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Performance of 16 *Stevia rebaudiana* seed cultigens for glycosides and yield in North Carolina

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ABSTRACT

Currently, stevia (*Stevia rebaudiana* Bertoni) seeds are available to growers from many sources, but the origin of these populations is often unknown. Since stevia is a natural outcrosser, populations are heterozygous and heterogeneous. We were interested to characterize germplasm from 16 sources of stevia seeds for traits including yield, glycosides, and plant morphology, and then identify trait correlations. The cultigens evaluated were obtained from garden seed companies and commercial sources, and the trials were conducted for two years at two field locations. To quantify plant morphological traits, objective measurements were collected at two intervals to determine stem height, branching width, and leaf area. In addition, we screened for lodging and disease resistance, and yield before flowering stage. The cultigens tested were highly variable for yield and steviol glycosides, suggesting that a diverse genetic base is found among the population which is readily available to growers and breeders. High-yielding cultigens for biomass and glycoside concentration were identified. Cultigens with the highest yield and stability over years were seed-derived progeny from 'Katupyry', sourced from Stevia Store, and represent genetics useful in breeding for increased biomass. Cultigens with the highest glycoside level were NC-1003 and NC-1022, seed grown from Seed Savers, and could be used to improve desirable glycosides. This study highlights readily available seed cultigens that can be used to develop elite breeding populations.

1. Introduction

Stevia rebaudiana (Bertoni) is an herbaceous perennial crop that can be used to produce non-caloric, plant-derived sweeteners (Yao et al., 1999). The leaves contain steviol glycosides, which have been hypothesized to deter herbivory (Metivier and Viana, 1979; Nanayakkara et al., 1987). The steviol glycosides found in stevia can vary in their level and ratio of accumulation. Major glycosides found in stevia include rebaudioside A (reb A) and stevioside, and minor glycosides include rebaudioside B (reb B), rebaudioside D (reb D) (Brandle et al., 1998), and rebaudioside M (reb M) (Prakesh et al., 2014). Stevioside comprises the majority of the steviol glycosides found in many cultivars of stevia (Brandle et al., 1998), and is known to have bitter flavor and a negative impact on consumer experience. However, reb A has a more desirable taste profile (DuBois, 2000), and currently, is the most studied steviol glycoside. In addition to the desired taste profile, Reb A is approved as a safe sweetener making it the main steviol glycoside used in stevia sweetened beverages. Recent studies suggest that reb D and reb M have better flavor and less bitterness than reb A (Allen et al., 2013; Nikiforov et al., 2013; Prakesh et al., 2014). However, the concentrations of reb D and M are lower in the stevia leaf (Hellfritsch et al., 2012). Cultivars being developed will have higher concentrations of desirable steviol glycosides for use in products. Recent plant patents have been granted for cultivars with enhanced levels of the minor glycosides such as reb D and M (WO 2018/165330, 2018), (USPP27902P3, 2014), (CN105850750A, 2015), (WO2014146084A1, 2014).

Stevia production is increasing around the world, including the US (Megeji et al., 2005), with a projected compound annual growth rate of 12 % (Research and Markets). However, adapted cultivars with consistent yields and sweetness are needed (Angelini et al., 2018). In addition, overwintering tolerance for temperate production areas is needed to support market expansion, as perennial yields increase after the first year through year four, and then yields tend to decline (Singh and Kaul, 2005). Around the world, cultivars have been developed with improved yield and reb A concentration (Tan et al., 2008; Yadav et al., 2011; Parris et al., 2016). However, more research is needed on the stability of these cultivars in different production regions.

Stevia is a naturally cross-pollinated species; therefore, open-

Abbreviations: TSG, Total Steviol Glycosides; reb, rebaudioside.

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pollinated cultivars are heterogeneous (Handro et al., 1977). Stevia is also self-incompatible (Miyagawa et al., 1986; Chalapathi, 1997). Researchers have reported large phenotypic variability for plant height and branch number (Abdullateef et al., 2015; Othman et al., 2015), and differences in yield and steviol glycosides profile among accessions and cultivars (Sakaguchi and Kan, 1982; Montoro et al., 2013; Barbet-Massin et al., 2015; Parris et al., 2016; Hastoy et al., 2019). There is a further need to study the performance of genotypes across environments (Hastoy et al., 2019).

Correlations have been estimated for pairs of traits. Reb A is correlated with leaf area, (Weng et al., 1996), leaf thickness (Shyu, 1994), and reb C (Nakamura and Tamura, 1985). However, reb A is negatively correlated with stevioside (Nakamura and Tamura, 1985) and dry leaf weight (Hastoy et al., 2019). Yield is positively correlated with branch number, leaf number (Buana and Goenadi, 1985; Shu and Wang, 1988; Buana, 1989; Yadav et al., 2011), and stevioside (Hastoy et al., 2019), but negatively correlated with reb C (Hastoy et al., 2019). However, other researchers reported that plant height was not correlated with leaf number or branch number (Yadav et al., 2011). More studies are needed to estimate genetic correlations.

In this study, we evaluated readily available seed sources of stevia for important agronomic traits and steviol glycosides. We also assessed the importance of genotype by environment effects for yield, glycosides content, and plant morphology. Cultigens identified in this study that have high glycoside content, high yield, and resistance to disease can be used in breeding programs

2. Materials and methods

2.1. Germplasm

Seeds of 16 stevia cultigens were obtained from seed companies and other organizations represented in Table 1. Since cultigens often were obtained as generic stevia from seed companies, those without cultivar names were assigned an accession number for each year.

Seeds were planted in early February in 2015 and 2016 using 72-cell seedling flats (550 plants m^2) in a greenhouse. Flats were filled with Fafard 4p (Sungro horticulture), a multipurpose potting soil, comprised of sphagnum peat moss (~48 %), bark (~30 %) perlite (~11 %), vermiculite (~11 %), and traces of 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. Each cultigen was planted in its own flat. Soil media was watered twice daily with an overhead mist system. A liquid-soluble fertilizer (20−10-20) at 100 ppm was applied starting at true-leaf stage and continuing weekly. The seedlings were thinned to one plant per cell at the true-leaf stage. Greenhouse temperatures were maintained at 28 °C day and 21 °C night under ambient lighting, with an average of 12 h daylight. Seedlings were grown for 12 weeks in the greenhouse before moving them to a cold frame outdoors with a 50 % shade cloth for acclimation one week before transplanting into the field.

2.2. Transplanting and field layout

Plants were transplanted in the field 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, with plots 3 m long having 30 plants each for destructive measurement of yield and leaf weight (fresh and dry). An adjacent planting had 6-plant plots of the same treatment combinations (year-location-replication-cultigen) for non-destructive measurement of traits such as glycosides, lodging, and disease resistance. The 30-plant plots represented a commercial density of 64,550 plants/ha. The 6-plant plot occupied the same area as the 30-plant plot, but with a density of 12,910 plants/ha. Plots were separated by a 1.5 m alley at each end to facilitate harvest. Plants were irrigated after transplanting and received 25 mm per week of irrigation as a minimal in the event of no rainfall. However, during periods of heavy rain, weekly rainfall totals could be 50 mm. Fertilizer was injected through the irrigation system. Sevin® (carbaryl) was used as a broad-spectrum pesticide to control insects in the early season. Plots were hand-weeded, since there are no labeled herbicides for stevia.

Weather data was collected at both research stations during the

Table 1
LS means for 16 cultigens of stevia for objective measures of plant size traits in August 2015-2016.

		2015			2016		
Cultigen (2015/2016)	Source	Stem height (mm)	Branching width (mm)	Leaf area (mm^2)	Stem height (mm)	Branching width (mm)	Leaf area (mm^2)
NC-1013/1032	Swallowtail	632	384	511	884	425	1522
NC-1001/1020	Baker Creek	721	473	585	832	393	1602
NC-1014/1033	Richters	662	423	566	784	408	736
NC-1009/1028	Jung Seed	640	424	460	749	434	680
NC-1015/1034	Eirete I	720	495	469	747	420	531
NC-1017/1036	Katupyry	740	473	462	740	481	663
NC-1004/1023	Johnny's	672	458	380	738	425	811
NC-1010/1029	R.H. Shumway	662	448	423	730	428	636
NC-1016/1035	Eirete II	760	502	434	720	396	761
NC-1011/1030	Everstevia	690	443	657	710	401	515
NC-1003/1022	Seed Savers	673	452	466	704	428	944
NC-1002/1021	Territorial	655	404	523	698	371	736
NC-1008/1027	Botanical Interest	604	382	558	683	348	664
NC-1005/1024	Park Seed	652	445	456	663	369	742
NC-1012/1031	Harris Seed	535	314	529	648	366	700
NC-1018/1037	Native	624	381	638	483	286	452
Mean		665	431	507	720	399	793
LSD 5%		76	68	102	89	44	238
Location		**	**	NS	**	**	NS
Year		**	NS	**	**	NS	**
Cultigen x location		NS	NS	NS	NS	NS	NS
Cultigen x year		**	**	**	**	**	**
r^2		0.51**	0.42**	−0.08	0.52**	0.44**	0.55**

Means for each cultigen were pooled over two locations and four replications.
r^2 is the correlation of 6-plant plot density vs. 30-plant plot density for each trait.
Location and year effect for each trait at 0.05 significance level.
Significant at 0.05 level of probability (*); Significantly at 0.01 level of probability (**).

growing season. Rainfall totals (May to the end of September) were 77 cm in 2015, and 90 cm in 2016 for Kinston, NC. Rainfall was 56 cm in 2015, and 73 cm in 2016 for Clinton, NC. Therefore, total irrigation including rainfall and irrigation ranged between 25–50 mm per week. From May to September, Kinston and Clinton had similar temperatures, averaging 30.3 °C day and 19.4 °C night for 2015 and 2016. The average photoperiod during the field experimental period was 13:42 h, spanning from 13:38 h (May), 14:30 (Peak), to 11:49 h (September). Both locations are in the coastal plain of NC and have sandy soil.

2.3. Plant morphology measurements

The morphological traits measured included stem height, plant branching width, and leaf area. Data were collected via objective measurements with a ruler in mm. In 2015, the objective measurement was made August 29. In 2016, the objective measurements were made on August 30. Leaf length and width were used to estimate leaf area (0.5 x leaf length x leaf width). The 30-plant plots were used for plant size traits to represent a commercial density. We used the third plant in the center row for morphological measurements to avoid border effect.

2.4. Quantifying yield

Yield (plant fresh wt., plant dry wt.), and leaf dry wt. were measured on the 30-plant plots after harvest and shown in Tons ha^{-1} due to large values (Ton = 1000 kg). 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. Plants from each plot were bagged and the fresh weight was taken. Dry weights were taken after two days in a dryer set at 60 °C. After drying, leaves were separated from the stems and weighed. Yield (plant fresh wt. and plant dry wt.) was calculated per ha and per plant. Finally, we calculated leaf dry wt. as a percentage of the total wt.

Lodging and disease severity were rated subjectively on a 0–9 scale (0 = none, 9=dead) in 2016 only. Lodging resistance was evaluated using a representative plant in the 6-plant plot. Disease severity was rated on a subjective scale from 0–9 (0 = none, 9=dead) following the scale of Jenkins and Wehner (1983). Lodging resistance was rated similarly.

2.5. Glycosides analysis

Glycoside content and concentration were measured using leaves taken five nodes from the top of the plant (Ceunen and Geuns, 2013; Bondarev et al., 2003). Leaf samples for glycoside measurements were taken from four plants per plot, from four replications, two years, and two locations, in mid-July before flower initiation to avoid the decline of steviol glycosides beyond this stage (Yadav et al., 2010). Leaves from each plant were kept separate, dried at 60 °C and ground to a fine powder prior to analysis. Leaf samples were sent to a third-party lab (PepsiCo), extracted and quantified using a proprietary method similar to that described by Shafii et al. (2012). Briefly, dry leaf samples are extracted in an ethanol mixture and quantified using LC–MS–MS. Glycosides (stevioside, and reb A–D) were reported in mg/g (concentration of dry leaf weight) as well as percentage of total steviol glycosides (TSG), the sum of all measured glycosides. Some additional glycosides making up TSG were not included in this study.

2.6. Data analysis

The experiment was a randomized complete block design with 16 cultigens, two years (2015, 2016), two locations (Clinton and Kinston, NC), and four replications for both sets of 6-plant and 30-plant plots. Cultigens were replanted each year and not left in the field to overwinter.

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 GLM. Cultigen was run as a fixed effect; location, block(rep), and year were treated as random effects. Least squares means were calculated to account for missing data. Data were analyzed using both years in the model except for traits added in 2016 (lodging, disease). However, due to multiple cultigen by year interactions, trait data was shown seperated by year. 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 whether one was reflective of the other. Significance level was p=0.05.

3. Results and discussion

3.1. Genetic and environmental effects

There were significant differences among cultigens for yield (plant fresh and dry wt.) in both years ($p \leq 0.001$) (Huber, 2017). Location had no effect on plant fresh and dry weight in our study, however year affected plant dry weight ($p = 0.01$) (Table 2). Cultigen had no effect on leaf dry wt., however location ($p \leq 0.001$) and year ($p = 0.01$) were significant (Table 2). Previous studies in the western US reported significant differences of location on dry weight (Parris et al., 2016). In this study, cultigen yield was significantly affected by genetics and environment.

Significant effects for reb A (mg/g) were observed for cultigen ($p \leq 0.001$), year ($p = 0.02$), and year by cultigen ($p \leq 0.001$); however, when measured as a percent of TSG was significant for cultigen ($p \leq 0.001$) and year by cultigen ($p = 0.008$) (Tables 3–6). Stevioside (mg/g) was significant for cultigen ($p = 0.02$) and year ($p = 0.005$); however, as a percent of TSG was only significant for cultigen ($p \leq 0.001$) and year by cultigen (Tables 3–6). Rebaudioside C was only affected by year ($p = 0.02$) when measured as a percent of TSG. In other studies, both genotype and environment have been found to affect reb A, reb C, and stevioside content (Tavarini et al., 2010; Parris et al., 2016). Rebaudioside D (mg/g) was significant for cultigen ($p = 0.003$), year ($p \leq 0.001$), and location ($p = 0.03$), however as a percentage of TSG was significant by cultigen ($p = 0.002$) and year ($p = 0.04$). Total steviol glycoside was significant for cultigen ($p \leq 0.001$) and year ($p \leq 0.001$), being 37 % higher in 2015 than 2016 in our study (Tables 3–6). Rebaudioside B (mg/g) was only significant for location ($p = 0.02$); however, as a percentage of TSG was significant for cultigen ($p = 0.03$) and location ($p = 0.03$). Therefore, steviol glycosides measured in our study were all affected by genetic and environmental effects, except for Reb C.

Stem height was highly significant for cultigen, year, location, year by location, and year by cultigen ($p \leq 0.001$) (Table 1). Branching width measured in August was significant for location ($p = 0.002$), year by cultigen, and year by location ($p \leq 0.001$), indicating mostly environmental effects. Leaf area measured in August was significant for year, cultigen, and year by cultigen ($p \leq 0.001$). Disease severity (measured only in 2016) was significant for location ($p \leq 0.001$), but not cultigen. Lodging effects were not found.

In this study, there were significant differences among cultigens for stem height, leaf area, yield, and glycoside concentration identified which can be used to identify superior germplasm for use in breeding programs.

3.2. Cultigen performance

Plant size was measured using objective measurements (Table 1). NC-1016 (‘Eirete II’) was the tallest cultigen in 2015 (0.76 m), and NC-1032 (Swallowtail) was the tallest in 2016 (Table 1) (0.88 m). However due to year and interaction effects, it is important to note cultigens that were consistently large from year to year such as those sourced from Baker Creek (NC-1001, NC-1020). For branching width, NC-1016 (’Eirete II’) was the largest in 2015 (0.50 m), and NC-1036

Table 2
LS means for 16 cultigens of stevia for yield traits in September 2015-2016.

		2015			2016		
Cultigen 2015/2016	Source	Plant fresh wt. (Ton ha^{-1})	Plant dry wt. (Ton ha^{-1})	Leaf dry wt. (Ton ha^{-1})	Plant fresh wt. (Ton ha^{-1})	Plant dry wt. (Ton ha^{-1})	Leaf dry wt. (Ton ha^{-1})
NC-1016/1035	Eirete II	23.1	9.6	2.7	19.5	4.7	2.0
NC-1015/1034	Eirete I	22.9	9.5	1.9	15.4	4.4	1.6
NC-1018/1037	Native	21.4	8.3	2.3	10.0	1.8	1.1
NC-1001/1020	Baker Creek	19.9	7.9	1.8	14.5	3.4	0.7
NC-1017/1036	Katupyry	20.8	7.8	2.0	24.3	6.8	1.6
NC-1005/1024	Park Seed	18.1	7.4	1.9	13.0	3.2	1.1
NC-1010/1029	R.H. Shumway	18.7	7.4	1.9	22.3	6.4	2.0
NC-1014/1033	Richters	17.2	7.2	1.7	15.7	4.0	1.6
NC-1004/1023	Johnny's	18.8	7.1	1.8	20.2	5.6	2.0
NC-1002/1021	Territorial	16.0	6.5	1.7	13.9	4.1	2.0
NC-1003/1022	Seed Savers	16.4	6.2	1.9	15.6	3.5	1.1
NC-1011/1030	Everstevia	14.9	6.1	1.9	15.4	4.1	1.8
NC-1009/1028	Jung Seed	13.4	5.6	1.4	19.8	5.2	2.0
NC-1008/1027	Botanical Interest	12.5	4.6	1.6	13.6	3.6	1.7
NC-1013/1032	Swallowtail	13.0	4.5	1.3	19.7	5.2	1.6
NC-1012/1031	Harris Seed	9.6	2.5	0.8	13.7	3.3	1.4
Mean		17.3	6.8	1.9	16.7	4.3	1.6
LSD 5%		2.1	1.1	0.1	2	0.6	0.1
Location		NS	NS	**	NS	NS	**
Year		NS	**	**	NS	**	**
Cultigen x location		NS	NS	NS	NS	NS	NS
Cultigen x year		**	**	NS	**	**	NS

Means for each cultigen were pooled over two locations and four replications.
Ton ha^{-1} = Ton per hectare.
Location and year effect for each trait at 0.05 significance level.
Significant at 0.05 level of probability (*); Significantly at 0.01 level of probability (**).

Table 3
LS means for 16 cultigens of stevia for glycoside amount 2015.

Cultigen	Source	Reb. A (mg/g)	Reb. B (mg/g)	Reb. C (mg/g)	Reb. D (mg/g)	Stevioside (mg/g)	TSG (mg/g)
NC-1010	R.H. Shumway	59.1	0.5	10.5	2.4	52.9	137
NC-1015	Eirete I	56.9	1.3	7.2	3.0	41.1	121
NC-1017	Katupyry	56.4	0.7	6.3	2.9	51.9	129
NC-1001	Baker Creek	55.9	0.5	7.2	2.8	59.3	137
NC-1003	Seed Savers	55.3	0.6	8.5	4.5	47.7	130
NC-1016	Eirete II	52.7	0.5	7.6	2.8	42.5	120
NC-1002	Territorial	51.4	0.6	12.8	2.3	54.2	133
NC-1005	Park Seed	51.2	0.6	6.4	2.6	67.1	138
NC-1009	Jung Seed	49.6	0.5	6.2	2.9	57.3	127
NC-1004	Johnny's	47.0	0.6	6.5	3.4	43.9	113
NC-1018	Native	46.2	0.5	8.2	2.6	52.5	121
NC-1014	Richters	44.3	0.5	11.7	2.5	62.4	133
NC-1011	Everstevia	42.5	0.6	6.2	2.6	60.4	123
NC-1012	Harris Seed	38.8	0.5	6.2	2.0	65.2	122
NC-1013	Swallowtail	35.0	0.4	6.2	2.1	63.2	117
NC-1008	Botanical Interest	31.9	0.5	7.8	1.5	44.3	112
Trait mean		48.4	0.6	7.8	2.7	54.1	126
LSD 5%		7.7	0.2	2.4	0.6	7.8	9
Location		NS	*	NS	*	NS	NS
Year		**	NS	NS	**	**	**
Cultigen x location		NS	NS	NS	*	NS	NS
Cultigen x year		**	NS	NS	NS	NS	**

Means for each cultigen were pooled over two locations and four replications.
Steviol glycosides reported as concentration (mg/g) of dry leaf weight.
TSG = Total steviol glycosides, sum of all steviol glycosides measured (Some not shown).
Location and year effect for each trait at 0.05 significance level.
Significant at 0.05 level of probability (*); Significantly at 0.01 level of probability (**).

('Katupyry') was the largest in 2016 (0.48 m) (Table 1); however, branching width was mainly influenced by environment. For leaf area, NC-1011 (Everstevia) had the largest leaf area in 2015 (657 mm^{-2}) and NC-1020 (Baker Creek) had the largest in 2016 (1602 mm^{-2}) (Table 1).

For disease resistance, NC-1037 ('Native'), NC-1021 (Territorial), and NC-1023 (Johnny's) had the highest resistance among cultigens; however, our data suggested that location had a large effect on disease severity, and cultigen had less effect (p = 0.08). Previous studies have shown screening to be effective in identifying disease resistance (Reeleder, 1999).

NC-1016 ('Eirete II') was the highest yielding cultigen consistently for plant fresh wt. and plant dry wt. in 2015, and NC-1036 ('Katupyry') was the highest yielding in 2016 (Table 2). Although there was a significant year x cultigen interaction, 'Katupyry' was stable and ranked in

Table 4
LS means for 16 cultigens of stevia for glycoside amount 2016.

Cultigen	Source	Reb. A (mg/g)	Reb. B (mg/g)	Reb. C (mg/g)	Reb. D (mg/g)	Stevioside (mg/g)	TSG (mg/g)
NC-1022	Seed Savers	65.1	0.8	10.1	2.8	32.5	123
NC-1030	Everstevia	52.5	0.7	9.0	2.3	32.3	107
NC-1035	Eirete II	43.9	0.5	8.5	2.2	37.6	102
NC-1029	R.H. Shumway	43.8	0.6	13.9	2.3	26.9	99
NC-1034	Eirete I	38.9	0.6	8.8	1.4	37.0	95
NC-1031	Harris Seed	38.2	0.6	8.8	1.7	43.4	102
NC-1023	Johnny's	36.8	0.6	8.5	2.0	40.8	100
NC-1028	Jung Seed	34.7	0.5	9.2	1.6	35.6	91
NC-1027	Botanical Interest	33.0	0.5	8.1	1.6	44.4	96
NC-1033	Richters	31.9	0.5	6.5	1.4	37.1	85
NC-1021	Territorial	28.4	0.4	8.1	1.5	40.4	88
NC-1037	Native	28.1	0.5	7.0	1.9	34.8	82
NC-1024	Park Seed	26.5	0.3	7.7	1.9	45.2	93
NC-1036	Katupyry	21.2	0.5	4.9	1.0	24.2	58
NC-1020	Baker Creek	21.2	0.3	6.8	0.9	43.6	81
NC-1032	Swallowtail	16.0	0.3	5.9	0.9	39.0	70
Trait mean		35.0	0.5	8.2	1.7	37.2	92
LSD 5%		7.8	0.1	1.6	0.7	4.6	9
Location		NS	*	NS	*	NS	NS
Year		**	NS	NS	**	**	**
Cultigen x location		NS	NS	NS	*	NS	NS
Cultigen x year		**	NS	NS	NS	NS	**

Means for each cultigen were pooled over two locations and four replications.
Steviol glycosides reported as concentration (mg/g) of dry leaf weight.
TSG = Total steviol glycosides, sum of all steviol glycosides measured (Some not shown).
Location and year effect for each trait at 0.05 significance level.
Significant at 0.05 level of probability (*); Significantly at 0.01 level of probability (**).

Table 5
LS means for 16 cultigens of stevia for glycoside percent in 2015.

Cultigen	Source	Reb. A %	Reb. B %	Reb. C %	Reb. D %	Stevioside %
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-1015	Eirete I	46.5	1.1	5.9	2.4	34.5
NC-1016	Eirete II	43.8	0.4	6.3	2.4	35.8
NC-1011	Everstevia	33.6	0.5	5.2	2.1	49.8
NC-1012	Harris Seed	30.9	0.4	5.1	1.6	53.9
NC-1004	Johnnys	41.4	0.5	5.6	3.0	38.9
NC-1009	Jung Seed	39.1	0.4	4.9	2.3	44.8
NC-1017	Katupyry	43.4	0.6	4.8	2.3	40.1
NC-1018	Native	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		38.2	0.5	6.3	2.2	43.7
LSD 5%		5.0	0.2	2.0	0.4	6.0
Location		NS	*	NS	NS	NS
Year		NS	NS	*	*	NS
Cultigen x location		NS	NS	NS	*	*
Cultigen x year		**	NS	NS	NS	*

Mean- Mean of trait across all cultigens.
LSD- Least significant difference between cultigens at the 0.05 confidence interval.
Location and year effect for each trait at 0.05 significance level.
Significant at 0.05 level of probability (*); Significantly at 0.01 level of probability (**).

the top five yielding cultigens for both years.

Leaf dry weight was highest for NC-1016 ('Eirete II') in 2015, and NC-1023 (Johnny's) in 2016 (Table 2). Leaf dry wt. average for cultigens was 1.9 Ton ha^{-1} in 2015 and 1.6 Ton ha^{-1} in 2016 (Table 2). Cultigens ranged from 0.7 to 2.7 Ton ha^{-1} with the highest being NC-1016 ('Eirete II'). Year ranged from 1.6 to 1.9 Ton ha^{-1} whereas locations ranged from 1.34 to 1.77 Ton ha^{-1}, similar to those reported in Paraguay of 1.5–2.5 Ton ha^{-1} per year, and China of 1.3–1.4 Ton ha^{-1} (Midmore and Rank, 2002; Ramesh et al., 2006). Our yields were lower than some locations reported in the US (3.6 Ton ha^{-1}), Malaysia (2.8 Ton ha^{-1}) (Tan et al., 2008), Canada (2.8 Ton ha^{-1}), Russia (1.4–5.5 Ton ha^{-1}), and India (4 Ton ha^{-1}) (Midmore and Rank, 2002; Parris et al., 2016). One possible explanation for higher yield found is the use of improved clonal varieties providing a uniform genetic background for expression of yield traits. In our study, we utilized populations that were seed-grown that would have more genetic variation compared to clones. For example, clonal lines such as 'SW 129' had yields of 2.02 Ton ha^{-1} in Yuma, AZ, and '1049' was 8.82 Ton ha^{-1} grown in Ontario, OR (Parris et al., 2016). In that study, they reported cultigen effects of 3.28–6.46 Ton ha^{-1} and location effects of 3.4 Ton ha^{-1} for Hanford, CA to 5.8 Ton ha^{-1} for Ontario, OR which are higher than most studies using seed grown lines. Although yields were affected by the environment and cultural practices, the use of vegetative propagated selections can provide higher, and more consistent yields. Cultigens in our study have little known about their genetic background and may not be the best for yield initially; however, they may serve as useful genetic material to further develop varieties.

Since there was significant cultigen x year effect specifically for reb A, we noted those with stable glycoside yield (mg/g dry leaf tissue) in both years, such as NC-1010, and NC-1029 (R.H. Shumway) (Tables 3 and 4). Similarly, there was a significant cultigen x year effect with reb A when measured as a percent of TSG; however, NC-1010, and NC-1029 (R.H. Shumway) still had consistently high percentage (43 %) of reb A between years (Table 5 and 6). The highest yielding cultigens for reb C were NC-1014 (Richters) in 2015 and NC-1029 (R.H. Shumway) in 2016 (Tables 3 and 4). Similarly, these cultigens had the highest reb C (9 and 15 %) when measured as a percent of TSG respectively (Table 5 and 6). For reb D, the highest yielding cultigens were NC-1003 (Seed Savers) in 2015 and NC-1022 (Seed Savers) in 2016 (Tables 3 and 4) which also had the highest percentage of reb D (2–3.5 %) (Tables 5 and 6). Stevioside (selected for low levels) was lowest in NC-1005 ('Eirete I') in

Table 6
LS means for 16 cultigens of stevia for glycoside percent in 2016.

Cultigen	Source	Reb. A %	Reb. B %	Reb. C %	Reb. D %	Stevioside %
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
NC-1035	Eirete I	40.4	0.7	9.2	1.5	39.5
NC-1034	Eirete II	42.1	0.5	8.3	2.0	37.6
NC-1035	Everstevia	48.3	0.7	8.5	2.2	31.2
NC-1031	Harris Seed	35.8	0.6	8.7	1.7	43.9
NC-1023	Johnnys	37.1	0.6	8.6	1.9	41.0
NC-1028	Jung Seed	37.6	0.6	10.5	1.8	39.0
NC-1036	Katupyry	40.5	0.9	8.3	1.6	38.5
NC-1037	Native	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-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		36.4	0.6	9.0	1.8	42.1
LSD 5%		6.0	0.2	2.0	0.5	6.0
Location		NS	*	NS	NS	NS
Year		NS	NS	*	*	NS
Cultigen x location		NS	NS	NS	*	*
Cultigen x year		**	NS	NS	NS	*

Mean- Mean of trait across all cultigens.
LSD- Least significant difference between cultigens at the 0.05 confidence interval.
Location and year effect for each trait at 0.05 significance level.
Significant at 0.05 level of probability (*); Significantly at 0.01 level of probability (**).

2015 and NC-1036 ('Katupyry') in 2016 (Tables 3 and 4). However, when measured as a percent, stevioside had a significant cultigen x year interaction. Therefore, a stable but low percentage of stevioside may be desired from NC-1003 (Seed Savers) in 2015 and NC-1022 (Seed Savers) in 2016 which on average contained 33 % stevioside (Tables 5 and 6). The glycoside ratio of reb A to stevioside is also desirable, and we found that NC-1015 ('Eirete I') was the highest in 2015 and NC-1022 (Seed Savers) in 2016. For TSG, NC-1003 and NC-1022 (Seed Savers) had the highest TSG over both years.

Of the glycosides measured in our trials, stevioside occurred in the greatest quantity (either concentration or percent of TSG), followed by reb A, reb C, reb D, and reb B. Stevioside is generally the most abundant of the steviol glycosides (Behera et al., 2013; Moraes et al., 2013; Pal et al., 2015; Serfaty et al., 2013; Vasilakoglou et al., 2016). However, in some cases where improved cultivars are used, reb A can have the highest concentration as observed in Parris et al. (2016). Another example is the cultivar 'Morita' with a 9:1 ratio of reb A to stevioside (Morita, 1987), indicating the potential breeding progress of increasing reb A. Minor glycosides such as reb D and reb M have become increasingly popular recently, but may be more challenging to exceed levels found of major glycosides as they naturally occur at low concentration (Allen, 2013; Prakash, 2014). For example, in our study reb D averaged 2% of the total steviol glycosides, much smaller than the reb A content (Tables 5 and 6). Improved cultivars with high purity of desirable glycosides would make extraction more economical, as currently, undesirable glycosides make it difficult to extract and purify minor glycosides. Recent alternatives to producing glycosides in plants would be through a synthetic process such as biofermentation used at Cargill to produce reb D and reb M for Eversweet™ (Cargill, 2015). This and similar methods of producing glycosides synthetically have been explored by others (Rumelhard et al., 2016).

Growers and breeders seeking germplasm for high yielding, and stable cultigens should consider the sources of 'Katupyry'. In addition, when seeking high concentration and stable glycoside lines for reb A and reb D, one should consider Seed Savers, and R.H. Shumway based on the cultigens we evaluated. Future studies should be conducted to compare these lines to existing varieties.

3.3. Correlations among traits

Fresh and dry yield were highly correlated (0.73) (Table 7), indicating that plant fresh weight is a good indicator of overall biomass without the need for drying and weighing plants. Correlations between yield as dry wt. and branching width were correlated, followed by stem height (0.29 and 0.15) (Table 7). A similar correlation between yield as a fresh wt. with branching width and stem height was observed (plant fresh wt.) (0.21 and 0.22, respectively) (Table 7). This suggests that high yield may be found by selecting for tall or well branched plants. Further, leaf area had only a minimal correlation (Table 7). Although quantifying total leaf area via a leaf area meter may be more effective, we were interested in a quick non-destructive screening method in the field. In our study stem height and leaf dry wt. were correlated with yield (fresh wt. and dry wt.) (Table 7), similar to previous findings (Buana and Goenadi, 1985; Chalapathi et al. 1998) which also suggested that taller plants are high yielding.

Percentage of steviol glycoside (of TSG) and concentration (mg/g) were highly correlated across all glycosides measured (Table 7). Correlations of the two traits were 0.84 for reb A, 0.93 for Reb B, 0.88 for reb C, 0.92 for reb D, and 0.77 for stevioside (Table 7); therefore, selection for plants with high concentration amounts (mg/g) of a steviol glycoside generally translates to a high percentage of TSG. Stevioside was negatively correlated with reb B, reb C, and reb D (-0.16 to -0.31) (Table 7) and percent stevioside had the highest negative correlation with percent reb A (-0.93), reb B (-0.56), reb C (-0.39), and reb D (-0.74) (Huber, 2017). These findings suggest that when selecting for high levels of stevioside either as total or a percent that reb A, reb B, reb C, and reb D tend to be found at low levels as a result. We also observed a negative correlation (-0.25) of stevioside concentration and yield (Table 7), similar to the report of Brandle and Rosa (1992), suggesting a trend of high yielding plants containing low stevioside. Although a low correlation was observed, there was a correlation (0.12−0.21) for reb A, reb B, and reb D with yield (dry wt.) (Table 7). Glycoside compounds that correlate higher to yield such as reb D (0.21), enable easier breeding progress to be made for both yield and the concentration of steviol glycoside simultaneously, based on the cultigens studied. We also observed that reb A concentration was correlated with yield (Table 7), as reported by Shyu (1994), suggesting that high yielding plants tend to have a high concentration of reb A. We observed that reb A and reb C concentration were correlated (0.29) (Table 7) as previously reported (Nakamura and Tamura, 1985; Brandle et al., 1998). We were unable to detect a correlation of leaf area and reb A unlike the findings of Weng et al. (1996), or a correlation of leaf area with stevioside unlike the findings of Truong et al. (1999), possibly due to genetic variation of our population.

Disease resistance had a significant correlation with yield (plant fresh wt. and dry wt.) and reb B, but a negative correlation with reb D (Table 7). The correlation between yield and disease resistance could be explained by reduced defoliation with resistance. Lodging tolerance is

Table 7
Pearson product-moment correlations for 16 cultigens of stevia for yield, resistance and glycoside amounts in 2015 and 2016.

	Stem height (mm)	Branching Width (mm)	Leaf area (mm^2)	Lodging severity (0−9)	Disease severity (0−9)	Plant dry wt. (Ton $ha^{-1)}$	Leaf dry wt. (Ton ha^{-1})	Reb. A (mg/g)	Reb. B (mg/g)	Reb. C (mg/g)	Reb. D (mg/g)
Branching width (mm)	0.32**										
Leaf area (mm^2)	0.32**	−0.08									
Lodging severity (0−9)	0.19	0.20	0.14								
Disease severity (0−9)	−0.24*	−0.15	0.21	0.17							
Plant dry wt. (Ton ha^{-1})	0.15**	0.29**	−0.24**	−0.10	0.21*						
Leaf dry wt. (Ton ha^{-1})	0.22*	−0.21*	0.01	−0.18	0.35**	0.53**					
Reb. A (mg/g)	−0.06	0.13*	−0.20**	−0.30*	−0.09	0.12*	−0.22**				
Reb. B (mg/g)	−0.17**	−0.01	−0.08	−0.33**	0.35**	0.18**	−0.01	0.54**			
Reb. C (mg/g)	0.00	0.02	0.07	−0.04	0.02	0.06	0.07	0.29**	0.11		
Reb. D (mg/g)	−0.11	0.10	−0.21**	−0.21	−0.24*	0.21**	−0.22**	0.59**	0.50**	0.04	
Stevioside (mg/g)	−0.03	0.08	−0.10	0.08	−0.14	−0.09*	−0.59**	−0.23**	−0.31**	−0.26**	−0.16*

Stem height = Stem height measured (mm) in August.
Branching width = Branching width measured (mm) in August.
Leaf area = Leaf area measured (mm^2) in August.
Lodging severity = Lodging severity rated subjectively 0−9 (0 = none, 1−3=low, 4−6=intermediate, 7−9=high).
Disease severity = Disease damage rated subjectively 0−9 (0 = none, 1−3=low, 4−6=intermediate, 7−9=high).
Plant dry wt. = Yield of plant dry weight (Ton ha^{-1}).
Leaf dry wt. = Leaf dry weight measured (Ton ha^{-1}).
Significant at 0.05 level of probability (*).
Significantly at 0.01 level of probability (**).

another important trait for mechanized harvests but was not correlated with yield in this study (Table 7). Further tests are needed using other germplasm as these correlations were generally low and varied with population and environment.

3.4. Correlations of traits over plot sizes

Plant size traits (stem height, branching width, leaf area) were measured in 30-plant plots (64,550 plants/ha), as well as in 6-plant plots (12,910 plants/ha) (Table 2). Since correlations between plot sizes were below 0.70, we concluded that plant size traits should be measured in the high-density plots for accuracy with commercial densities.

4. Conclusions

Genetic diversity had a major impact on yield, resistance, growth habit and steviol glycosides among the cultigens analyzed in this study over two years and two field locations. The highest yielding and environmentally stable cultigen were derived from 'Katupyry'. In general, high-yielding cultigens were observed to have large branching width and or large stem height. Therefore, correlations suggest that yield may be improved by selecting well-branched and or tall plants. The largest leaves were found in NC-1020 (Baker Creek) and NC-1011 (Everstevia), however plants with larger leaf area was not an effective indicator of yield. Although disease resistance is another important consideration for yield, environmental effects were mainly a factor in our study. The cultigens used in this study were variable in glycoside yield as a concentration and percent of TSG. High yielding cultigens for glycosides varied depending on the rebaudioside of interest. This study highlights readily available seed cultigens that can be used to develop elite breeding populations.

CRediT authorship contribution statement

Brandon M. Huber: Conceptualization, Writing - original draft, Investigation, Writing - review & editing. **Todd C. Wehner:** Conceptualization, Methodology, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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