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Effect of harvest timing on leaf production and yield of diterpene glycosides in *Stevia rebaudiana* Bert: A specialty perennial crop for Mississippi

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ABSTRACT

Stevia rebaudiana (Bertoni), a perennial herb of the Asteraceae, is one of the most important sources of non-caloric natural sweeteners. Stevia's plant extracts and glycosides have been used by indigenous cultures for centuries in Paraguay and Brazil. Several studies suggest that Stevia and its glycosides exert beneficial effects on human health, including anti-hyperglycemic and antihypertensive properties (Jeppesen et al., 2000). The objectives of this study were to evaluate Stevia's cold hardness in Mississippi and determine the effects of different harvest timing on leaf production and yield of diterpene glycosides. Plants purchased from Ritchers Herbs were the source of nodal explants for *in vitro* clonal propagation. Rooted plantlets adapted in trays were later transplanted in the field of the University of Mississippi Biological Field Station. Plant spacing was 30 cm between plants in a row and 60 cm between rows. Drip irrigation supplied water to the field at rate of ¼ to ½ in. per week during summer. To protect plants during the winter of 2010 a plastic in field low tunnel was used, while in 2011 plants remained uncovered. Leaf biomass production and yield of diterpene glycosides were evaluated based on three different harvest timings: (1) Three harvests at 60 day intervals, two leaf harvests at a 90-day interval and a single harvest after 180 days of cultivation. Results revealed that leaf production from plants harvested once a year yielded more than the yield of multiple harvests (2 and 3 harvests per growing season). A total of 13,896.37 kg per hectare of stem and leaves were harvested in a single harvest. The glycosides productivity obtained from a single harvest; rebaudioside A (398.80 kg ha^{-1}) and stevioside (512.21 kg ha^{-1}) was also greater than the yields of multiple harvests (2 and 3 harvests).

1. Introduction

The Center for Diseases Control and Prevention (CDC) reports that more than one third of adults in the United States are obese (Ogden et al., 2012). Obesity-related conditions, such as cardiovascular diseases, type 2 diabetes and certain cancers are some of the leading preventable causes of death. Medical costs associated with obesity in 2008 were estimated to be $147 billion (Finkelstein et al., 2009). This is particularly important for the States with higher obesity levels like Mississippi, with a rate of 34.9% in 2011, the highest in the nation, while the South with an average rate of 29.5% showed the highest incidence among regions (CDC, 2011).

Kersh et al. (2011) reported that there is urgency to solve childhood obesity problems, and public policies are limited. Among public policies are community strategies that include enhancing the availability of healthier food. However, reduction of sugar-sweetened foods is not yet listed. In accordance with the CDC program recommendations to prevent obesity by replacing sugar with natural sweeteners, we investigated the development of *Stevia rebaudiana* (Bertoni) as a specialty crop for Mississippi, a species that belongs to the Asteraceae family which has been used as a natural sweetener and traditional medicine. The possible industrial applications for *Stevia* and its glycosides are extensive including bakery and dairy products, non-caloric food sweeteners, food supplements and pharmaceuticals.

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S. rebaudiana is native to Paraguay and Brazil where it grows at altitudes of 200–500 m above sea level, where temperatures range from −6.0 to 43 °C with the average of 23 °C (Brandle and Rosa, 1992), its leaves contain diterpene glycosides including stevioside, rebaudioside A, B, C, D, E, F, dulcoside A and C and steviolbioside (Kinghorn, 1987; Dacome et al., 2005). Pharmacological studies suggest that stevia glycosides have broad spectrum therapeutic properties including antihypertensive, antihyperglycemic, antioxidant, anticancer and anti-inflammatory (Yadav et al., 2011; Shivanna et al., 2012). Thus, due to potentially the extensive applications of stevia compounds, two major leaf compounds stevioside and rebaudioside A, were evaluated for toxicological properties. The studies revealed that oral intakes of stevioside and rebaudioside A are safe at a rate of 5 mg kg^{-1} body weight, and no carcinogenic and teratogenic effects were detected (Urban et al., 2013).

Recently, this neglected and still underutilized crop was included into domestication programs in many countries including Canada, Brazil, India and Italy. Stevia is mainly produced in China (Lavini et al., 2008). According to Ramesh et al. (2007), *S. rebaudiana* was planted in hilly, frost prone areas in India where it becomes dormant during winter (Megeji et al., 2005). Large corporations such as Stevia Corp., a farm management company focused on the economic development of stevia, are currently partnering with companies in China and Vietnam because of low labor costs (Stevia Corp., 2003). We have evaluated *S. rebaudiana*'s growth and development in the Northen part of Mississippi hoping to develop this plant as a specialty crop that may help to turn obesity problems into an economic opportunity for Mississippi farmers.

2. Materials and methods

2.1. Plant material and growing conditions

Stevia plants purchased from Ritchers Herbs, Canada were used as the source of nodal explants for *in vitro* clonal propagation following the protocol described by Ahmed et al. (2007). Rooted plantlets were planted in trays using Pro-Mix BX (Rivière-du-Loup, Québec) peat-based as growing medium. The planting trays were placed on heating mat at 22–25 °C, watered daily. The trays were covered with a plastic dome for 2 weeks to maintain humidity.

Plants were transplanted into the field located at the University of Mississippi Biological Station, Abbeville, Mississippi during the second week of April 2010. The UM Biological Field Station is located at the north part of the State of Mississippi. According to the Office of Mississippi Climatologist (MS Climate Office, 2012), Mississippi is located in the humid subtropical climate region, characterized by temperate winters; absence of severe cold in the winter. Table 1 includes the history of January weather in Abbeville MS (http://weathersource.com).

Plant spacing was ∼30 cm between plants in a row and 60 cm between rows, a total of 30 plants per row. Plants were watered by drip irrigation at a rate of 6.35–12.7 mm per week. Growth slowed during June and July when temperatures were above 38 °C. Late October 2010 the plants were covered with plastic in a field low tunnel (Fig. 1A). Straw mulch was placed around the plants before the winters. In 2011, plants were not covered with plastic to evaluate the cold hardness of *Stevia* in Mississippi (Fig. 1B). Plants were foliar fed with 4-1-1 (NPK) fertilizer at a rate of 1 gal/acre in early spring. Foliar fertilizations were repeated after each harvest (Fig. 1C).

In 2010, plants were harvested manually twice a year in a 90-day intervals, July and October, leaving 8–10 cm stem height from the collar (Megeji et al., 2005). The procedure was repeated in 2011 and 2012. In 2012, we examined three harvest schedules as treatments in a complete randomized design; three harvests at a 60-day interval, two harvests at a 90-day interval and a single harvest after 180 days after spring re-growth (Fig. 1D). All plants were foliar fed with 4-1-1 (NPK) fertilizer at a rate of 1 gal/acre after 60, 90 and 120-day intervals keeping the same conditions to all treatments. Thus the treatments TR1, TR3 and TR4 were harvests from one set of plants done at an interval of 60 days; TR2 and TR5 were harvests from a second set of plants done at an interval of 90 days, while TR6 were just harvested one time during the growing season. Plants harvest in treatments TR1, TR2 and TR3 were harvested during vegetative stage while TR4, TR5 and TR6 were at flowering stage. After the 2012 harvests, the plants were maintained in the field and were watered once. *Stevia* plants were evaluated for spring re-growth in April 22, 2013 (Fig. 2A and B).

2.2. Extraction of sweet diterpene glycosides from S. rebaudiana

Dried and ground leaf samples (1 g) were placed in 250 mL Erlenmeyer flasks filled with 100 mL of aqueous EtOH 70% (w/w). Flasks were incubated in a 70 °C water bath for 30 min with shaking. After the extracts were cooled, extracted samples were filtered and analyzed by HPLC using an NH_2 column (250 mm × 4.6 mm) and a mixture of acetonitrile/water (80:20, v/v) as mobile phase, pH 5 adjusted with acetic acid. The detection was in the UV range at 210 nm (0.04 AUFS) following the method described by Kolb et al. (2001).

Quantitative analyses were performed by means of an external standard calibration curve for each analyte prepared from four concentration points (0.15, 0.3, 0.6 and 1.0 mg mL^{-1}) of standard solutions of pure stevioside and rebaudioside A purchased from ChromaDex Inc. (Irvine, CA). Each standard was used to formulate separate calibration curves. Linearity was imposed by using response factors and regression coefficients independently. Response factors were calculated using the equation $RF = DR/C$, where DR was the detector response in peak area (PA) and C was the standard concentration. The target peaks were confirmed by retention time and mass spectra, when needed. Confirmed integrated peaks were then used to determine the percentage of each chemical constituent in the sample. The R.F. of the target chemical constituent was used to determine the "percent" for each sample using the equation: $PA/R.F./C \times 100 = \%$ (peak area/response factor/concentration) in the fresh weight. To estimate the yield of each diterpene glycoside (stevioside and rebaudioside A) in leaf tissues, stem biomass representing half of the total dried weight was discarded. According to Yadav et al. (2011) plant organs contain different amounts of diterpene glycosides, leaves and flowers containing the most and stem, seeds and roots low levels.

Table 1
January weather (temperature and precipitation) in Abbeville MS from 1900 to 2012 according to Weather Source, LLC.

Temperature	°F	°C	Precipitation	in.	mm
Highest temp.	80	26.66	Highest precip.	16.15	410.20
Lowest temp.	−13	−25.00	Average precip.	4.96	125.98
Average high temp	51	10.55	Lowest precip.	2.10	53.34
Average low temp.	31	−0.55	Highest daily precip.	4.60	116.84

The history of January weather for Abbeville MS from weathersource© Weather Source, LLC.

Fig. 1. Stevia field located at UM biological Field Station, Abbeville Mississippi during winter of 2010–11 (A and B) and winter of 2011–12 (C). Stevia re_growth, shoot emerging from the crown in April of 2012 (D). Stevia actively growing in May and middle August of 2012 (E and F).

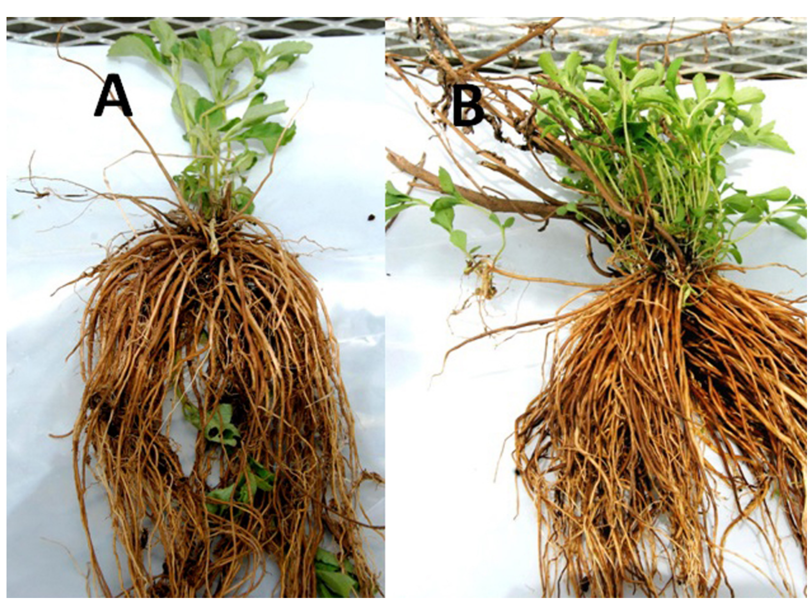

Fig. 2. *Stevia* forms a cluster of shoots at the stems bases during re-growth (A), steams that are in contact with the ground may also establish fibrous roots (B). This is a cloning mechanism that Stevia presents.

Table 2
Winter weather (temperature and precipitation) during December, January and February of 2010 to 2013 in Abbeville MS.

Temperature and precipitation	Winter 2010–11			Winter 2011–12			Winter 2012–13		
	December (°C)	January (°C)	February (°C)	December (°C)	January (°C)	February (°C)	December (°C)	January (°C)	February (°C)
Highest Temp	21.0	23.8	25.5	20.5	22.2	28.3	25.0	25.5	20.5
Lowest Temp	−7.0	−14.4	−13.9	−2.8	−6.0	−9.4	−8.3	−7.2	−5.0
Average High	10.2	8.9	14.7	12.4	14.9	14.8	15.7	12.8	13.1
Average Low	−2.0	−1.9	2.2	−2.2	−1.7	3.1	2.8	0.3	−0.1
Monthly precipitation (mm)	57.7	61.7	93.5	93.5	81.8	77.5	159.8	193.3	101.1

Data collected from Weather Source, LLC.

2.3. Statistical analyses

Data were subjected to statistical analysis using the Statistical Analysis System – (SAS Institute Inc., Cary, NC) (SAS, 2003).

3. Results and discussion

The temperature in North Mississippi during 2010, 2011, 2012 and 2013 varied between 40 °C in July, when plants slowed their growth, to a complete cold damage of the leaves after temperatures dropped below 0 °C even under low plastic tunnel (Fig. 1A and B) in January 2011 or without the tunnel protection (Fig. 1C) in January 2012 and 2013. January is one of the coldest months in Mississippi reaching −0.55 °C as the lowest average temperature in 100 years period (Table 1). During the winters of 2010–11, 2011–12 and 2012–13, the lowest average temperatures were in December of 2010 and January 2011, temperature reached −1.9 °C and −2.0 °C (Table 2). All plants recovered from winter cold temperatures (Fig. 1D, E and F) and were harvested during the growing season of 2012. Plants were maintained in the field after the 2012 fall harvest, but did not receive any mulch or fertilizer applications. In the spring of 2013, all plants showed re-growth that started in second week of April. According to Brandle and Rosa (1992), *Stevia* is native to a region of South America, where the temperature may reach −6 °C in winter months. The plastic mini tunnel and the straw mulch may have protected the plants during the first and second winters, however these practices were not necessary during winter of 2012–13. Nevertheless, Stevia survived the 2012–13 winter and we noticed that *Stevia* shoots during re-growth forms a cluster of shoots at the stem basis where fibrous (adventitious roots) are attached. This may be a cloning mechanism that *Stevia* plants present, which allows the species to survive at temperature below 0 °C (Fig. 2A and B), in locations such as Mississippi where the ground rarely freezes.

3.1. Biomass yields

In 2010 and 2011, plants harvested during the first and the second growing season produced a total of 3080.92 kg ha^{-1} of dry aerial part, including stems and leaves (data not shown). Our yield in the first year was less than the yields reported by Andolfi et al. (2006) in Central Italy (3600 kg ha^{-1} of dry leaf) and Megeji et al. (2005) in India with a production of 3500 kg ha^{-1} of dry stems and leaves. The total fresh biomass produced during the third growing season varied significantly depending on the treatment, which were number of harvests per growing season. The overall fresh biomass production harvested in a single harvest was 34,950.90 kg ha^{-1}, yielding 13,896.37 kg ha^{-1} of dry stems and leaves (Table 3). Two or three cuts per season in intervals of 60 and 90 days produced 4790.28 kg ha^{-1} and 5384.68 kg ha^{-1} respectively, yields that were significantly lower than plants harvested once a year. Andolfi et al. (2006) reported that 2 cuts per year resulted in higher yields for both tested genotypes. They also reported significant increases until the 6th year reaching a peak of 6100 kg ha^{-1} of dry leaves and our data are similar to those reports (Table 3).

3.2. Quality of biomass

Our average leaf content of stevioside produced in 2010 and 2011 was higher (10.58% and 11.05%, w/w basis) than the content reported by Lavini et al. (2008) in Italy (8.36%) or in India with 6.09% (Megeji et al., 2005). Leaves produced in India were not analyzed for rebaudioside A but crop produced in Italy showed levels of 5.67% (w/w) (Lavini et al., 2008). The rebaudioside A content found in leaves harvested in Mississippi during the first and the second growing season was 3.1% and 4.5% (w/w) basis respectively. Our stevioside content in plants harvested once a year was lower than that in the combined biomass of plants harvested several times, while for the rebaudioside A, the content of a single harvest was higher than in the biomass from plants harvested twice a year. Although, one leaf harvest per year produced biomass with significantly less stevioside than biomass from multiple harvests, the biomass yield of aerial parts in a single harvest was so much greater (Table 3) that overall glycoside total yield was higher for the one leaf harvest, 534 kg of stevioside and 398.8 kg of rebaudiside A per hectare (Table 4).

Table 3
Biomass production and yield of diterpene glycosides of 2 year old *Stevia* plants cultivated in north Mississippi.

Harvest schedule	Aerial biomass F.W. (kg ha^{-1})	Aerial biomass D.W (kg ha^{-1})	D.W. leaf biomass (kg ha^{-1})	% of Stevios.	% of Reb. A	Yield of Stevios. (kg ha^{-1})	Yield of Reb. A (kg ha^{-1})
TR1 (60 days)	13,437.61bc*	2208.08 bc	1104.04 bc	11.16 ab	3.12 ab 3.63 ab	126.93 bc	32.22 bc
TR2 (90 days)	16,050.98 b	2889.08 bc	1444.54 bc	12.75 a	6.44 a	178.48 b	59.96 bc
TR3 (60 days)	9159.88 cd	2215.20 bc	1107.60 bc	11.68 a	4.34 ab	130.20 bc	69.10 b
TR4 (60 days)	4408.85 d	367.00 c	183.50 c	11.53 a	1.98 b	77.57 c	30.35 bc
TR 5 (90 days)	7266.97d	2495.60 bc	1247.80 bc	11.91 a	5.68 a	148.53 b	24.66 c
TR6 (180 days)	34,950.90 a	13,896.37 a	6948.18 a	7.51 b		512.22 a	398.80 a

TR1, TR3 and TR4 are the same plants harvested in interval of 60 days; TR2 and TR5 are the same plants that were harvested 90 days period, while TR6 were just harvested one time during the growing season.

* Means followed by the same letter in the column are not significantly different by the Scott–Knott test $P < 0.05$. Stevios. is same as stevioside and Reb. A. is rebaudioside A.

Table 4
Yields of *Stevia* biomass and diterpene glycosides.

Number of cuts	Harvest interval	Total yield (kg ha^{-1})	Stevioside (kg ha^{-1})	Rebaudioside. A (kg ha^{-1})
3	60 days	27,006.3 ab*	334.6	131.7
2	90 days	23,318.0 b	327.0	84.6
1	180 days	34,950.9 a	512.2	398.8

* Means followed by the equal letter in the column are not significantly different by the Scott–Knott test $P < 0.05$.

Leaf stevioside content of this study was higher than the content reported by Lavini et al. (2008) and Megeji et al. (2005). According to Lemus-Mondaca et al. (2012), stevioside content ranges between 4 and 13% on leaf dry weight basis. The ratio between stevioside and rebaudioside A is an indicator of the quality of the biomass. Thus, if leaves contain equal amounts of rebaudioside A and stevioside the aftertaste is greatly diminished. The sweetness quality increases the greater the relative concentration of rebaudioside A (Yadav et al., 2011).

The rebaudioside content was greatly affected by the number of cuts per growing season. Higher levels were found in plants harvested once at flowering stage (TR6). In fact the ratio between stevioside and rebaudioside A was reduced with a single cut (TR6). Singh and Rao (2005) reported that once flowering is initiated glycoside concentration in the leaves starts to decline. Our data on stevioside concentration agree with Singh and Rao (2005); however the concentration of rebaudioside A in leaves appears to have increased (TR6; Table 3).

Many of the studies on glycoside production only include data on stevioside concentration as the measurement of quality (Megeji et al., 2005), even though this glycoside is largely responsible for *Stevia*'s aftertaste. Lavini et al. (2008) study on glycoside productivity included the concentration of rebaudioside A which was 5.72%. In our study the content of rebaudioside A varied between 1.98 and 6.44% on leaf dry weight basis (Table 3) in plants having two or three cuts, while one cut produced leaves containing 5.68%. The rebaudioside A overall yield varied between 84.6 and 398 kg ha^{-1} depending on the number of harvests per season. Rebaudioside A production in Central Italy was 0.26 t ha^{-1} (Lavini et al., 2008).

4. Conclusion

S. rebaudiana plants grew well in the northern part of Mississippi, producing quality biomass containing stevioside and rebaudioside A. Further studies are necessary to evaluate several factors; including plant density, influence of different types of fertilizer on growth, yield and quality and selection trials for varieties that perform well in Mississippi and have the best quality for consumer satisfaction.

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