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SEASONAL COMPARATIVE ANALYSIS OF GROWTH PARAMETERS AND STEVIOSIDE CONTENT OF *STEVIA REBAUDIANA* VAR. MORITA II

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ABSTRACT

The genus *Stevia* is known for its natural sweetening property because of the presence of different ent-kaurene glycosides. Among these compounds stevioside is the highest and mainly responsible for the sweetening property. The most important characteristics of stevioside is its non-calorific nature, due to which it is highly preferable for using in food and medicines. The antimicrobial properties of stevioside also makes it a superior constituent of the medicines used for different skin diseases. Because of these special characteristics, cultivation and commercialization of *Stevia* have become widespread throughout the world. Many Indian states have already successfully cultivated this plant species in large scale. But, being a country having different regions with prominent seasonal variation, the yield of stevioside can be different throughout the year, which will affect the commercial value of the compound and hence can directly affect our farmers. This seasonal variation is also persistent in the northeast Indian state, Assam, which is highly affected by extensive rain in the monsoon. Therefore, an investigation was under taken to analyze the stevioside content in three different seasons: summer, rainy and winter, taking *Stevia rebaudiana* var. MoritaII as the plant of study. The results showed a distinct variation in the stevioside concentration in different seasons. At the p=0.01 level, there was a significant positive association between the number of branches per plant and leaf fresh weight (r=0.87*), leaf dry weight (r=0.90**), stevioside concentration (r=0.90**) and stevioside percentage (r=0.88**). This study will be helpful to the farmers for selecting the suitable season for the cultivation of *Stevia rebaudiana* var. Morita II for an increased stevioside content.

***Key words*:** Stevia; Stevioside; Seasonal variation; Morphological parameters Stevioside content; Correlation

Introduction

Stevia, a small perennial herb, belonging to the family Asteraceae, is known for its rich content of natural sugar. Being a plant of semi-humid, subtropical environment, *Stevia* can be easily grown in a well-drained sandy loamy soil where pH should be in the range of 6.5 to 7.5 (Jadhao *et al*., 2011; Zaman *et al*., 2015). Although the plant is native to Central and South America, yet, because of its characteristic sweetening property, cultivation of *Stevia* has become popular in different parts of the world including India, China and some other parts of Southeast Asia (Wolwer-Rieck, 2012). Indian states like Rajasthan, Maharashtra, Kerala and Orissa have already successfully cultivated this plant species in recent years. According to a report by Kinghorn *et al*., (1984), 110 species investigated for sweetness, only 18 were found to possess its characteristic sweetening property. The most important part of a *Stevia* plant is its leaves as they are the reservoir of the constituents responsible for the sweetening. *Stevia rebaudiana* is the sweetest of all *Stevia* species, containing all the eight ent-kaurene or steviol glycosides in its leaves with stevioside as the major constituent (9.1% by weight) (Kinghorn *et al*., 1984, Goyal, 2010). *Stevia rebaudiana* is considered as “the

calorie free bio sweetener" for containing phytochemicals which have various health benefits and is used as natural taste and flavor enhancer for its antibacterial effect (Preethi *et al*., 2011; Misra, 2011; Ruiz-Ruiz *et al*., 2015). The compounds that are responsible for these significant properties are the ent-kaurene-type of diterpene glycosides, such as stevioside, rebaudiosides A, D, and E and dulcosides A and B. Stevioside (5-10%) and rebaudioside A (2-4 %) are the most abundant diterpenes found in the species of *Stevia*. Rebaudioside C (1-2%) and dulcoside (0.4-0.7%) are the minor components in this plant (Chatsudthipong and Muanprsat, 2009). Stevioside is a non-nutritive sweetener derived from the leaves of different species of *Stevia*. It is a white, crystalline, odorless powder that is 300 times sweeter than sucrose. Stevioside has already been approved for using as sweeteners in a variety of foods and beverages in many countries, including Japan, Australia, Argentina, Brazil, China, India, Israel, Japan, New Zealand, Paraguay, Russia, South Korea and in many South American countries (Kinghorn *et al.*, 1984; Kroyer, 2010, Wolwer-Rieck, 2012). In the year 2009, highly purified steviol glycosides were granted GRAS (Generally Recognized As Safe) classification in the United States (Wolwer-Rieck, 2012). Stevioside (13-[2-*O*-β-D-glucopyranosyl-α-glucopyranosyl)oxy]kaur-16-en-19-oic-acid- β-D-glucopyranosyl ester) is a glycoside which have a glucosyl and a sophorosyl residue attached to steviol aglycone, having a cyclopentanonhydrophenanthrene skeleton. Apart from the sweetening property, stevioside is also a source of vitamins, minerals and anti-oxidants, and possess antimicrobial properties (Guo *et al*., 2019). The increasing rate of different chronic diseases like diabetes mellitus, cardiovascular problems, high cholesterol level or high blood pressure has become a serious problem in today's world. One of the most important reasons of these aliments is the increasing ingestion of calorie rich foods. Stevioside in this case can be considered as a good alternative of sugar possessing high calorie, because of its low calorie content and requirement of a small amount for sweetening purpose. In South America too extracts of *Stevia rebaudiana* have long been used to treat diabetes (Chatsudthipong and Muanprasat, 2009). For the treatment of skin diseases stevioside serves as a very good medicine due to its antimicrobial property. Because of these significant characteristics, *Stevia* is an important topic of recent research. The increasing demands of natural sweetener has also driven the farmers for the cultivation of *Stevia* to a large extent. In India, different states have already started growing *Stevia* for commercial purpose. Being a seasonally diverse country and having a large number of states, India experience different climatic and environmental conditions throughout the year. The seasonal variation is an important factor that affects the accumulation of different phytochemical content in plants. The effect of different seasons on the production of *Stevia* is a matter of concern as it can affect the quality and yield of stevioside and hence the commercial value. Therefore, a study was undertaken to evaluate the stevioside content in the leaves of *Stevia rebaudiana* var. Morita II grown in three different seasons (summer, rainy and winter).

Material and Methods

Growth and Collection of Plant Samples

Stevia rebaudiana var. Morita II was selected as the plant of study. The plants were grown and harvested in three different seasons (summer, rainy and winter). The time of growth and collection of plant samples were divided as per the climatic condition and seasonal variation in Assam. The period between March to May was considered as summer, period between June to September as rainy and period between October to February as winter. Leaf samples were collected in an interval of three months after plantation. The plants grown and the sample collected from the summer season was named as T1, from the rainy season as T2 and from the winter as T3. Collected leaves were dried and ground into powder. The trial was conducted in Horticultural Research Station, Assam Agricultural University, Kahikuchi, Guwahati (26°06'12"N latitude, 91°35'43"E longitude with an altitude of 72 m above MSL).

Fig. 1: Cultivation of *Stevia rebaudiana* var. Morita II in field: **A:** Plants after transplantation; **B:** Growing plants in the matured stage; **C:** A matured plant before harvesting.

Assessment of Morphological Parameters

Different morphological parameters for estimation of growth of the plants (*Stevia rebaudiana* var. Morita II) were measured. Plant height, leaf breadth, number of branches per plant, fresh and dry weight of the leaves per plant were evaluated.

HPLC analysis and estimation of stevioside

High performance liquid chromatography was performed for the estimation of stevioside content using HPLC-grade water as the mobile phase. Hitache primaide HPLC system with UV-VIS detector and 5C18-MS-II (4.6IDX250 mm) column was used. The absorption was measured at 219 nm for stevioside. The chromatographic data was recorded and processed with Primaide software.

Preparation of leaf Extract

Preparation of leaf extract was done using the method of Jadhao *et al*., (2011). 5 g of dried leave powder was extracted with 50 ml of Mili-Q grade water and allowed to shake for 2 hr at 55°C in the rotary shaker (120 rpm). For the complete extraction of stevioside the process was repeated one more time. The two fractions were pooled together and concentrated using the rotary vacuum evaporator. The extracted sample was then prepared for further analysis adopting the method of Jadhao *et al*., (2011).

Standard solution preparation

Standard stevioside was procured from Sigma-Aldrich. 10 mg of standard was accurately weighed and made a stock solution of 10 ml with HPLC grade water. Five different solutions of 10, 20, 30, 40 and 50 ìg/ml were made by transferring the aliquot from stock solution and the volume was adjusted with HPLC grade water in each case.

Test sample preparation

25 mg of water extract was accurately weighed and transferred to a 25 ml volumetric flask and the volume was adjusted by HPLC grade water. The final stock solution of 100 µg/ml concentration was made by transferring 1 ml of above solution to 10 ml volumetric flask adjusting the volume with HPLC grade water. All the test samples and the standard solutions were filtered with 0.45 µm filter before injecting into the HPLC column. 10 µl from each of the solutions was injected for HPLC analysis.

Pearson correlation coefficient

Pearson correlation coefficient was conducted taking the data set from different morphological parameters, concentration and percentage of stevioside. To conduct this analysis, performance analytics packages and R was used.

Results and Discussion

Morphological parameters

The results of morphological parameters are shown in Fig. 2, 3, 4, 5 and 6 and Table 1. A distinct variation was observed in the morphological parameters in different seasons. The plants (*S. rebaudiana* var. Morita II) in the summer season (T1) showed a height of 58 cm,

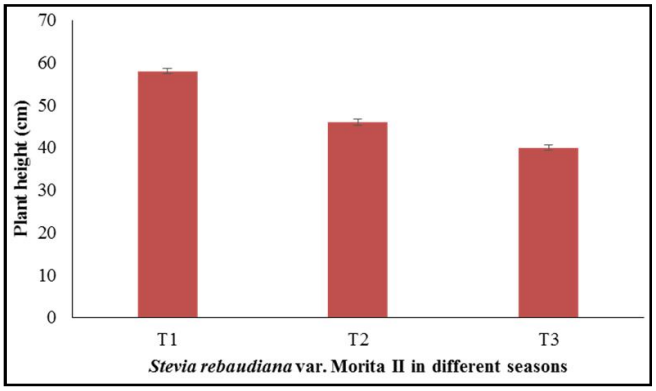

Fig. 2: Plant height (cm) of *Stevia rebaudiana* var. MoritaII in three different seasons (summer, rainy and winter).

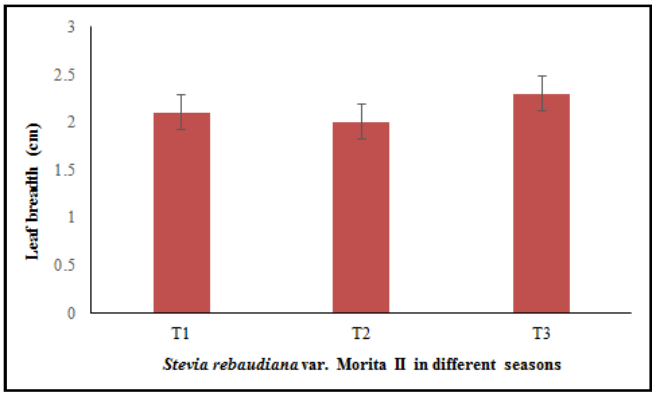

Fig. 3: Leaf breadth per plant (cm) of *Stevia rebaudiana* var. MoritaII in three different seasons (summer, rainy and winter).

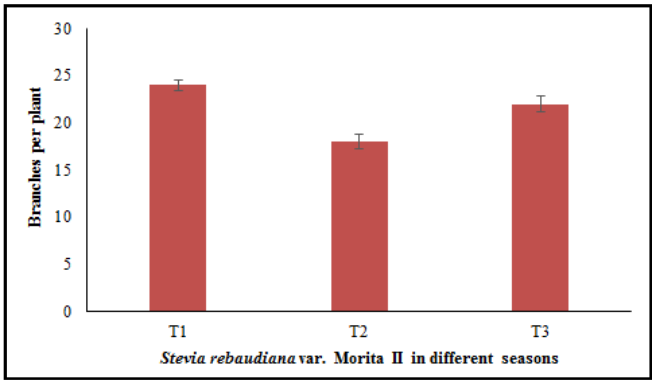

Fig. 4: Number of branches per plant of *Stevia rebaudiana* var. Morita II in three different seasons (summer, rainy and winter).

Table 1: Morphological parameters and stevioside content in three different seasons (T1=summer, T2=rainy, T3=winter).

Sl. No.	Plant sample	Plant height (cm)	Leaf breadth (cm)	No. of branches	Leaf fresh weight (g)	Leaf dry weight (g)	Conc. of stevioside (µg/g)	Percentage of stevioside (%)
1	T1	58±0.58	2.1±0.18	24±0.58	105±0.58	29.5±0.56	5.63±0.16	13.24±0.25
2	T2	46±0.82	2±0.16	18±0.82	85±0.80	15±0.86	0.67±0.07	1.57±0.10
3	T3	40±0.57	2.3±0.18	22±0.82	94±0.57	22±0.82	4.95±0.48	11.92±0.70

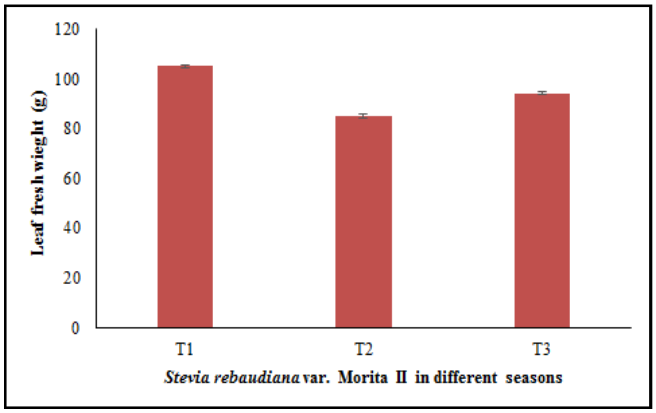

Fig. 5: Leaf fresh weight (g) of *Stevia rebaudiana* var. MoritaII in three different seasons (summer, rainy and winter).

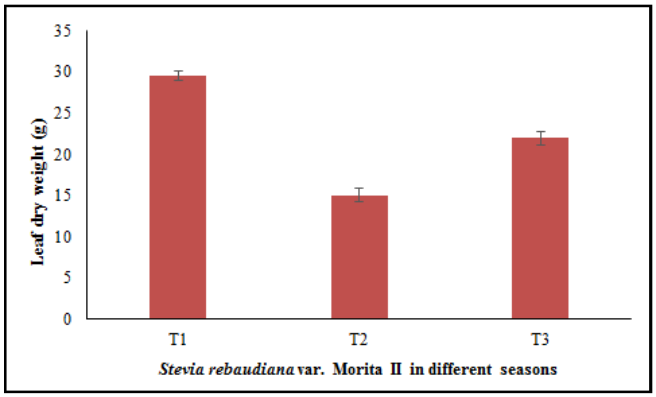

Fig. 6: Leaf dry weight (g) of *Stevia rebaudiana* var. MoritaII in three different seasons (summer, rainy and winter).

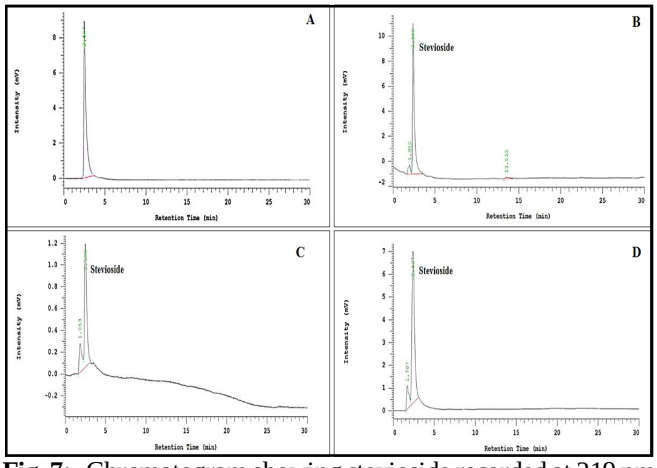

Fig. 7: Chromatogram showing stevioside recorded at 219 nm wavelength and retention time 2.4 min.: **A:** Standard stevioside (50 µg/ml); **B:** Leaf extract from the sample collected in summer season; **C:** Leaf extract from the sample collected in rainy season; **D:** Leaf extract from the sample collected in winter season.

whereas in rainy (T2) and winter seasons (T3), the plants were found to reach a height of 46 cm and 40 cm respectively. Leaf breadth was also found to be varied with respect to the seasons. Leaves in the winter season (T3) showed maximum breadth, 2.3 cm, followed by 2.1 cm breadth of the plants grown in summer (T1). The lowest leaf breadth was recorded from the plants grown in rainy season (T2), 2.0 cm. Number of branches per plant was highest (24 number per plant) in the summer grown plants (T1). But, this number decreased (22 number per plant) in the winter grown plants (T3). The plants grown in the rainy season (T2) produced lowest number of branches per plant (18 numbers per plant).

Leaf fresh weight per plant was recorded highest (105 g) in summer (T1), lowest (85 g) in rain (T2) and intermediate (94 g) in winter (T3). It was observed that leaf dry weight per plant was maximum in T1 (29.5 g) followed by T3 (22 g). T2 showed the lowest leaf dry weight per plant (15 g).

Data analysis and quantification of stevioside

The results of high performance liquid chromatography are shown in Fig. 7, 8 and 9 and Table 1. A visible difference was seen in the stevioside concentration with respect to their growing season. For estimating the concentration of stevioside, external standard method was used. To perform this method, solution of known concentrations of the compound of interest were prepared with one standard that is similar in concentration to the unknown. A fixed amount of sample is injected. The concentration of stevioside was calculated based on the calibration curve equation. Leaf

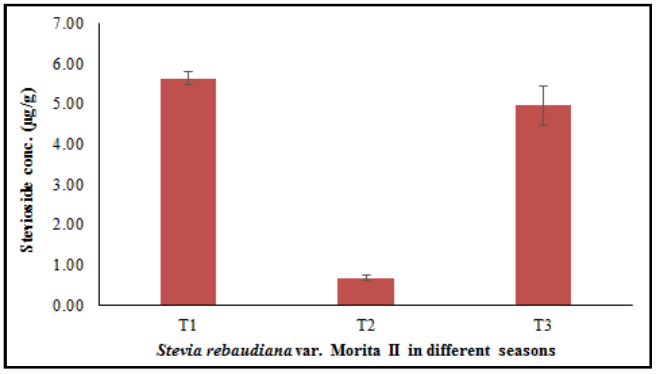

Fig. 8: Stevioside content (µg/g) in the leaves of *Stevia rebaudiana* var. MoritaII collected in three different seasons (summer, rainy and winter).

sample collected in the summer (T1) showed 5.63 µg/ g of stevioside content, whereas samples collected in the rainy (T2) and winter (T3) season showed 0.67 µg/ g and 4.95 µg/ g stevioside content respectively. The percentage (w/w) of stevioside content was found to be highest (13.24%) in the summer season (T1), being intermediate (1.57%) in the rainy season (T2) and the lowest (11.92%) in winter (T3).

Pearson correlation co-efficient

The result of Pearson correlation coefficient is shown in Fig. 10. A significant positive correlation was found between the number of branch per plant and leaf fresh weight (r=0.87*) at p=0.05 level, leaf dry weight (r=0.90**), concentration of stevioside (r=0.90**) and percentage of stevioside (r=0.88**) at p=0.01 level. Plant height was negatively correlated with leaf breadth (r=-0.88**) at p=0.01 level. At p=0.1 level, positive correlation was found between leaf breadth and percentage of stevioside (r=0.67˙).

The above observations clearly show a distinct relation between the morphological parameters and stevioside content in the leaves of *Stevia rebaudiana* var. Morita II. The highest amount of stevioside was found in the plants grown in summer season (5.63 µg/g; 13.24%) followed by winter (4.95 µg/g; 11.92%) and rainy seasons (0.67 µg/g; 1.57%). This might be due to the day length and light intensity, as a long day and bright light favors the accumulation of stevioside (Hernández *et al*., 2022). The growth and biomass of *Stevia* is directly associated with the accumulation of stevioiside. According to the work of Dhange and Gopinath (2020), the period of growth and the harvesting time of *Stevia* plays an important role in determining the concentration of stevioside. Many previous workers like Ramesh *et al*., (2006) and Madan *et al*., (2010) have already mentioned about the sensitivity of *Stevia* to the photoperiod. Being a short day plant, the species of *Stevia* requires light for flowering for a shorter period. But, reports have shown that a photoperiod of 12-16 h in the summer season is favorable for the vegetative growth of the plants which facilitate the accumulation of stevioside. Basharat (2021) in their work also found a decrease in the stevioside yield in the leaves of *Stevia rebaudiana* var. Bertoni, planted in the autumn prevailed by cold and rain. In this study, the morphological growth parameters of plants (the plant height, number of branches) and biomass of the leaves (leaf fresh and dry weight) were found to be highest in the summer grown plants, whereas it was found intermediate in the winter and lowest in rainy season, except the plant height, where winter grown plants were found to be the lowest. However, the leaf breadth was highest in the winter season, lowest in the rainy season and intermediate in the summer. The winter season in Assam is governed by less light intensity and short day condition. These factors can be related to the finding of our study, as a decline of these parameters was noticed in the winter grown plants. Apart from the photoperiod, another factor is responsible for the decrease of plant growth, i.e. water availability. In Assam, a rainless or almost no rain condition is sustained in the winter, which might be a cause of reduction of the amount of ground water required for the growth of *Stevia* plants. Moreover, all these environmental factors are associated with the secondary metabolite accumulation in leaves. Hence being a secondary metabolite (Junaedi *et al*., 2020), amount of stevioside is also affected by the above mentioned factors. It has been reported from studies of Rachmawati *et al*.,

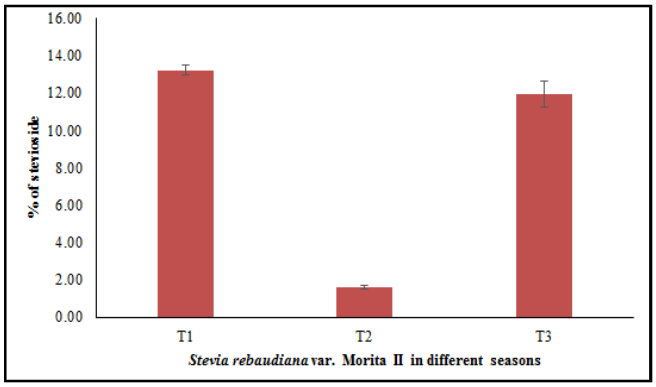

Fig. 9: Percentage (w/w) of stevioside in the leaves of *Stevia rebaudiana* var. Morita II collected in three different seasons (summer, rainy and winter).

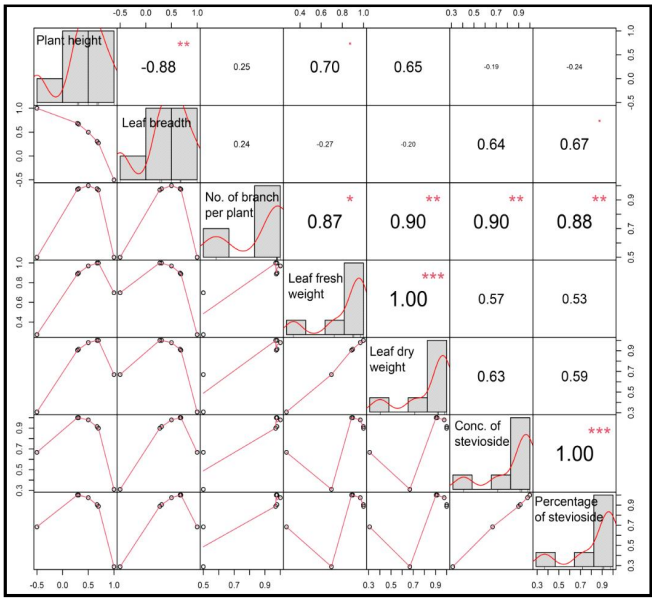

Fig. 10: Pearson correlation matrix showing correlation among different morphological parameters and the concentration and percentage of stevioside. (Level of significance: P=0.001, 0.01, 0.05, 0.1<=> symbols: " ***" , " **" , " *" , ").

(2017) that the morphology and the biochemical composition of the plants can be altered with light radiation changes. Generally, in the conditions of light with low intensity, plants maximize their efficacy to capture light, but, in the conditions of high light intensity, the plants try to increase the level of saturation of light for photosynthesis (Shulgina, 2021). In low temperature, due to a comparatively low rate of photosynthesis, the production of glyceraldehyde-3-phosphate will be low, which is a precursor for the biosynthesis of steviol glycosides including stevioside (Yadav *et al*., 2011; Ishabella *et al.*, 2023). Furthermore, leaves are the major site of stevioside accumulation (Bondarev *et al*., 2003). So, reduction in the leaf biomass, leaf breadth and number of branches containing leaves affect the amount of stevioside present in a plant. Therefore, the minimum concentration of stevioside in the rainy season can be attributed to the above mentioned reasons. A positive correlation between the plant height, number of branch per plant, leaf fresh and dry weight and the concentration of stevioside supports this statement. On the other hand, a negative correlation was found between plant height and leaf breadth. As the height of the plants reduced (from summer, rainy to winter), the leaf breadth was found increasing, the (from winter, summer to rainy). The Pearson correlation coefficients showed a positive correlation of leaf breadth with percentage of stevioside at a very low level (P=0.1*). As a higher leaf breadth corresponds to a higher leaf area indicating a higher chloroplast number, which is an organelle involved in the biosynthesis of stevioside (Yadav *et al*., 2011). But, in spite of this correlation, the winter grown plants were found to have a lower stevioside amount than the summer grown plants.

Conclusion

From the above study it was observed that in the summer season *Stevia rebudiana* var. Morita II showed promising results in the vegetative growth as well as in the concentration of stevioside. As this plant species possesses stevioside, the very important and potent compound from the point of human health, day by day the cultivation practices of *Stevia rebudiana* has been arising throughout the world including Assam. So, the cultivation time with the required environmental conditions are a matter of concern for the farmers to attain a higher yield of stevioside. From the present study, it can be concluded that summer season with a high light intensity for a longer period is suitable for the cultivation of *Stevia rebudiana* var. Morita II in Assam. This investigation hopefully will help the farmers for a better production of stevioside which in turn will contribute to the health sciences and economy of our country.

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