	Botanical Interest	NC-1027	Botanical Interest
13	NC-1009	Jung Seed	NC-1028	Jung Seed
14	NC-1010	R.H. Shumway	NC-1029	R.H. Shumway
15	NC-1011	Everstevia	NC-1030	Everstevia
16	NC-1012	Harris Seed	NC-1031	Harris Seed
17	NC-1013	Swallowtail	NC-1032	Swallowtail
18	NC-1014	Richters	NC-1033	Richters
19	NC-1015	David's Garden	NC-1034	Gurneys
20	NC-1016	Colonial Creek	NC-1035	Seed rack
21	NC-1017	Fragrant Fields	NC-1036	Rare Exotic seeds
22	NC-1018	China (Amazon)	NC-1037	Urban Farmer
23	NC-1019	Super Sweet (Amazon)	NC-1038	Super Sweet (Amazon)

Garden seed cultivars were given a "NC" accession number, unique for each year. Cultivars from "Stevia store" were left as the cultivar name.

Cultivars from "Stevia store" were left as their cultivar name.

Table 2.2. Analysis of variance of yield for a cultigen trial of 18 cultivars at a 30-plant plot density for 2015-2016 in Clinton and Kinston NC.

Source	df	Yield (Mg/ha fresh wt.)	Yield (Mg/ha dry wt.)	Yield (Mg/ha dry stem wt.)	Yield (Mg/ha dry leaf wt.)	Dry wt./dry leaf wt.	% Survival
Year (Y)	1	8.25	429.86*	20.16*	9.9*	4703.9*	0.65**
Location (L)	1	468.6*	38.16	1.94	0.61	90.1	0.8**
Y x L	1	7.41	17.51	0.0001	2.45	38.1	0.05
Rep (Y x L)	12	92.82**	45.93**	0.07	1.62	144.6	0.02*
Cultigen (C)	17	190.75**	29.75**	2.76	1.59	55.7	0.04**
Y x C	15	152.81**	26.33**	2.62	1.8	53.4	0.03**
L x C	17	44.32*	4.95	1.03	0.58	47.1	0.02
Y x L x C	14	92.77**	7.83*	1.16	0.51	41.5	0.02
Error	174	26.04	5.83	6.04	0.43	36.9	0.014

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Yield (Mg/ha fresh wt.) =Yield for fresh weight after harvest measured in Mg/ha Yield.

Yield (Mg/ha dry wt.) =Yield for dry weight after drying in Mg/ha Yield.

Yield (Mg/ha dry stem wt.) =Yield for dry stem weight after drying in Mg/ha Yield.

Yield (Mg/ha dry leaf wt.) =Yield for dry leaf weight after drying in Mg/ha Yield.

Dry wt./dry leaf wt. =dry leaf weight percent to total dry weight.

% survival= percent of stand loss, plants alive at harvest/ plants planted in Spring.

Table 2.3. Analysis of variance of glycoside amount of a cultigen trial of 18 cultivars for 2015-2016 in Clinton and Kinston NC.

Source	df	Reb A	Reb B	Reb C	Reb D	Stevioside	TSG
Year (Y)	1	6018.7*	0.5	0.1	44.8**	12370.8*	43793.3**
Location (L)	1	643.4	2.29*	33.5	4.0	56.9	688.7
Y x L	1	1095.1	0.2	0.7	14.9**	150.8	1754.9
Rep (Y x L)	8	837.9**	0.29*	27.5	1.3	1215.5**	3712.6**
Cultigen (C)	17	1034.8**	0.2	14.3	3.75**	769.0**	646.2**
Y x C	15	550.8**	0.1	15.9	2.0*	323.5*	1047.9**
L xC	17	233.0	0.1	13.4	1.69*	188.0	331.9
Y x L x C	14	186.9	0.1	6.4	0.9	209.9	355.5
Error	131	197.8	0.2	17.3	1.0	157.2	290.7

* Significantly different at 0.05 level of probability
** Significantly different at 0.01 level of probability.
Glycoside estimates are for content are measured in mg/g
All glycosides measured as amount of mg/g
TSG= total steviol glycosides in mg/g

Table 2.4. Analysis of variance of glycoside percent of total of a cultigen trial of 18 cultivars for 2015-2016 in Clinton and Kinston NC.

Source	df	% Reb A	% Reb B	% Reb C	% Reb D	% Stevioside
Year (Y)	1	0.7	0.2	193.0*	8.2	311.5
Location (L)	1	131.1	2.0**	3.0	1.0	249.5
Y x L	1	194.0	0.0	0.1	4.9	588.3
Rep (Y x L)	8	93.2	0.3**	19.6	2.3	141.0
Cultigen (C)	17	603.9**	0.21**	20*	2.3**	855.5**
Y x C	15	230.4**	0.1	5.4	1.7**	252.5**
L xC	17	108.6	0.0	15.7	0.9	193.3*
Y x L x C	14	96.0	0.1	8.3	0.4	126.8
Error	131	90.6	0.1	11.3	0.6	98.2

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Glycoside estimates represent percent of glycoside of the total steviol glycosides measured.

Table 2.5. Analysis of variance of agronomic subjective traits for a cultigen trial of 18 cultivars at a 6-plant plot density for 2015-2016 in Clinton and Kinston NC.

Source	df	Plant size (June)	Stem height (June)	Plant width (June)	Leaf area (June)
Year (Y)	1	0.1	5.3**	14.5**	0.1
Location (L)	1	5.4**	35.1**	90.2**	1.4
Y x L	1	1.7	33.2**	58.2**	11.1**
Rep (Y x L)	12	0.4*	0.4	1.0	0.6
Cultigen (C)	17	2.1**	4.3**	5.5**	1.7**
Y x C	15	1.7**	4.1**	4.5**	1.8**
L xC	17	0.4	1.6**	1.7**	1.2**
Y x L x C	14	0.54*	1.21*	2.82**	0.94*
Error	193	0.3	0.6	0.7	0.4

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Subjective ratings measured on a 1-9 subjective scale. Measured in June and August 2015-2016

Table 2.5 (continued)

Source	df	Plant size (August)	Stem height (August)	Plant width (August)	Leaf area (August)
Year (Y)	1	4.1*	0.2	10.7*	10.6**
Location (L)	1	0.8	0.0	0.6	2.9
Y x L	1	8.4**	7.9**	12.1*	5.6*
Rep (Y x L)	12	0.6**	0.7*	1.5**	0.7**
Cultigen (C)	17	1.8**	2.2**	2.7**	2.8**
Y x C	15	1.1**	1.7**	1.6**	1.3**
L xC	17	0.2	0.4	0.3	0.2
Y x L x C	14	0.4*	0.4	1.11*	0.4
Error	193	0.2	0.3	0.5	0.3

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Subjective ratings measured on a 1-9 subjective scale. Measured in June and August 2015-2016

Table 2.6. Analysis of variance of agronomic objective traits for a cultigen trial of 18 cultivars at a 6-plant plot density for 2015-2016 in Clinton and Kinston NC.

Source	df	Plant size	Stem height	Plant width	Leaf area	Disease	Lodging
Year (Y)	1	114058**	51163*	81087*	1145893**	-	-
Location (L)	1	58403**	971370**	460710**	964471**	259.7**	0.06
Y x L	1	246086**	61501*	36861	1094542**	-	-
Rep (Y x L)	12	3137	10702	16798*	29313	1.9	0.78
Cultigen (C)	17	49135**	38307**	49450**	217248**	6.1**	3.5**
Y x C	15	36139**	31171**	30523**	200857**	-	-
L x C	17	9191	6220	2605	49786	3.04**	3.1**
Y x L x C	14	8427	1888	10399	43909	-	-
Error	193	7354	6045	8329	39899	1.24	1.06

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Note- Disease and lodging ratings only measured in 2016, year estimates are unavailable.

Objective measurements measured in millimeters measured in August 2015-2016

Table 2.7. LS means for 20 cultigens for subjective rating 1, June 2015. 1-9 subjective scale by cultigen for 2015 at two densities.

Cultigen	Source	Plant size (1-9)	Stem height (1-9)	Plant width (1-9)	Leaf area (1-9)
NC-1008	Botanical Interest	5.9	5.9	6.2	5.6
Eirete II	Stevia Store	5.7	5.9	6.0	5.3
NC-1001	Baker Creek	5.5	5.9	5.1	5.7
NC-1004	Johnny's	5.4	5.9	5.6	4.8
Katupyry	Stevia Store	5.4	5.8	5.4	5.1
Native	Stevia Store	5.4	5.9	5.4	4.9
NC-1005	Park Seed	5.3	5.2	5.2	5.5
Eirete I	Stevia Store	5.3	5.6	5.4	5.0
NC-1011	Everstevia	5.3	5.4	4.1	6.2
NC-1007	Hirt's Seed	5.2	6.5	5.1	4.1
NC-1009	Jung Seed	5.1	5.1	5.4	4.8
NC-1014	Richters	5.1	5.3	4.7	5.3
NC-1003	Seed Savers	5.0	5.0	4.9	5.3
NC-1002	Territorial	4.9	5.2	4.7	4.9
NC-1015	David's Garden	4.7	5.0	4.6	4.4
NC-1010	R.H. Shumway	4.6	4.9	4.7	4.3
NC-1013	Swallowtail	4.5	4.9	3.6	5.0
NC-1017	Fragrant Fields	4.4	6.1	4.3	2.9
NC-1012	Harris Seed	4.2	4.3	3.6	4.9
NC-1016	Colonial Creek	4.0	4.9	3.4	3.7
Mean		5.0	5.4	4.7	4.8
LSD 5%		0.5	0.6	0.7	0.5
r		0.54***	0.5***	0.58***	0.52***

Mean- Mean of trait across all cultigens

LSD- Least significant difference between cultigens at the 0.05 confidence interval

r= Correlation of trait measured in a 6-plant plot density to a 30-plant plot density. Rating 1 measured in June 2015.

Table 2.8. LS means for 19 cultigens for subjective ratings, June 2016. 1-9 subjective scale by cultigen at two densities.

Cultigen	Source	Plant size (1-9)	Stem height (1-9)	Plant width (1-9)	Leaf area (1-9)
Eirete II	Stevia Store	5.9	6.9	5.7	5.2
Katupyry	Stevia Store	5.9	7.4	5.6	4.7
NC-1032	Swallowtail	5.6	5.9	4.5	6.6
NC-1033	Richters	5.4	5.5	4.7	6.0
NC-1020	Baker Creek	5.3	5.7	4.4	6.0
NC-1023	Johnny's	5.2	5.5	4.8	5.4
NC-1028	Jung Seed	5.2	5.6	4.7	5.2
NC-1029	R.H. Shumway	5.1	5.3	4.8	5.3
NC-1021	Territorial	5.0	5.4	4.1	5.5
NC-1034	Gurneys	4.8	4.9	3.9	5.5
Eirete I	Stevia Store	4.8	5.3	3.8	5.2
NC-1031	Harris Seed	4.8	4.9	4.1	5.3
NC-1027	Botanical Interest	4.7	5.0	3.9	5.2
NC-1030	Everstevia	4.5	5.1	3.8	4.7
NC-1022	Seed Savers	4.4	4.7	3.3	5.3
NC-1024	Park Seed	4.4	4.6	3.6	4.9
NC-1025	Seedway	4.4	4.6	3.6	4.8
NC-1026	Stokes	4.2	4.4	3.7	4.5
Native	Stevia Store	3.8	4.0	4.1	3.4
Mean		4.9	5.3	4.3	5.1
LSD 5%		0.6	0.7	0.7	0.6
r		0.54**	0.5**	0.58**	0.52**

Mean- Mean of trait across all cultigens

LSD- Least significant difference between cultigens at the 0.05 confidence interval

r= Correlation of trait measured in a 6-plant plot density to a 30-plant plot density.

Rating 1 measured in June 2016, Rating 2 measured in August 2016

Table 2.9. LS means for 20 cultigens for subjective rating 2, August 2015. 1-9 subjective scale by cultigen for 2015 at two densities.

Cultigen	Source	Plant size (1-9)	Stem height (1-9)	Plant width (1-9)	Leaf area (1-9)
NC-1011	Everstevia	6.3	7.0	5.4	6.4
NC-1001	Baker Creek	6.1	6.9	5.6	5.9
Eirete II	Stevia Store	6.1	6.7	6.3	5.3
Eirete I	Stevia Store	6.1	6.8	6.1	5.3
Katupyry	Stevia Store	6.1	6.7	6.2	5.3
NC-1014	Richters	6.0	6.6	5.7	5.6
Native	Stevia Store	6.0	6.9	5.6	5.3
NC-1005	Park Seed	5.8	6.3	5.7	5.5
NC-1004	Johnny's	5.8	6.5	5.8	5.2
NC-1008	Botanical Interest	5.7	5.8	6.1	5.2
NC-1010	R.H. Shumway	5.7	6.3	5.7	5.0
NC-1003	Seed Savers	5.7	6.3	5.5	5.2
NC-1002	Territorial	5.4	5.8	5.0	5.4
NC-1013	Swallowtail	5.4	5.7	4.9	5.6
NC-1015	David’s Garden	5.4	6.1	5.0	5.0
NC-1009	Jung Seed	5.2	5.7	5.3	4.7
NC-1007	Hirt’s Seed	5.2	5.9	5.2	4.4
NC-1012	Harris Seed	5.1	5.5	4.4	5.6
NC-1016	Colonial Creek	4.8	5.1	5.0	4.3
NC-1017	Fragrant Fields	4.4	4.9	4.8	3.5
Mean		5.6	6.2	5.4	5.2
LSD 5%		0.4	0.5	0.6	0.4
r		0.01	0.51**	0.42**	-0.08

Mean- Mean of trait across all cultigens
LSD- Least significant difference between cultigens at the 0.05 confidence interval
r= Correlation of trait measured in a 6-plant plot density to a 30-plant plot density.

Table 2.10. LS means for 19 cultigens for subjective rating 2, August 2016. 1-9 subjective scale by cultigen for 2016 at two densities.

Cultigen	Source	Plant size (1-9)	Stem height. (1-9)	Plant width (1-9)	Leaf area (1-9)
NC-1020	Baker Creek	7.0	7.5	6.0	7.5
NC-1032	Swallowtail	6.9	7.5	6.0	7.3
NC-1022	Seed Savers	6.3	6.5	6.2	6.2
NC-1033	Richters	6.2	6.7	5.8	6.1
NC-1028	Jung Seed	6.1	6.7	6.0	5.6
NC-1029	R.H. Shumway	6.1	6.6	6.0	5.6
Katupyry	Stevia Store	6.1	6.5	6.3	5.4
Eirete II	Stevia Store	6.1	6.2	6.6	5.4
NC-1023	Johnny's	6.1	6.6	6.1	5.5
NC-1034	Gurneys	5.8	6.1	5.5	5.8
Eirete I	Stevia Store	5.8	6.2	5.5	5.7
NC-1030	Everstevia	5.8	6.3	5.7	5.4
NC-1021	Territorial	5.7	5.7	5.7	5.6
NC-1026	Stokes	5.7	5.8	5.4	5.8
NC-1031	Harris Seed	5.7	5.9	5.5	5.6
NC-1024	Park Seed	5.6	5.9	5.4	5.6
NC-1027	Botanical Interest	5.6	5.9	5.4	5.4
NC-1025	Seedway	5.5	5.8	5.2	5.4
Native	Stevia Store	4.6	4.4	4.8	4.5
Mean		5.9	6.2	5.7	5.7
LSD 5%		0.9	0.4	0.3	0.4
r		0.01	0.51**	0.42**	-0.08

Mean- Mean of trait across all cultigens

LSD- Least significant difference between cultigens at the 0.05 confidence interval

r= Correlation of trait measured in a 6-plant plot density to a 30-plant plot density.

Table 2.11. LS means for 20 cultigens for objective measurement, August 2015. Measurements in millimeters for 2015 at two densities.

Cultigen	Source	Plant size mm	Stem height mm	Plant width mm	Leaf area mm^2
NC-1011	Everstevia	597	690	443	657
NC-1001	Baker Creek	593	721	473	585
Native	Stevia Store	565	760	502	434
Eirete I	Stevia Store	561	720	495	469
Eirete II	Stevia Store	558	740	473	462
NC-1014	Richters	550	662	423	566
NC-1003	Seed Savers	530	673	452	466
NC-1002	Territorial	527	655	404	523
Katupyry	Stevia Store	526	727	462	388
NC-1007	Hirt's Seed	524	671	409	490
NC-1005	Park Seed	518	652	445	456
NC-1015	David's Garden	517	659	474	416
NC-1008	Botanical Interest	515	604	382	558
NC-1010	R.H. Shumway	511	662	448	423
NC-1013	Swallowtail	509	632	384	511
NC-1009	Jung Seed	508	640	424	460
NC-1004	Johnny's	503	672	458	380
NC-1012	Harris Seed	459	535	314	529
NC-1017	Fragrant Fields	426	617	422	240
NC-1016	Colonial Creek	406	537	377	303
Mean		518	658	426	471
LSD 5%		52	76	68	102
r		0.59**	0.52**	0.44**	0.55**

Mean- Mean of trait across all cultigens

LSD- Least significant difference between cultigens at the 0.05 confidence interval

r= Correlation of trait measured in a 6-plant plot density to a 30-plant plot density.

Table 2.12. LS means for 19 cultigens for objective measurement, August 2016. Measurements in millimeters for 2016 at two densities.

Cultigen	Source	Plant size mm	Stem ht. mm	Branch width mm	Leaf area mm^2	Disease (1-9)	Lodging (1-9)
NC-1032	Swallowtail	944	884	425	1522	6.5	4.2
NC-1020	Baker Creek	942	832	393	1602	6.8	4.3
NC-1022	Seed Savers	692	704	428	944	6.8	5.3
NC-1023	Johnny's	658	738	425	811	5.5	3.8
NC-1033	Richters	643	784	408	736	5.7	5.1
Eirete II	Stevia Store	628	740	481	663	7.0	4.3
Eirete I	Stevia Store	625	720	396	761	6.9	6.6
NC-1028	Jung Seed	621	749	434	680	5.6	3.3
NC-1034	Gurneys	604	699	363	750	7.2	5.1
NC-1021	Territorial	602	698	371	736	6.5	5.0
NC-1029	R.H. Shumway	598	730	428	636	6.3	4.0
NC-1024	Park Seed	591	663	369	742	6.6	4.9
NC-1025	Seedway	572	664	338	715	6.3	5.5
NC-1031	Harris Seed	571	648	366	700	5.5	3.8
NC-1026	Stokes	571	643	367	703	6.5	4.8
Katupyry	Stevia Store	566	747	420	531	-	-
NC-1027	Botanical Interest	565	683	348	664	5.3	3.5
NC-1030	Everstevia	542	710	401	515	4.8	3.0
Native	Stevia Store	407	483	286	452	4.6	2.4
Mean		616	709	390	750	6.1	4.5
LSD 5%		95	89	44	238	0.6	0.7
r		0.59**	0.5**	0.4**	0.55**	-	-

Mean- Mean of trait across all cultigens

Disease and lodging on a subjective 1-9 scale.

LSD- Least significant difference between cultigens at the 0.05 confidence interval

r= Correlation of trait measured in a 6-plant plot density to a 30-plant plot density.

Table 2.13. LS means for 20 cultigens for yield in mg/ha, September 2015.

Cultigen	Source	Fresh wt. Mg/ha	Dry wt. Mg/ha	Dry stem wt. Mg/ha	Dry leaf wt. Mg/ha	% dry leaf
Eirete II	Stevia Store	23.1	9.6	4.6	2.7	0.28
Eirete I	Stevia Store	22.9	9.5	4.2	1.9	0.20
Native	Stevia Store	21.4	8.3	5.0	2.3	0.28
NC-1001	Baker Creek	19.9	7.9	3.5	1.8	0.23
Katupyry	Stevia Store	20.8	7.8	2.5	2.0	0.25
NC-1005	Park Seed	18.1	7.4	3.6	1.9	0.25
NC-1010	R.H. Shumway	18.7	7.4	3.2	1.9	0.26
NC-1014	Richters	17.2	7.2	3.4	1.7	0.24
NC-1004	Johnny's	18.8	7.1	3.6	1.8	0.25
NC-1002	Territorial	16.0	6.5	4.4	1.7	0.26
NC-1015	David’s Garden	16.9	6.3	2.7	1.5	0.23
NC-1003	Seed Savers	16.4	6.2	2.9	1.9	0.31
NC-1011	Everstevia	14.9	6.1	3.4	1.9	0.32
NC-1009	Jung Seed	13.4	5.6	1.7	1.4	0.24
NC-1008	Botanical Interest	12.5	4.6	1.6	1.6	0.35
NC-1013	Swallowtail	13.0	4.5	1.3	1.3	0.28
NC-1017	Fragrant Fields	10.2	4.1	-	-	-
NC-1007	Hirt’s Seed	12.6	4.1	-	-	-
NC-1016	Colonial Creek	4.3	2.9	1.0	0.8	0.29
NC-1012	Harris Seed	9.6	2.5	-	-	-
Mean		15.5	6.1	2.9	1.7	0.27
LSD 5%		2.1	1.1	0.5	0.1	0.17

Mean- Mean of trait across all cultigens
LSD- Least significant difference between cultigens at the 0.05 confidence interval
*Some loss of material or gain due to humidity occurred during processing dry samples

Table 2.14. LS means for 20 cultigens for yield in mg/ha, September 2016.

Cultigen	Source	Fresh wt. Mg/ha	Dry wt. Mg/ha	Dry stem wt. Mg/ha	Dry leaf wt. Mg/ha	% dry leaf
Katupyry	Stevia Store	24.3	6.8	5.0	1.6	0.24
NC-1029	R.H. Shumway	22.3	6.4	3.5	2.0	0.31
NC-1023	Johnny's	20.2	5.6	4.2	2.0	0.37
NC-1028	Jung Seed	19.8	5.2	3.1	2.0	0.38
NC-1032	Swallowtail	19.7	5.2	3.1	1.6	0.32
Eirete II	Stevia Store	19.5	4.7	4.4	2.0	0.42
Eirete I	Stevia Store	15.4	4.4	3.2	1.6	0.38
NC-1030	Everstevia	15.4	4.1	3.3	1.8	0.44
NC-1021	Territorial	13.9	4.1	3.0	2.0	0.50
NC-1033	Richters	15.7	4.0	2.6	1.6	0.39
NC-1027	Botanical Interest	13.6	3.6	2.4	1.7	0.47
NC-1022	Seed Savers	15.6	3.5	2.2	1.1	0.32
NC-1020	Baker Creek	14.5	3.4	1.3	0.7	0.21
NC-1031	Harris Seed	13.7	3.3	2.1	1.4	0.43
NC-1025	Seedway	13.1	3.3	2.6	1.4	0.41
NC-1024	Park Seed	13.0	3.2	2.2	1.1	0.33
NC-1034	Gurneys	14.9	3.1	2.9	1.7	0.55
NC-1026	Stokes	11.6	2.6	1.7	1.1	0.41
Native	Stevia Store	10.0	1.8	1.8	1.1	0.65
Mean		14.6	3.7	2.6	1.4	0.39
LSD 5%		2.0	0.6	0.4	0.1	0.27

Mean- Mean of trait across all cultigens

LSD- Least significant difference between cultigens at the 0.05 confidence interval

*Some loss of material or gain due to humidity occurred during processing dry samples

Table 2.15. LS means of glycoside amount (mg/g) of total for 20 cultigens in 2015.

Cultigen	Source	RebA mg/g	Reb B mg/g	Reb C mg/g	Reb D mg/g	Stevioside mg/g	RebA : Stv	TSG
NC-1005	Park Seed	51.2	0.6	6.4	2.6	67.1	0.8	138
NC-1010	R.H. Shumway	59.1	0.5	10.5	2.4	52.9	1.1	137
NC-1001	Baker Creek	55.9	0.5	7.2	2.8	59.3	0.9	137
NC-1002	Territorial	51.4	0.6	12.8	2.3	54.2	0.9	133
NC-1014	Richters	44.3	0.5	11.7	2.5	62.4	0.7	133
NC-1003	Seed Savers	55.3	0.6	8.5	4.5	47.7	1.2	130
Katupyry	Stevia Store	56.4	0.7	6.3	2.9	51.9	1.1	129
NC-1016	Colonial Creek	41.7	0.5	4.9	2.6	67.0	0.6	129
NC-1009	Jung Seed	49.6	0.5	6.2	2.9	57.3	0.9	127
NC-1007	Hirt’s Seed	21.4	0.2	1.9	1.0	91.8	0.2	126
NC-1011	Everstevia	42.5	0.6	6.2	2.6	60.4	0.7	123
NC-1012	Harris Seed	38.8	0.5	6.2	2.0	65.2	0.6	122
Native	Stevia Store	46.2	0.5	8.2	2.6	52.5	0.9	121
NC-1015	David’s Garden	48.9	0.5	5.9	2.1	53.9	0.9	121
Eirete I	Stevia Store	56.9	1.3	7.2	3.0	41.1	1.4	121
Eirete II	Stevia Store	52.7	0.5	7.6	2.8	42.5	1.2	120
NC-1013	Swallowtail	35.0	0.4	6.2	2.1	63.2	0.6	117
NC-1004	Johnny's	47.0	0.6	6.5	3.4	43.9	1.1	113
NC-1008	Botanical Interest	31.9	0.5	7.8	1.5	44.3	0.7	112
NC-1017	Fragrant Fields	3.7	0.1	0.1	0.3	93.3	0.0	105
Trait mean		45.9	0.6	6.9	2.6	55.8	1.0	124
LSD 5%		7.7	0.2	2.4	0.6	7.8	0.4	9

Mean= Mean of trait across all cultigens
LSD=Least significant difference between cultigens at the 0.05 confidence interval
TSG=Total steviol glycosides.
*Some glycosides are not listed that amount to the total steviol glycosides

Table 2.16. LS means of glycoside amount (mg/g) of total for 19 cultigens in 2016.

Cultigen	Source	Reb A mg/g	Reb B mg/g	Reb C mg/g	Reb D mg/g	Stevioside mg/g	RebA : Stv	TSG
NC-1022	Seed Savers	65.1	0.8	10.1	2.8	32.5	2.0	123
NC-1030	Everstevia	52.5	0.7	9.0	2.3	32.3	1.6	107
NC-1025	Seedway	40.8	0.6	8.9	1.8	43.4	0.9	106
Eirete II	Stevia Store	43.9	0.5	8.5	2.2	37.6	1.2	102
NC-1031	Harris Seed	38.2	0.6	8.8	1.7	43.4	0.9	102
NC-1023	Johnny's	36.8	0.6	8.5	2.0	40.8	0.9	100
NC-1029	R.H. Shumway	43.8	0.6	13.9	2.3	26.9	1.6	99
NC-1027	Botanical Interest	33.0	0.5	8.1	1.6	44.4	0.7	96
Eirete I	Stevia Store	38.9	0.6	8.8	1.4	37.0	1.1	95
NC-1024	Park Seed	26.5	0.3	7.7	1.9	45.2	0.6	93
NC-1028	Jung Seed	34.7	0.5	9.2	1.6	35.6	1.0	91
NC-1021	Territorial	28.4	0.4	8.1	1.5	40.4	0.7	88
NC-1033	Richters	31.9	0.5	6.5	1.4	37.1	0.9	85
NC-1026	Stokes	21.0	0.4	7.6	1.1	44.6	0.5	83
Native	Stevia Store	28.1	0.5	7.0	1.9	34.8	0.8	82
NC-1020	Baker Creek	21.2	0.3	6.8	0.9	43.6	0.5	81
NC-1034	Gurneys	14.8	0.3	7.6	1.2	46.3	0.3	79
NC-1032	Swallowtail	16.0	0.3	5.9	0.9	39.0	0.4	70
Katupyry	Stevia Store	21.2	0.5	4.9	1.0	24.2	0.9	58
Trait mean		34.3	0.5	8.2	1.7	38.0	1.0	92
LSD 5%		7.8	0.1	1.6	0.7	4.6	0.4	9

Mean= Mean of trait across all cultigens
LSD= Least significant difference between cultigens at the 0.05 confidence interval
TSG=Total steviol glycosides
*Some glycosides are not listed that amount to the total steviol glycosides

Table 2.17. LS means of glycoside percent of total for 20 cultigens in 2015.

Cultigen	Source	RebA % total	Reb B % total	Reb C % total	Reb D % total	Stevioside % total
NC-1001	Baker Creek	40.8	0.5	5.3	2.1	43.1
NC-1008	Botanical Interest	33.8	0.1	8.0	2.0	47.0
NC-1016	Colonial Creek	32.0	0.3	3.9	2.0	52.0
NC-1015	David's Garden	41.3	0.4	5.0	1.8	43.4
Eirete I	Stevia Store	46.5	1.1	5.9	2.4	34.5
Eirete II	Stevia Store	43.8	0.4	6.3	2.4	35.8
NC-1011	Everstevia	33.6	0.5	5.2	2.1	49.8
NC-1017	Fragrant Fields	6.0	0.0	0.0	0.4	85.5
NC-1012	Harris Seed	30.9	0.4	5.1	1.6	53.9
NC-1007	Hirt's Seed	15.2	0.1	1.1	0.7	75.6
NC-1004	Johnny's	41.4	0.5	5.6	3.0	38.9
NC-1009	Jung Seed	39.1	0.4	4.9	2.3	44.8
Katupyry	Stevia Store	43.4	0.6	4.8	2.3	40.1
Native	Stevia Store	38.2	0.4	7.0	2.2	42.6
NC-1005	Park Seed	36.8	0.4	4.7	1.8	48.6
NC-1010	R.H. Shumway	42.1	0.4	7.7	1.9	39.1
NC-1014	Richters	32.9	0.4	9.2	1.9	47.0
NC-1003	Seed Savers	41.1	0.5	6.5	3.5	38.1
NC-1013	Swallowtail	29.7	0.3	5.2	1.9	54.3
NC-1002	Territorial	37.6	0.4	9.1	1.7	42.4
Trait mean		36.1	0.4	5.5	2.0	46.9
LSD 5%		5.0	0.2	2.0	0.4	6.0

Mean= Mean of trait across all cultigens
LSD= Least significant difference between cultigens at the 0.05 confidence interval

Table 2.18. LS means of glycoside percent of total for 21 cultigens in 2016.

Cultigen	Source	RebA % total	Reb B % total	Reb C % total	Reb D % total	Stevioside % total
NC-1020	Baker Creek	25.1	0.4	8.3	1.1	55.1
NC-1027	Botanical Interest	32.6	0.5	8.5	1.6	47.7
Eirete I	Stevia Store	40.4	0.7	9.2	1.5	39.5
Eirete II	Stevia Store	42.1	0.5	8.3	2.0	37.6
NC-1030	Everstevia	48.3	0.7	8.5	2.2	31.2
NC-1034	Gurneys	17.9	0.3	9.6	1.5	59.3
NC-1031	Harris Seed	35.8	0.6	8.7	1.7	43.9
NC-1023	Johnny's	37.1	0.6	8.6	1.9	41.0
NC-1028	Jung Seed	37.6	0.6	10.5	1.8	39.0
Katupyry	Stevia Store	40.5	0.9	8.3	1.6	38.5
Native	Stevia Store	33.3	0.6	8.6	2.3	43.3
NC-1024	Park Seed	27.2	0.3	8.5	1.8	50.6
NC-1029	R.H. Shumway	42.9	0.7	15.3	2.2	27.2
NC-1033	Richters	36.5	0.6	7.9	1.5	45.1
NC-1022	Seed Savers	51.2	0.7	8.4	2.3	28.0
NC-1025	Seedway	37.7	0.6	8.5	1.7	41.9
NC-1026	Stokes	23.3	0.4	9.3	1.3	55.7
NC-1032	Swallowtail	20.8	0.4	7.9	1.1	58.4
NC-1021	Territorial	31.1	0.5	9.3	1.7	47.0
Trait mean		35.5	0.6	9.0	1.8	43.0
LSD 5%		6.0	0.2	2.0	0.5	6.0

Mean= Mean of trait across all cultigens

LSD= Least significant difference between cultigens at the 0.05 confidence interval

Table 2.19. Pearson correlations for agronomic and glycoside traits of 25 stevia cultigens grown at two locations in 2015 and 2016.

	Plant size avg.	Stem ht. Sub. June	Width Sub. June	Leaf Sub. June	Stem ht. Sub August	Width Sub August	Leaf Sub August.
Plant size avg							
Stem ht. Sub1	0.75**						
Width Sub1	0.71**	0.63**					
Leaf Sub1	0.68**	0.41**	0.31**				
Stem ht. Sub2	0.67**	0.29**	0.22**	0.36**			
Width Sub2	0.52**	0.21**	0.24**	0.13**	0.44**		
Leaf Sub2	0.44**	0.00	-0.1*	0.43**	0.52**	0.24**	
Plant sz Obj	0.40**	0.17**	0.01	0.40**	0.46**	0.22**	0.54**
Stem ht. Obj	0.36**	0.17**	0.05	0.31**	0.47**	0.24**	0.36**
Width Obj	0.19**	0.11**	0.04	-0.02	0.25**	0.36**	0.10*
Leaf Obj	0.30**	0.12**	-0.03	0.38**	0.31**	0.07	0.50**
Lodging	0.13	0.01	-0.06	0.09	0.30*	0.21	0.27*
Disease	0.31**	0.38**	0.48**	0.25*	-0.04	-0.22	-0.27*
Plant wt. fresh	0.35**	0.19**	0.24**	0.15*	0.33**	0.39**	0.06
Plant wt. dry	0.30**	0.11	0.25**	0.09	0.36**	0.28**	0.04
Dry stem wt.	0.30**	0.26*	0.18	0.28**	0.28**	0.11	-0.05
Dry leaf wt.	0.44**	0.38**	0.35**	0.26*	0.23*	0.28**	0.04
Reb A	0.10	0.01	0.12	0.03	0.13*	0.10	-0.01
Reb B	0.11	0.06	0.17**	0.02	0.11	0.10	-0.04
Reb C	0.00	-0.10	-0.04	0.03	0.03	0.02	0.08
Reb D	0.11	0.05	0.14*	0.00	0.17**	0.09	-0.06
Stev	-0.13*	0.08	0.04	-0.08	-0.10	-0.05	-0.16*
%Reb A	0.11*	0.00	0.16**	0.05	0.10*	0.11*	0.00
%Reb B	0.18**	0.12**	0.23**	0.08	0.11*	0.10*	0.02
%Reb C	0.01	-0.08	-0.05	0.04	0.06	0.04	0.13**
%Reb D	0.08	-0.03	0.17**	0.06	0.06	0.08	-0.08
%Stev	0.12**	0.02	-0.16**	-0.06	-0.12**	-0.14**	-0.03

Plant size avg= Index for plant size subjective average
Stem ht. Sub1= Stem height subjective rating measured in June on a 1-9 scale.
Width Sub1= Plant width subjective measured in June on a 1-9 scale.
Leaf Sub1= Leaf area subjective measurement measured in June on a 1-9 scale.
Stem ht. Sub2= Stem height subjective rating measured in August on a 1-9 scale.
Width Sub2= Branching width subjective measured in August on a 1-9 scale.
Leaf Sub2= Leaf area subjective measurement measured in August on a 1-9 scale.
Plant sz Obj= Index for plant size average for objective measurements in mm.
Stem ht. Obj = Stem height for objective measurements in August in mm.
Width Obj = Branching width objective measurements in August in mm.
Leaf Obj = Leaf area measured objectively in August in mm.
Lodging= Lodging resistance measured subjectively on a 0-9 scale.
Disease= Disease resistance measured subjectively on a 0-9 scale.
Yield (FW) = Yield of fresh weight measured in Mg/ha.

Yield (DW) = Yield of dry weight measured in Mg/ha.
Yield (DSW) = Yield of dry stem weight measured in Mg/ha.
Yield (DLW) = Yield of dry leaf weight measured in Mg/ha.
Glycosides measured in total amount of mg/g.
Glycosides % measured in percent of glycoside/ total steviol glycosides.
* Significant at 0.05 level of probability
** Significantly at 0.01 level of probability.
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Table 2.19. (continued)

	Plant size Objective August	Stem ht. Objective August	Width Objective August	Leaf Objective August	Lodging	Disease
Stem ht. Obj	0.67**					
Width Obj	0.28**	0.32**				
Leaf Obj	0.89**	0.32**	-0.08			
Lodging	0.24*	0.19	0.20	0.14		
Disease	0.07	-0.24*	-0.15	0.21	0.17	
Plant wt. fresh	0.06	0.22**	0.21**	-0.05	0.02	-0.01
Plant wt. dry	-0.11	0.15**	0.29**	-0.24**	-0.10	0.21*
Dry stem wt.	-0.20	0.01	0.21*	-0.29**	-0.17	0.30*
Dry leaf wt.	0.03	0.22*	-0.21*	0.01	-0.18	0.35**
Reb A	-0.11	-0.06	0.13*	-0.20**	-0.30*	-0.09
Reb B	-0.12	-0.17**	-0.01	-0.08	-0.33**	0.35**
Reb C	0.06	0.00	0.02	0.07	-0.04	0.02
Reb D	-0.15*	-0.11	0.10	-0.21**	-0.21	-0.24*
Stevioside	-0.05	-0.03	0.08	-0.10	0.08	-0.14
%Reb A	-0.06	-0.05	0.01	-0.06	-0.18*	-0.05
%Reb B	-0.07	-0.12**	-0.09*	0.00	-0.23**	0.37**
%Reb C	0.11*	0.05	-0.07	0.15**	0.07	0.13
%Reb D	-0.17**	-0.14**	0.01	-0.16**	-0.08	-0.12
%Stevioside	0.04	0.06	0.03	0.01	0.15	0.04

Plant sz Obj= Index for plant size average for objective measurements in mm.
Stem ht. Obj = Stem height for objective measurements in August in mm.
Width Obj = Branching width objective measurements in August in mm.
Leaf Obj = Leaf area measured objectively in August in mm.
Lodging= Lodging resistance measured subjectively on a 0-9 scale.
Disease= Disease resistance measured subjectively on a 0-9 scale.
Yield (FW) = Yield of fresh weight measured in Mg/ha.
Yield (DW) = Yield of dry weight measured in Mg/ha.
Yield (DSW) = Yield of dry stem weight measured in Mg/ha.
Yield (DLW) = Yield of dry leaf weight measured in Mg/ha.
Glycosides measured in total amount of mg/g.
Glycosides % measured in percent of glycoside/ total steviol glycosides.
* Significant at 0.05 level of probability
** Significantly at 0.01 level of probability.

Table 2.19. (continued)

	Plant wt. fresh	Plant wt. dry	Dry stem wt.	Dry leaf wt.
Plant wt. dry	0.73**			
Dry stem wt.	0.53**	0.82**		
Dry leaf wt.	0.60**	0.53**	-0.01	
Reb A	0.12*	0.12*	0.34**	-0.22**
Reb B	0.13**	0.18**	0.28**	-0.01
Reb C	0.05	0.06	0.08	0.07
Reb D	0.09*	0.21**	0.51**	-0.22**
Stevioside	-0.23**	-0.09*	0.16*	-0.59**
%Reb A	0.23**	0.16**	0.17*	0.15
%Reb B	0.18**	0.19**	0.10	0.35**
%Reb C	0.08	-0.04	-0.10	0.34**
%Reb D	0.13**	0.24**	0.35**	0.04
%Stevioside	-0.25**	-0.17**	-0.11	-0.30**

Yield (FW) = Yield of fresh weight measured in Mg/ha.
Yield (DW) = Yield of dry weight measured in Mg/ha.
Yield (DSW) = Yield of dry stem weight measured in Mg/ha.
Yield (DLW) = Yield of dry leaf weight measured in Mg/ha.
Glycosides measured in total amount of mg/g.
Glycosides % measured in percent of glycoside/ total steviol glycosides.
* Significant at 0.05 level of probability
** Significantly at 0.01 level of probability.

Table 2.19. (continued)

	Reb A	Reb B	Reb C	Reb D
Reb B	0.54**			
Reb C	0.29**	0.11		
Reb D	0.59**	0.50**	0.04	
Stevioside	-0.23**	-0.31**	-0.26**	-0.16*
% Reb A	0.84**	0.54**	0.22**	0.47**
% Reb B	0.35**	0.93**	0.06	0.36**
% Reb C	-0.02	0.00	0.88**	-0.16**
% Reb D	0.39**	0.45**	-0.03	0.92**
% Stevioside	-0.74**	-0.54**	-0.45**	-0.50**

Glycosides measured in total amount of mg/g.
Glycosides % measured in percent of glycoside/ total steviol glycosides.
* Significant at 0.05 level of probability
** Significantly at 0.01 level of probability.

Table 2.19. (continued)

	%Reb A	% Reb B	%Reb C	% Reb D
%Reb B	0.52**			
%Reb C	0.10	0.08		
%Reb D	0.47**	0.43**	-0.1*	
%Stev	-0.93**	-0.56**	-0.39**	-0.54**

Glycosides measured in total amount of mg/g.
Glycosides % measured in percent of glycoside/ total steviol glycosides.
* Significant at 0.05 level of probability
** Significantly at 0.01 level of probability.

Fig. 2.1. Unbranched selection of stevia cultigen NC-1014 (from Richters seeds purchased in 2015).

Fig. 2.2. Large leaves of stevia cultigen NC-1001 (Baker Creek).

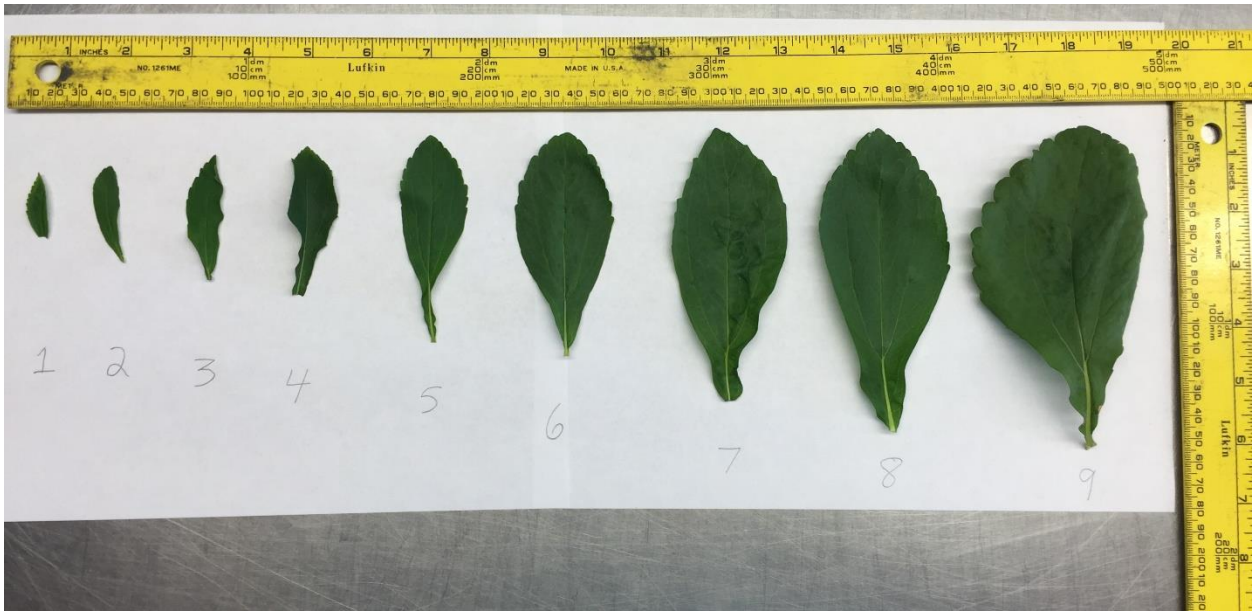

Fig. 2.3. Subjective rating scale (1-9) for leaf size.

Fig 2.4. A highly-branched plant of stevia on ‘Eirete-II’.

Fig. 2.5. Disease resistant and susceptible plants of stevia.

Fig. 2.6. Lodging susceptible cultigen of stevia.

Fig. 2.7. Lodging resistant cultigen of stevia.

CHAPTER 3: HERITABILITY AND GENETIC VARIANCE ESTIMATES FOR AGRONOMIC TRAITS AND GLYCOSIDE YIELD IN FOUR ELITE STEVIA BREEDING POPULATIONS

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Abstract

Narrow sense heritability of agronomic traits and glycoside concentration were measured in four elite stevia (*Stevia rebaudiana* Bertoni) breeding populations. Heritability was estimated using half-sib family analysis. Data were from North Carolina field trials conducted in 2016. StvEP1, StvEP2, StvEP3, and StvEP4 are elite breeding populations with superior traits. Heritability estimates for traits of interest from useful populations will provide breeders with information for planning their cultivar development programs.

There were significant differences among the cultigens for most of the agronomic and glycoside traits evaluated. Stem height, plant width, and leaf size were assessed subjectively as well as objectively measured. Cultigen was significant for all subjective ratings, and for all objective measurements except for plant width measured in August. Yield (as fresh and dry weight) was significant, as lodging resistance, disease resistance, and percent survival were. All glycosides had significant differences across cultigens except for rebaudioside B as a concentration of steviol glycosides.

Pearson correlations were generated to assess relationship between yield and glycoside traits: rebaudioside C concentration had the highest correlation with yield. Correlations were low between

subjective ratings and objective measurements, indicating that subjective assessments did not turn out useful and objective measurements may be more precise.

Plant growth traits were measured, including stem height, and plant width and leaf size, all of which may contribute to yield. Heritability estimates pooled over the four populations were moderate for yield as dry weight (0.41) and low for lodging resistance (0.10). Stem height had high heritability, plant width had moderate heritability, and leaf size had low heritability.

Rebaudiosides A, C, D, stevioside (and total steviol glycosides (TSG) had high heritability: Rebaudioside B had a heritability near zero.

Gain from selection at 20% selection pressure was estimated for agronomic and glycoside traits. Growth traits, measured subjectively (1 to 9 scale) included plant size (0.4/year), stem height (0.3/year), plant width (0.4/year), and leaf size (0.2/year). The gain from selection for growth traits, measured objectively in June was estimated for plant size (127 mm/year), stem height (86 mm/year), plant width (37 mm/year) , and leaf area(289 mm^2). The gain from selection for growth traits assessed subjectively in August was estimated for plant size(0.2/year), stem height(0.3/year), plant width(0.04/year), and leaf size(0.1/year). The gain from selection for growth traits measured objectively in August for plant size(65mm/year), stem height (24 mm/year), and leaf area (131mm^2 /year). The gain from selection for yield (dry weight) was 1.2 Mg/ha per year. Gain from selection per breeding cycle for percent survival (2.1%) and lodging resistance (0.1) were low.

The gain from selection per each of two breeding cycles was estimated for concentration of rebaudioside A (26.44mg/g) and (14.51%), for rebaudioside C (20.20mg/g) and (20.17%), for rebaudioside D (1.70mg/g) and(1.49%), for stevioside (6.73mg/) and(10.72%), and for TSG (20.90mg/g) . Glycosides like rebaudioside D could double its initial concentration in one breeding cycle. In conclusion, it should be possible to make good progress in selecting stevia for improved agronomic and glycoside traits.

Introduction

Improved cultivars of stevia are available for production around the world. However, there is limited research available on the inheritance of traits among available accessions and cultivars. Cultivars have been improved for leaf yield, glycoside content, and disease resistance (Kafle, 2011). However, additional gains should be possible with novel breeding methods and other traits have yet to be evaluated. In addition, there is limited research on effective breeding methods and correlation among traits of interest.

Stevia leaves typically have 5 to 10% stevioside and 3 to 5% rebaudioside A of dry leaf weight (Brandle & Rosa, 1992). Some reports show stevioside ranging even as high as 20% in some cultivars, depending on environment (Kennely 2002; Starrat, 2002). Other minor glycosides such as rebaudioside C typically range 1-2% of dry leaf matter (Sekaran, 2007). However, other glycoside compounds exist in stevia that have been less explored. Despite rebaudioside A being most commonly used, it was found to be more bitter when compared to rebaudioside D which was the least bitter (Hellfritsch et al., 2012). Recent studies have indicated the potential for another minor glycoside, rebaudioside M, as a sweeter and less bitter compound (Prakesh, 2014). Rebaudioside C is another desirable glycoside that GLG filed a patent for a plant with high content in 2014. This difference in bitterness makes rebaudioside C, rebaudioside D, and rebaudioside M a more useful trait in stevia breeding programs than rebaudioside A. Current breeding programs have shown success in reducing stevioside content and increasing rebaudioside fractions, specifically rebaudioside A, therefore indicating that selection for specific rebaudiosides may be effective. Therefore, stevia can be improved to make a higher quality sweetener and potential reduce the need for heavy processing needed to extract only desirable compounds.

Genetic inheritance of glycosides is useful for improvement of breeding efficiency. Some of the genes from the steviol glycoside pathway have been identified (Brandle et al., 2002), however many still remain unknown. Most stevia accessions were reported as morphologically different from one another, indicating a good amount of phenotypic diversity available for breeding (Othman, 2015).

Genotype and environment play a role in the content of steviol glycosides and leaf yield in stevia, as well as the effect of cultural practice and propagation method on stevioside and rebaudioside A content. Open pollinated seedlings were found to have much more variation than plants produced by clonal propagation (Tavarini et al., 2010). Some trials have been conducted on stevia yield and glycosides, using multiple cultivars and locations. Leaf yield for cultivars differed depending on location. Rebaudioside A differences were found among cultivar x location (Parris et al., 2016). A cultivar x location interaction indicates that some cultivars perform differently depending on the test environment. Further, some cultivars may be more adapted to particular environments.

Genetic gain for quantitative traits requires dependable estimates of heritability to improve efficiency in breeding programs (Dudley and Moll, 1969). Broad-sense heritability was measured for yield and for quality across ten cultivars, one location, and three replications (Gaurav et al., 2008). Heritability for leaf yield was high (99%), for stevioside content it was 93%, and for plant height it was 97%. Negative correlations were noted with selection for leaf length and leaf yield at -0.85. Leaf yield had a positive correlation with flower number at 0.63. In addition, plant height was found to be positively correlated with number of leaves per plant at 0.88. Brandle and Rosa (1992) reported that yield, leaf:stem ratio, and stevioside content had high heritability. These findings provide an estimate of heritability and suggest potential for genetic gain. However, the same experiments should be conducted with more locations and multiple years for a better estimate of heritability by attempting to control error.

The objective of this study was to identify if differences were found among 72 elite half-sib cultigens such as yield, high glycoside, and large plant size by genotype and environmental effects. Environmental effects in this study include year, location, and replicate. Traits that affect yield such as plant height, plant width, leaf size, plant weight, lodging, and disease resistance were evaluated at different times in the season to see if significant correlations exist among the elite population. In addition, an estimation for narrow-sense heritability was estimated for important traits of stevia,

including traits for yield, plant growth, and glycoside content. Other objectives were to estimate additive and environmental effects, and to determine if the population evaluated were suitable for improvement for traits of interest. Estimation of genetic variance components and heritability of traits will be used to improve the efficiency of stevia breeding and to predict gain for traits of interest. This study was intended to help growers and researchers explore the breeding potential in stevia.

Materials and Methods

Populations Tested

Four stevia populations were used in this study: stevia elite population 1, 2, 3, and 4, respectively (StvEP1, StvEP2, StvEP3, and StvEP4). StvEP1 was developed by intercrossing 16 half-sib families selected from a cultigen trial in 2015 for a desirable glycosides. StvEP2 was developed by intercrossing 20 half-sib families selected from a cultigen trial in 2015 for desirable glycosides. StvEP3 was developed by intercrossing 22 half-sib families selected from a cultigen trial in 2015 for desirable glycosides. StvEP4 population was developed by intercrossing 16 half-sib families selected from a cultigen trial in 2015 for yield. All four populations were assembled in the first year of selection by collecting open pollinated seeds from the 2015 cultigen trial from select plants with desirable glycosides and or yield. Those seeds, from half-sib families were then used to assemble the populations. Intercrossing 16 to 22 half-sib families each cycle should enable continuous gain for a decade or more, depending on genetic variance.

Experiment Design

These experiments were conducted in 2016, using four populations tested in two locations, Clinton and Kinston in North Carolina. Each population had from 16 to 22 half-sib families in a randomized complete block design with two replications.

Seeds were germinated in flats in February. Soil media was kept moist by watering twice daily by overhead irrigation. A liquid soluble fertilizer (20-10-20) was applied weekly at 100 ppm once seedlings reached the true-leaf stage. The seedlings were thinned at the true-leaf stage to one

plant per cell. Greenhouse temperature was maintained at 29°C during the day and 21°C at night. After eight weeks in the greenhouse, flats were moved to open cold frames for one week where temperatures ranged from 10-25°C, and transplanted to the field in May.

Plants were transplanted in the field on raised beds one meter wide with drip irrigation under black plastic. Rows were on 1.5 m center-to-center. At each location plots consisted of either six plants or 30 plants. Each cultigen was planted into a 6-plant per plot density and 30-plant per plot density, with plant data taken on the 6-plant plot (glycosides, lodging, disease), and yield data (yield, stem, leaf fresh and dry weight) taken on the 30-plant plot. The thirty-plant plots had three rows on a bed with 0.31 m between rows and 0.31 m between plants resulting in a population density of 64,500 plants/ha. The 6-plant plot was planted in a single row with 1.5 m between rows and 0.62 m between plants resulting in a population density of 12,900 plants/ha. Alleys 1.5 m wide were used to separate plots at each end in both cases. Plants were watered for a combined 25 mm of irrigation when rainfall was deficient throughout the season. Weeds were removed by hand throughout the season.

Stand-count was measured two weeks after transplant at the end of May in 2016. At harvest, the stand-count was measured again to calculate percent survival over the summer.

Data Collection

The plants were evaluated for size using two subjective ratings and two objective measurements during the growing season. Subjective rating one was assessed June 14 and objective measurement one June 21. Subjective rating two was taken August 23, and objective measurement two August 30.

The subjective scale was on a 1 to 9 continuous visual rating scale for stem height, plant width, and leaf size (1-3=small, 4-6=medium, and 7-9=large). Stevia can grow to about 1 m in stem height (Shock, 1982), so a 9 rating is based from that height. For plant width, ratings would be based from the widest plant reaching 0.6 m. For leaf size, the ratings would be based on a leaf size maximum of 10 cm. Leaf size was usually largest early to mid-season as new leaves develop smaller later in the season. Objective size was measured directly in mm.

Objective measurements were taken on three traits: stem height, plant width, and leaf area. Leaf length and width were used to estimate leaf area (leaf area = 0.5 x leaf length x leaf width). Plants in plots of both planting densities were measured both subjectively and objectively. For the six-plant plot, each plant within a plot was measured. For the thirty-plant plot, we used the third plant from the front in the center row.

The thirty-plant plots were harvested at the end of September. A stand count was made as plants were harvested. Percent survival (100 x final stand / initial stand) was affected by plant vigor and resistance to diseases such as southern blight (*Sclerotium rolfsii*) Sacc. At harvest plants were cut and placed in tagged burlap bags. Yield as fresh weight was measured and the leaves dried in a tobacco curing barn in the burlap bags for five days at 60°C before yield as dry weight was measured.

Glycosides were measured using leaves taken 5 nodes from the top of the plant, where glycosides accumulate in highest quantity (Ceunen, 2013) in the 6-plant plot density in mid-July before flower initiation as steviol glycosides have reached their maximum at this stage (Yadav et al., 2010) Leaves from each plant were placed into labeled coin envelopes. They were then dried at 60°C for two days in seed drier. Leaves were ground to a fine powder and shipped for analysis. Glycosides were measured in mg/g and the percentage of the total steviol glycosides by High-performance liquid chromatography-mass spectrometry (HPLC/MS) and alcohol extraction at PepsiCo through a proprietary method.

Analysis of Field Data

Data were analyzed using SAS v9.4 (SAS Institute, Cary, N.C.). As part of analysis, data were checked for normality and errors. Analysis of variance and least squares means were calculated using the “general linear model” of SAS. Tests of hypothesis were estimated by using type III mean squares for block (study by location) as the error term for study and location. Variance components were estimated for calculation of heritabilities and gain from selection. Variance components were estimated using the TYPE=MIVQUE0 option which is an unbiased estimate for fixed effects in

PROC VARCOMP for general linear models. This method does not correct negative calculations to zero, therefore negative estimates can occur (Dudley & Moll, 1969).

The covariance of half-sibs estimates one quarter of the additive variance which we used along with the phenotypic variance to estimate narrow-sense heritability (Isik et al., 2017):

$$h^2_N = V_{HS} / (V_{HS} + (V_{HSxLoc}/L) + (V_{Er}/rxL))$$

Results and Discussion

Analysis of Variance

Plant size was calculated as an index starting from stem height, plant width, and leaf size for subjective rating one in June, and was significant for location and cultigen (Table 3.1). Stem height measured subjectively in June was significant for location and cultigen. Branching width measured subjectively in June was significant for location, cultigen, and location by cultigen. Leaf size measured subjectively in June was significant for cultigen, and location by cultigen.

Plant size measured subjectively as an index in August was significant for location, cultigen, and location by cultigen (Table 3.2). Stem height measured subjectively in August was significant for location, cultigen, and location by cultigen. Branching width measured subjectively was significant for cultigen, and location. Leaf area measured subjectively was significant for cultigen, and location by cultigen. The analysis of variance showed that the effect of cultigen was highly significant for all subjective traits therefore large differences exist between the cultigens evaluated.

Plant size measured objectively in June was significant for location, and cultigen (Table 3.3). Stem height measured objectively was significant for location and cultigen. Branching width measured objectively in June was significant for location and cultigen. Leaf size was found significant for location and cultigen. Plant size measured objectively in August was found significant for location, cultigen, and location by cultigen (Table 3.4). Stem height measured objectively in August was significant for location, cultigen, and location by cultigen. Branching width measured

objectively in August was only significant for location. Leaf area measured objectively in August was significant for cultigen and location by cultigen.

Plant growth measures that were significant offer similar significant differences, whether measured in June or August (measured by cultigen F ratio). The subjective and objective methods were both effective in estimating plant size, stem height, plant width, and leaf size. The subjective ratings were less time consuming but potentially less consistent. Objective measurements are by definition more accurate but require hand measurement using a ruler which is more time consuming than a visual rating. Digital imaging technology could be used in future studies to measure plants with high accuracy and efficiency. Correlations between subjective and objective measurements were used to indicate how closely the subjective and objective traits measured growth.

Yield as total fresh weight (Mg/ha) was significant for location, cultigen, and location by cultigen (Table 3.5). Yield as total dry weight (Mg/ha) was significant for location, cultigen and location x cultigen. Yield of dry leaf weight was not estimated in this study but would have been interesting to compare to other variety trials who reported yield as dry leaf weight (Ramesh et al., 2006; Parris et al., 2016).

Disease resistance was significant for location, cultigen, and location by cultigen (Table 3.5). Lodging resistance was significant for cultigen and location by cultigen. Percent survival was significant for location and cultigen. Significance for percent survival suggests that cultigens vary in ability to become established after planting due to various factors which we cannot clearly identify based on our data. However, some plants appeared to die initially after transplanting, before becoming established. Screening for *Septoria steviae* Ishiba. is effective where significance by cultigen was found, as it was for the screening method to find disease resistance for both field and greenhouse tests in clone 598-1 (Reeled, 1999). Breeding for disease resistance will be an important focus in stevia especially for production areas where high disease pressure causes defoliation.

Rebaudioside A concentration (mg/g) was significant for location and cultigen (Table 3.6), similar to findings of others (Tavarini et al., 2010; Parris et al., 2016) who, contrary to us, also

detected significance for location by cultigen interactions. Concentration of rebaudiosides B and C was significant for cultigen and location by cultigen. Rebaudioside D was significant for location and cultigen. Stevioside was significant only for cultigen in our study but other researchers found significance for environment effects (Tavarini et al.,2010). Total Steviol Glycosides (TSG) was significant for cultigen and location.

Rebaudioside A as percent of TSG was significant for location and cultigen (Table 3.7). Rebaudioside B as percent of TSG was significant for location and location by cultigen. Previous research has suggested that rebaudioside B may be a byproduct during the extraction process for glycosides (Brandle, 1998). Rebaudioside C as percent of TSG) was significant for cultigen and location. Rebaudioside D as percent of TSG was significant only for cultigen. Stevioside as percent of TSG was significant by location and cultigen. Glycoside concentration (mg/g and percent of TSG) was significant for location. All glycosides measured had significance for cultigen effects and indicate a strong genetic component determining the concentration of these compounds despite some environmental factors that can affect the content measured at a given time. This suggests potential for selection and breeding for these glycosides although some environmental variation would be found on glycoside accumulation. Further studies could be conducted to include unique glycoside selections such as rebaudioside D and rebaudioside M which have not been well studied and to test how each of these compounds vary by environment.

This study showed that there are significant differences among cultigens across agronomic traits including yield and glycoside profiles in an elite population of 72 half-sib selections, indicating sufficient genetic variation for breeding. Genetic diversity was observed in this study, contributed by the out-crossing nature of stevia (Handro et al., 1993). Other researchers (Othman et al. 2015) found similar significance for traits when using improved cultivars.

Correlations Among Traits

Pearson correlations were estimated among all traits. Most correlations were low, although significant. We were interested in those that were 0.7 or greater (as well as -0.7 or less), which indicates a high correlation, one that allows you to predict more than half of the other traits estimate. Branch width subjectively rated early in the season and objective stem height measurements were highly correlated (0.70). Overall plant size (subjective) were highly correlated with stem height subjective rated in the fall (0.73). Branch width objective measurement early in season was highly correlated with plant width subjective rating (0.75) and stem height objective measured early (0.82). Plant size objective measurement measured early in season was highly correlated to early season leaf area objective measurements (0.93). Yield as fresh weight and yield as dry weight were highly correlated (0.86); thus, suggesting that for breeding purposes fresh yield could be the sole yield measurement needed. Although correlations between yield and subjective or objective traits were not high, significant correlations were observed. Branch width and stem height had the highest correlation to yield fresh and yield dry which might suggest that plant height and width are more important to yield than leaf size. Screening for yield could be done by selecting plants tall and wide. Stem height showed the highest correlation to yield as dry weight as an objective measurement either measured in June (0.50) or August (0.44). This also indicated that early season ratings in June when measured objectively had a higher correlation than when measured in August, suggesting that early season ratings were also effective. Branch width correlations were also similarly high with yield as dry weight when measured in June (0.44). However, August ratings decreased drastically (0.15). Leaf area generally had insignificant correlations with yield. Subjective rating correlations were also significant with yield for stem and plant width in some cases, although generally lower correlation than objective measurements. Objective measurements appear more effective than subjective ratings based on the significance and higher correlations. Overall estimates measured in June correlated to yield similarly to those same measurements in August, indicating that both early and late season estimates are marginal estimates.

Correlations between subjective and objective measurements should offer similar results when measured at the same time with high correlation because in theory you are estimating the same trait. However, correlations for stem height measured in August (0.38), plant width (0.53), and leaf area (0.01) were moderate to low. These correlations, although significant for stem height and plant width, indicate that our subjective and objective measurements are not interchangeable. Leaf area did not have correlation between the subjective and objective measurements taken in August.

In our trial, we harvested our plots by hand due to limited harvesting systems for small plots. Hand harvesting provides more precise measurement than machine harvest, but it is more labor intensive. Selection for yield would occur naturally if using a mechanized harvesting system, where plants that do not fit the harvest system would be eliminated in selection. However mechanized systems allow for larger populations to be sampled. With the high correlation of yield as fresh and dry weight (0.86) found in our study, breeders could eliminate the drying process of stevia to save resources and time.

Correlations between glycoside concentration as percent of TSG and mg/g was high across all glycosides measured: 0.79 for rebaudioside A, 0.86 for rebaudioside B, 0.82 for rebaudioside C, 0.90 for rebaudioside D, and 0.95 for stevioside. Stevioside concentration was negatively correlated with the total of rebaudiosides A, C, and D (-0.16, -0.26, -0.30). A positive correlation was in concentration between stevioside and rebaudioside B (0.07). As a concentration stevioside total had negative correlation with the total of rebaudioside A at -0.51, but also negatively correlated with percent rebaudioside B (-0.03), and percent rebaudioside D (-0.35). A slightly positive correlation was noted between rebaudioside C and stevioside concentrations (0.16) while for all other glycosides concentrations were negatively correlated. A negative correlation of concentration of rebaudioside A was noted for stevioside as percent of TSG (-0.33). When measured as concentration in mg/g, stevioside and rebaudioside C were significantly negatively correlated (-0.26). However, when measured in percent of TSG, they were positively correlated (0.16). All other traits measured did not have high correlation (0.7) with each other.

Glycoside correlation with yield could offer phenotypic screening methods to find selections with desirable glycoside levels instead of randomly selecting plants to test for glycosides, which is costly due to expensive testing methods. In our population, we were only able to observe a significant correlation with rebaudioside C and yield (0.22). Rebaudioside B, rebaudioside D, rebaudioside A, and stevioside concentrations had zero or slightly negative correlation with yield. Stevioside concentration had negative correlation with yield as previously indicated (Brandle and Rosa, 1992). Leaf area was not found to have correlation with stevioside concentration unlike other researchers who found a positive correlation (Truong et al. 1999). We found evidence that rebaudioside A concentration was negatively correlated with yield (-0.51) unlike the positive correlation findings of Shyu in 1994. We found strong evidence that rebaudioside A and rebaudioside C concentration were negatively correlated (-0.51) but others found a positive correlation (Nakamura and Tamura, 1985; Brandle, 1998). Weng et al., in 1996 found that plants with high rebaudioside A concentration correlated with large leaves but we did not. Conflicting findings between our and other studies may be caused by our medium population size, the advanced population itself and environment.

Desirable glycoside compounds that correlate to yield enable breeding progress to be made for both traits simultaneously. Depending on the glycoside compound being bred for will have an effect on the yield. With some crops quality of a product and yield result in a negative correlation, like tomato for example where taste quality and yield, shelf life, and fruit size are found negatively correlated (Connor, 2005). The breeder then has a boundary that exists for high yield versus quality. Although more data is needed, breeding for a specific glycoside such as rebaudioside D may have its limitations where yield or biomass is sacrificed at high glycoside accumulation. If selecting for yield and glycoside proves difficult, a solution may be to select an intermediate yielding plant with medium glycoside concentration, rather than selecting the highest glycoside line and loosing yield. Regardless of how high yielding a line might be, one with minimal glycoside concentration would not be grown. The same being true for a high glycoside plant that has poor yield.

Disease incidence had a low but significant correlation with yield as fresh and dry weight but no significant correlation with glycosides. Lodging resistance is also an important trait, especially for mechanized harvesting. Lodging resistance had no significant correlation with all glycoside compounds or yield, but may vary in a mechanized harvest system.

Heritability and Variance Components

Narrow-sense heritability estimates will help in planning a breeding program for efficient improvement of important traits of stevia. Plant size, stem height, and plant width (subjective rating in June) had moderate heritability (0.44, 0.35, and 0.38 respectively) (Table 3.9). Heritability of leaf size measured in June was low (0.14).

Plant size, stem height, plant width, and leaf size measured in June had moderate to high heritability (0.61, 0.68, 0.59, and 0.54 respectively) (Table 3.9).

Plant size, stem height, plant width, and leaf area subjective ratings in August had moderate low to heritability (0.37, 0.30, 0.08, and 0.13 respectively) (Table 3.9).

Measurements for plant size in August had moderate heritability (0.38) (Table 3.9). Stem height measured in August had a low heritability (0.21) although others found heritability to be as high as 0.93 (Gaurav et al., 2008). Branching width measured in August had a negative heritability and was therefore assumed to be null (Johnson et al., 1955). Leaf size measured in August had moderate heritability (0.31). Percentage survival had low heritability (0.05) as did lodging resistance (0.10) (Table 3.9). Yield as dry weight had moderate heritability (0.41). Although heritability of leaf yield was not estimated in our study, previous research has shown that heritability of leaf yield ranged from 0.75 (Brandle and Rosa, 1992) to 0.99 (Guarav et al., 2008). We also did not measure leaf to stem ratio but it was found to be highly heritable by others at 0.86 (Brandle and Rosa, 1992).

Heritabilities for the concentration of glycosides were calculated. Rebaudioside A as a concentration of TSG was highly heritable (0.69) and mg/g (0.71) (Table 3.11). Rebaudioside B as a concentration was found to be negative and therefore assumed to be null. Rebaudioside C concentration was highly heritable (0.83) as mg/g and (0.89) as percent of TSG. Rebaudioside D was

highly heritable (0.62) as mg/g and (0.64) as percent of TSG. Stevioside was moderately heritable (0.53) as mg/g and (0.65) as percent of TSG. Guarav et al. in 2008 found stevioside heritability to be much higher at 0.93, and Brandle and Rosa in 1992 found it to be 0.83. Total steviol glycosides (TSG) was highly heritable (0.69). Further studies of heritability should be conducted on these glycosides, in addition to other glycosides that were not included in this study such as rebaudioside M which is a recent focus for sweetener processors such (Cargill, 2015).

Gain from selection for a 20% selection intensity was estimated for agronomic traits and glycosides. Gain from selection per breeding cycle for percent survival is 2.10% (Table 3.9). Gain from selection for subjective ratings in June was moderate for plant size (0.39), stem height (0.31), and plant width (0.36), and low for leaf size (0.17) per cycle. Gain from selection for measurements in June was high for plant size (127.36 mm), stem height (86.49 mm), plant width (36.86 mm), and leaf area (289.20 mm^2). Realized gain would be lower if selecting for multiple traits at once, such as selection for stem height and plant width together. The gain from selection for subjective ratings in August were moderate for plant size (0.24) and stem height (0.29), but low for plant width (0.04) and leaf size (0.09), Gain from selection for measurements in August were moderate for plant size (64.94 mm), stem height (23.82 mm), and leaf area (131.09 mm^2). Gain in yield as dry weight were estimated at 1.19 Mg/ha per breeding cycle, which is quite high. Gain from selection per breeding cycle for lodging resistance was low (0.10).

Gain from selection for glycoside concentration were high for rebaudioside A as mg/g (26.44) and percent of TSG (14.51) per breeding cycle. Gain for rebaudioside B concentration was null. Rebaudioside C concentration had gains of 20.20 mg/g, and 20.17 percent of concentration per breeding cycle. Rebaudioside D had gains of 1.70 mg/g, and 1.49 percent of concentration per breeding cycle. Gain from selection for stevioside was 6.73 mg/g, and 10.72 percentage of concentration per breeding cycle. Finally, TSG had a moderate gain per breeding cycle (20.90).

Heritability and gain from selection estimated based on our population showed that moderate to high gain could be made by breeding. Rebaudioside C, followed by rebaudioside A and stevioside

showed the highest gain per cycle for major glycosides while minor glycosides such as rebaudioside D showed lower gains per cycle. It was unusual that rebaudioside C showed higher gain per cycle than rebaudioside A, considering that it is found to be in lower accumulation than rebaudioside A and stevioside (Parris et al., 2016). These gains for minor glycosides are large considering how minimal these compounds are present in a normal population. Typically, rebaudioside D is found at 1-2% of a glycoside profile and these gains show that you could increase them as much as 1.49 percent per breeding cycle, which would almost double the amount the first breeding cycle. With minor glycosides, it would be interesting to see how much can be improved. High levels of rebaudioside D and rebaudioside M are preferred by sweetener processors such as Cargill, however due to the complexity of the biopathway in stevia you would not get near 100% in a plant. Therefore, due to the limited concentration found in plants they have resorted to production via yeast fermentation (Cargill, 2015).

Overall, glycosides show the potential for gain per breeding cycle. Agronomic measurements are more moderately heritable with moderate gain potential per breeding cycle. Our heritabilities appeared lower when compared to those estimated by other researchers (Guarav et al., 2008; Brandle and Rosa, 1992). Expensive procedures to measure glycoside profiles across our population limit our ability to test the entire population for glycoside analysis. Instead we randomly select plants to test within each cultigen planted and therefore many plants go untested each season. Although our sample size was moderate for the glycosides we measured, a larger sample size is always preferred when estimating heritability and genetic components. Heritability for some traits was null (estimates were negative), likely due to a limited sample size for accurate estimation of those traits. To calculate our heritabilities for half-sib families we resorted to the method by Isik et al. (2017) for narrow-sense Heritabilities rather than the conventional genotype divided by phenotype.

Conclusions

Significant differences were identified, where cultigen, or in this study 72 elite half-sib lines was significant for majority of the traits estimated. This comes as no surprise, as these lines were selected for superior performance of yield or various desirable glycosides. Plant breeders prefer a diverse population for long term breeding gains, due to wide base of genes. Further evaluations should be conducted within specific populations, to develop phenotypic screening techniques for high glycoside compounds. New technology such as high-throughput phenotyping could likely assist in screening methods that could save resources in expensive glycoside testing via HPLC-MS where limitations in glycoside sampling exist due to cost. Correlations between glycosides and yield were generally low, however a significant correlation was found for rebaudioside C, which may suggest selection for yield and rebaudioside C may occur simultaneously. However, the correlation although significant was not high. Therefore, more evidence would be needed to support this finding.

Location was significant for most traits; however, block effect was only significant for lodging and rebaudioside C. Despite significance of location, additional locations covering a wider range of environments should be used for future studies, as it would be interesting to detect if more variation would occur. Additionally, less replications are needed within a location due to insignificant findings as an attempt to save resources.

Significant environmental effects and interactions of cultigen were identified for location, which suggest that some varieties prefer specific environments. For example, some lines may be more drought tolerant, and perform better than others in a dry environment. Although varieties that show a genotype x environment are unstable across various environments, they can be quite useful if they excel in a specific region.

Although two densities were used in this study, we gathered agronomic data on the 30-plant plot and glycoside data on the six-plant plot, therefore we cannot determine the effect on density for traits such as glycosides and yield, but they could be useful to explore in future studies. Although there are various densities reported for stevia experiments, studies are needed to determine optimal

plant density for commercial stevia production. Glycoside amounts shown in the table are from a low planting density, which glycosides could decrease when grown in a more compact commercial density, due to plant stress. Future studies, may want to sample glycosides directly in the commercial density, however the findings even at a smaller density should still indicate the best glycoside lines.

Stem height and plant width had the highest correlation to yield in both early and late season estimates for fresh or dry weight, where leaf size, showed no correlation to. Therefore, one can evaluate a population for high yield and select lines just by looking at plant width and height, even as early as June. Early screening techniques can be useful to save resources in breeding programs.

Subjective and objective measurements were both effective in estimating stevia traits, however had low correlation between each other when measured at the same time. Subjective measures are commonly used in breeding programs, and can offer quick estimation, however were too variable for estimating stevia yield traits. Therefore, objective measurements, although time consuming were more reliable. Digital imaging and advanced technology may be useful to help automate or speed up data collection for future trials.

All glycosides measured were significant by cultigen except for rebaudioside B measured as a percentage of TSG. It other words rebaudioside B concentration is similar across all 72 half-sib lines, but the amount varies based on how high the total steviol glycosides are. Additionally, rebaudioside B had no detected heritability in the study. Previous research has suggested that rebaudioside B may be a byproduct during the extraction process for glycosides, but has not yet been confirmed. A larger population should be sampled to estimate heritabilities for rebaudioside B and to explore this compound further.

Narrow-sense heritabilities were consistently high for glycosides as well as for TSG which indicates that progress can be made in breeding for glycosides. Additionally, gain from selection (20%) based on our population, shows that compounds can be effectively improved each breeding cycle. In rebaudioside D for example, one could double its concentration in one breeding cycle, however this would not likely continue for many generations. Heritability estimates are valuable to

plant breeders as it can help them optimize their breeding program. Where heritability estimates are low, breeders may need to utilize a larger population size to find genes of interest, or explore additional sources of germplasm. They also may need to select fewer plants each breeding cycle, but ones that are most superior, however at a cost of genetic diversity.

Yield in dry weight had moderate heritability, showing gain from selection of 1.19 Mg/ha/breeding cycle which suggests that initial gain when selecting for yield could be high. This refers to the gain based on a 20% selection criteria, where only the top 20% of the population is selected per cycle. Had a lower selection criteria been used such as 40%, lower gains overall would be expected with a higher standard deviation. Gains estimated give an idea on the initial expected gain, but will likely decrease per cycle as the traits improve. Yield of fresh weight was not determined due to negative estimates. A larger population size for detecting heritabilities should be used to further explore these traits, which might potentially eliminate negative estimates. However, these heritabilities and gains estimated, give plant breeders an idea of how to make progress and design field experiments to breed stevia.

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Table 3.1 Analysis of variance for agronomic traits measured subjectively of four elite, half-sib populations in June 2016 Clinton and Kinston NC.

Source	df	Plant size	Stem height	Plant Width	Leaf area
Location	1	17.74**	52.31**	57.82**	4.51
Rep (Loc)	2	0.16	0.93	0.07	0.34
Cultigen	72	0.63**	0.74**	0.77**	1.92**
Loc*Cultigen	61	0.38	0.47	0.55**	1.81*
Error	122	0.34	0.41	0.27	1.17

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Subjective rating 1-9 scale in June 2016.

Table 3.2 Analysis of variance for agronomic traits measured subjectively of four elite, half-sib populations in August 2016 Clinton and Kinston NC.

Source	df	Plant size	Stem height	Branch	Leaf area
Location	1	7.74**	16.09**	19.45**	0.01
Rep (Loc)	2	0.15	0.62	0.08	0.99
Cultigen	72	0.37**	0.87**	0.43*	0.70**
Loc* Cultigen	61	0.26**	0.71*	0.39	0.62*
Error	122	0.15	0.43	0.27	0.39

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Subjective rating 1-9 scale in August 2016.

Table 3.3. Analysis of variance for agronomic traits measured objectively of four elite, half-sib populations in June 2016 Clinton and Kinston NC.

Source	df	Plant size	Stem height	Branch	Leaf area
Location	1	686599**	1853851**	823093**	22606658**
Rep (Loc)	2	5563	3684	1298	23848
Cultigen	72	29383**	9050**	2425**	198190**
Loc* Cultigen	61	13241	4131	1363	112434
Error	122	12650	3782	1015	100628

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Table 3.4. Analysis of variance for agronomic traits measured objectively of four elite, half-sib populations in August 2016 Clinton and Kinston NC.

Source	df	Plant size	Stem height	Branch	Leaf area
Location	1	286076**	2411500**	146689**	109771
Rep (Loc)	2	21024	1574	10444	86919
Cultigen	72	25527**	14886*	6690	169683**
Loc* Cultigen	61	18744**	15303*	7403	135024**
Error	122	8148	9598	5696	62135

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Table 3.5 Analysis of variance for yield measurements of four elite, half-sib populations in 2016 Clinton and Kinston NC.

Source	df	Yield (Fresh)	Yield (Dry)	Disease	Lodging	% Survival
Location	1	1473.3**	248.75**	519.64**	5.27	2885.02**
Rep (Loc)	2	61.23	7.74	1.95	6.69**	12.80
Cultigen	72	99.63**	6.44**	3.75**	4.28**	335.05**
Loc* Cultigen	61	63.40**	4.48*	3.67*	3.88**	182.17
Error	122	34.84	2.90	2.51	2.29	175.15

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Yield (Fresh) = weight at harvest for yield in Mg/ha.

Yield (Dry) = weight after drying in Mg/ha.

Disease= subjective 0-9 rating for disease susceptibility

Lodging= subjective 0-9 rating for lodging susceptibility

Table 3.6 Analysis of variance for glycoside content mg/g measurements of four elite, half-sib populations in 2016 Clinton and Kinston NC.

Source	df	Reb A	Reb B	Reb C	Reb D	Stevioside	TSG
Location	1	13691.6**	0.1	0.4	32.5**	101.0	18099.7**
Rep (Loc)	2	534.8	0.0	92.5	4.0	8.8	268.9
Cultigen	72	816.6**	0.08**	317.9**	5.1**	122.3**	772.2**
Loc* Cultigen	61	283.3	0.09**	58.7**	2.1	56.9	380.0
Error	122	232.7	0.0	35.8	1.5	42.9	322.6

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Glycosides measured as total content in mg/g

Table 3.7 Analysis of variance for glycoside percent of total steviol glycosides of four elite, half-sib populations in 2016 Clinton and Kinston NC.

Source	df	% Reb A	%Reb B	% Reb C	% Reb D	% Stevioside
Location	1	1447.43**	0.59**	138.23*	3.75	351.80**
Rep (Loc)	2	103.43	0.05	107.83*	1.28	2.95
Cultigen	72	276.99**	0.04	267.33**	3.64**	179.45**
Loc* Cultigen	61	93.72	0.05*	36.21	1.32	66.31
Error	122	67.65	0.03	28.07	0.99	51.34

* Significantly different at 0.05 level of probability

** Significantly different at 0.01 level of probability.

Table 3.8. Variance components and heritability estimate means for agronomic components across all four half-sib populations in 2016.

Trait	Var Loc	Var Rep	Var Clt	Var LocxClt	Var Er
% survival	73.53	-8.67	39.08	17.76	2924.20
Plant size sub. June	0.18	0.00	0.07	0.01	0.34
Branch sub. June	0.52	0.00	0.08	0.13	0.27
Leaf sub. June	0.01	-0.01	0.07	0.24	1.17
Stem height sub. June	0.49	0.01	0.07	0.06	0.41
Plant size obj. June	5934.0	-110.3	4859.2	86.3	12650.0
Stem height obj. June	16851.1	20.3	1856.1	-88.2	3782.5
Branch size obj. June	7385.3	5.5	429.0	145.5	1015.6
Leaf area obj. June	200600.0	1327.6	29892.0	-101.1	100628.2
Plant size sub. August	0.07	0.00	0.04	0.05	0.15
Stem height sub. August	0.16	0.00	0.07	0.14	0.43
Branch sub. August	0.17	0.00	0.01	0.06	0.27
Leaf sub. August	-0.01	0.00	0.02	0.11	0.39
Plant size obj. August	2770.6	176.7	2662.3	4911.3	8148.3
Stem height obj. August	24465.0	-83.9	839.0	3004.3	9598.7
Branch size obj. August	1099.7	119.2	-125.7	1193.6	5696.2
Leaf area obj. August	-480.2	406.6	14634.2	35000.9	62135.4
Yield (fresh wt.)	76.94	966.0	-376.7	550.78	54687.5
Yield (dry wt.)	2.02	0.02	0.79	0.81	2.96
Lodging	-0.01	0.00	0.05	0.29	1.17
Disease	1.79	-0.01	-0.02	0.11	1.23

Sub.-subjective measurement rating 1-9
Obj.- objective measurements in mm
Var Loc- Variance component for location
Var Block- Variance component for block
Var Clt- Variance component for cultigen
Var Loc xClt- Variance component for interaction of location*cultigen
Var Error- Variance component for error

Table 3.9. Variance components and heritability estimate means for agronomic components across all four half-sib populations in 2016.

Trait	N	Mean	Var A	Var P	h^2_N	G_S 20%
% survival	258	92.8	156.33	896.27	0.05	2.10
Plant size sub. June	259	3.2	0.29	0.39	0.44	0.39
Branch sub. June	259	2.2	0.33	0.46	0.38	0.36
Leaf sub. June	259	5.1	0.29	0.71	0.14	0.17
Stem height sub. June	258	2.1	0.27	0.4	0.35	0.31
Plant size obj. June	257	444	19438.08	22610.8	0.61	127.36
Stem height obj. June	257	329.2	7425.8	8308.43	0.68	86.49
Branch size obj. June	257	205.9	1717.79	2021.87	0.59	36.86
Leaf area obj. June	257	796.8	119569.2	144728.6	0.54	289.20
Plant size sub. August	257	5.9	0.15	0.21	0.37	0.24
Stem height sub. August	257	6.3	0.3	0.47	0.30	0.29
Branch sub. August	257	5.6	0.03	0.13	0.08	0.04
Leaf sub. August	257	5.9	0.1	0.25	0.13	0.09
Plant size obj. August	258	576.8	10649.39	15020.94	0.38	64.94
Stem height obj. August	258	680.4	3356.17	6489.45	0.21	23.82
Branch size obj. August	258	396.8	-503.15	1399.92	-	-
Leaf area obj. August	258	653.4	58536.93	91065.23	0.31	131.09
Yield (fresh wt.)	258	36.09	-1506.95	12440.3	-	-
Yield (dry wt.)	258	5.31	3.15	4.3	0.41	1.19
Lodging	265	5.37	0.18	0.62	0.10	0.10
Disease	264	3.8	-0.08	0.28	-	-

N=number of individuals sampled for each trait
Mean= mean for trait across the whole population
Var A= additive variance
Var P= Phenotypic variance
h^2_N= Narrow sense heritability of half-sib population
G_S (20%) = Gain from selection with 20% selection intensity

Table 3.10. Variance components and heritability estimate for glycosides means across all four half-sib populations in 2016.

Trait	Var Loc	Var Rep	Var Clt	Var Loc x Clt	Var Er
Reb A	114.56	3.88	159.81	14.63	232.7
Reb B	0.00	0.00	0.00	0.02	0.04
Reb C	-0.50	1.00	71.18	9.65	35.8
Reb D	0.21	0.03	0.84	0.27	1.53
Stevioside	-0.10	-0.43	16.76	8.26	42.9
TSG	160.79	-1.81	130.39	9.94	322.6
% Reb A	11.50	0.59	50.88	12.08	67.6
% Reb B	0.00	0.00	0.00	0.01	0.03
% Reb C	-0.79	1.31	64.00	1.84	28.1
% Reb D	0.01	0.00	0.60	0.18	0.99
% Stevioside	5.39	-0.69	30.96	9.00	51.34

Var Loc- Variance component for location
Var Block- Variance component for block
Var Clt- Variance component for cultigen
Var Loc*Clt- Variance component for interaction of location*cultigen
Var Error- Variance component for error

Table 3.11. Variance components and heritability estimate for glycosides means across all four half-sib populations in 2016.

Trait	N	Var A	Var P	h2N	GS %20
Rebaudioside A	266	639.23	703.92	0.71	26.44
Rebaudioside B	266	-0.01	0.01	-	-
Rebaudioside C	266	284.7	298.79	0.83	20.20
Rebaudioside D	266	3.35	3.87	0.62	1.70
Stevioside	266	67.03	81.81	0.53	6.73
TSG	266	521.56	606.25	0.61	20.90
% Rebaudioside A	266	203.52	226.5	0.69	14.51
% Rebaudioside B	266	-0.02	-0.01	-	-
% Rebaudioside C	266	255.98	264.18	0.89	20.17
% Rebaudioside D	266	2.41	2.75	0.64	1.49
% Stevioside	266	123.85	140.88	0.65	10.72

N=sample size

Mean= mean for trait across the whole population

Var Additive= Additive variance

Var Phenotype- Phenotypic variance

h2N= Narrow sense heritability of half-sib population

GS (20%)= Gain from selection at 20%