Enrichment of Stevioside and Rebaudioside A in *Stevia rebaudiana* through Optimal Nitrogen Fertilizer Application

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Abstract

Stevia rebaudiana, a member of the Asteraceae family, is widely recognized for its medicinal properties and its production of steviol glycosides, particularly Stevioside and Rebaudioside A. Optimizing the production of these compounds is crucial for providing intense sweetness with minimal caloric content, making Stevia highly popular in the food, beverage, pharmaceutical and agriculture industries. In this study, we investigated the effect of various concentrations (0, 200, 400, 600 and 1000 kg N/ha) of nitrogen (N) fertilizer on plant growth, biomass, chlorophyll content, carotenoid, stevioside and rebaudioside A. The ideal concentration of N fertilizer for plant growth was 200 kg N/ha, while higher concentrations had a detrimental effect on plant vitality.

Plant height was recorded as 26.04 cm and biomass of leaves showed 9.2 g fresh weight (FW) as well as 3.2 g dry weight (DW). Chlorophyll a, b and carotenoid were highest in lower node leaves and recorded as 9.1mg/g FW, 6.4mg/g FW and 1.3 mg/g respectively. High-pressure liquid chromatography (HPLC) analysis also revealed that the highest concentration of stevioside and rebaudioside A was observed in lower node leaves, followed by middle leaves, upper leaves, stem and root. This study provides important insights regarding the use of N fertilizers for the cultivation of this medicinal and industrially important plant.

Keywords: *Stevia rebaudiana*, nitrogen fertilizer, urea, Rebaudioside A, Stevioside.

Introduction

Stevia rebaudiana is a perennial herbaceous plant belonging to the Eupatorieae tribe within the Asteraceae family and is a notable producer of diterpenoid steviol glycosides (SG), particularly stevioside and rebaudioside A (Reb A). These compounds have low-calorie sweetening properties, making them 200-300 times sweeter than sucrose, also providing notable nutritional and pharmacological benefits[19]. The Food and Drug Administration (FDA) has classified *Stevia* as GRAS (Generally Recognized as Safe), according to Basharat et al[5]. Scientific research has consistently demonstrated the sweetening properties of *Stevia*, emphasizing its significance for cultivation[34]. Plants display diverse reactions to different forms of stress. While stress can decrease the overall yield in crops, it also enhances the production of bioactive compounds in therapeutic plants[30]. Multiple stressors, such as salinity, heat, cold, drought, oxidative stress and chemical fertilizers, enhance the production of secondary metabolites in plants[7,16]. Soil fertilizers, particularly nitrogen (N) fertilizers, are vital for controlling the development of plants as well as the formation of secondary metabolites[4,12].

Despite the critical role of agronomic methods in enhancing the yield of SG, there has been limited research on this aspect. N is essential for the production of nucleic acids, proteins and chlorophyll, thereby enhancing plant expansion, productivity and basic metabolism. Its effect on secondary metabolism, however, is less obvious[31]. N fertilizers have been demonstrated to boost biomass in *Stevia*[27], but it is still unclear how this affects SG synthesis. For example, Tavarini et al[32] found that after application of N fertilizers, leaves had higher levels of stevioside and reb A.

Furthermore, Pal et al[22] found that N fertilizers increased the amount of SG in leaves. Conversely, different growth stages or conditions have shown varied effects of N on SG synthesis [14]. This highlights the need for further research to elucidate the influence of agronomic practices, particularly application of N fertilizers, on the yield of SG in *Stevia*.

In this study, we carried out a pot experiment to investigate the mechanisms responsible for nitrogen use efficiency and uptake in *Stevia*, specifically in relation to SG synthesis. This study aimed to examine, how N fertilizer affected the growth and biochemical characteristics of the whole *Stevia* plant including the stem, roots and various leaf positions (upper, middle and lower nodes).

We hypothesized that optimizing the maximum nitrogen (N) uptake by the plant and enhancing its use efficiency could increase the steviol glycoside (SG) content in *Stevia*. This enhancement would likely be associated with changes in leaf growth and metabolic activity.

By understanding the relationship between nitrogen fertilizer use efficiency and SG synthesis, this research can contribute to optimizing cultivation practices, thereby enhancing the yield and quality of *Stevia* as a natural sweetener. The findings could have broader implications for enhancing sustainable agricultural practices and boosting the economic value of *Stevia* crops.

Material and Methods

Materials: Ethanol (SRL Pvt. Ltd., India), Methnol (Thermo Fisher Scientific Ltd.), Acetone (SRL Pvt. Ltd., India), Methanol (HPLC grade; SRL Pvt. Ltd., India), Acetonitrile (HPLC grade; SRL Pvt. Ltd., India), Water (HPLC Grade; SRL Pvt. Ltd., India) and Urea (Sigma Aldrich, Germany) were used.

Plant material and N fertilizer treatments: In order to examine the impact of N fertilizer on the yield of SG in *Stevia*, experiments were conducted in April 2024 at the field of study located in Gaya, Bihar, India (24.891689 N latitude; 84.860611 E longitude). The study was conducted in the Department of Biotechnology, Central University of South Bihar (CUSB). The experiment began with two-week-old SA-178 variety saplings of *Stevia*, obtained from Jamuna Biotech Farm in Pune, India. After planting into a peat-moss vessel, the saplings were placed in a greenhouse to select robust plantlets. After four weeks, cuttings of consistent size from these chosen plantlets were transferred and placed in individual pots measuring 15.5 cm x 13.5 cm. These pots were filled with sterilized potting soil with a pH range of 5.5 to 7.5 and contained 30% organic materials.

A single plant was placed in each pot and the soil was compacted to a depth of 2-3cm. In April 2024, the plants were transferred to an outdoor area. Throughout the period of plant growth, the average highest and lowest temperatures recorded were 29.9°C and 14.1°C respectively. The study employed different treatments of N fertilizer on *Stevia* plants including 0, 200kg, 400kg, 600 kg, 800kg and 1000 kg N per hectare (N/ha). The choices of N fertilizer and their concentrations were based on preliminary studies by Halitligil et al[11]. The N fertilizer levels were determined based on prior research that established the specific nutritional requirements of growing *Stevia* in an open-field environment.

A completely random block design was used, with three replicates of each of the five treatments. N fertilizer, in the form of urea, was administered in 18 separate applications, each occurring every 20 days throughout the vegetative growth phase. Watering was administered to facilitate the recuperation of the transplants and maintain the soil's moisture level at 80-85% of the pot's capacity during the entire experiment. This configuration enabled the evaluation of N fertilizer role in SG production within controlled yet realistic cultivation conditions.

Monitoring of physiological and morphological characteristics: Prior to harvesting, the height of the plant (cm) and its fresh and dry weights (FW and DW) were recorded for each component organ of the plant including upper node leaves (UnL), middle node leaves (MnL), lower node leaves (LnL), stems (S) and roots (R). The F/W was determined as the average weight per plant, while the DW was obtained by washing the plant materials with tap water, drying them in an oven at 55°C for one day and then weighing them. The leaves were then pulverized to make a finely ground powder, using a pestle and mortar, concealed in parchment paper, then kept in airtight bags at 4°C until further use.

20 days after treatment, whole plant organs (UnL, MnL, LnL, S, R) were collected for analysis of chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (Car) content. For this analysis, 100 mg of samples were blended in a mortar using 3 ml of 80% acetone. The mixture was then vortexed and filtered and 6 ml volume had been configured using 80% (v/v) acetone. The absorbance of Chl a, Chl b and Car was evaluated utilizing spectrophotometry at 663.0 nm, 645.0 nm and 440.0 nm respectively, applying the procedure outlined by Lichtenthaler[18]. The following equations were used to calculate the contents:

Chl A(mg/g) = (12.7*A 662) – (2.69 *A 645)*V/1000*W
Chl B (mg/g) = (22.9*A 645) – (4.68*A 662)*V/1000*W
Total carotenoid (mg/g) = [(A440+(0.114 A 663) – (0.638*A 645)*V/1000*W

where V is final chlorophyll extraction volume in acetone and W is fresh weight of the plant extract used.

Determination of Steviol Glycosides using Reverse Phase HPLC: Samples were taken from both control and treated plants to determine the amount of SG present within *Stevia* leaves. The samples were subjected to an HPLC analysis utilizing a similar altered version of the Ahmad et al[2] technique. The leaves were dehydrated at 45°C for approximately 48 hrs to produce a fine powder in order to prepare them for HPLC analysis. Then, 1 ml of high-purity (HPLC grade) methanol was combined with 20 mg of the powdered substance. For 1.5 hours, this mixture underwent sonication at 50°C in a water bath.

The samples were sonicated and then they were centrifuged for 10 minutes at 10,000 rpm. Prior to HPLC analysis, using a 0.22 µm syringe, filter carefully. ThermoFisher Vanquish HPLC system with C18 Accucore column (4.6*150mm, 2.6µm) set at 210nm wavelength was used for HPLC analysis.

A combination of water and acetonitrile in a ratio of 80:20 (v/v) made up the mobile phase. An isocratic a 1 ml/min flow rate was kept throughout the analysis to guarantee reliable and consistent target compound elution. In order to introduce a sufficient amount of extract and maintain ideal chromatographic conditions, the sample injection volume was set at 20 µl. Every analysis was conducted thrice and the outcomes were displayed as mean ± standard deviation. Using the Statistical Package for Social Sciences (IBM SPSS 2021) software, Two-tailed Student's t-test, $P < 0.05$. Data are shown as mean ± SEM to identify the significant differences in the variables using Chromelion 7.3 software to calculate the concentration of SG and the antioxidant content using Microsoft Excel 2008.

Results and Discussion

Impact of Nitrogen fertilizer treatments on growth, biomass, pigment content and SG accumulation in potted *Stevia* plants: In the current study, the impact of N fertilizer in the form of urea at concentrations of 0 (control), 200 kg, 400 kg, 600 kg, 800 kg and 1000 kg (N/ha) on the growth, biomass, chlorophyll and carotenoid content of *Stevia* was evaluated. In this study, we determined the upper limit on the effectiveness of using N fertilizer and its adoption in response to crop improvement.

Plant growth: The application of N fertilizer treatments has been observed to significantly impact both the development and growth of plants. Data analysis revealed a notable trend: concentration increased from 200 kg N/ha to 1000 kg N/ha, there was a corresponding decrease in plant height, along with detrimental impacts on the quantity of branches and leaves per plant. Interestingly, the treatment with 200 kg N/ha recorded a significantly higher plant height of 26.04 cm compared to the control at 22 cm after a 20-day treatment period.

Our results indicate that higher concentrations of N fertilizer significantly reduced plant height, while lower concentrations (200kg N/ha) were associated with increased height. These findings suggest a dose-dependent response of *Stevia* to N fertilizer levels, consistent with the principles of nutrient uptake and plant growth. At higher N concentrations, it is possible that N toxicity inhibited growth, leading to stunted plant height.

However, although N fertilizer greatly influences plant growth, too much N fertilizer can have detrimental effects on the aerial parts of the plant by negatively affecting the plant's root growth[17].

This phenomenon was also reported in *Stevia*, where excessive N fertilizer can lead to an imbalance in nutrient uptake and potential toxicity symptoms[13,26]. Rakesh et al[24] had additionally recorded an elevated amount of *Stevia* leaves and branches per plant with greater amounts N (263kg N/ha upto 300kg N/ha) application. Conversely, the increase in height observed at lower N fertilizer concentrations (60 to 105 kg N/ha) suggests that a moderate level of N fertilizer is beneficial for *Stevia* growth [20].

Plant Biomass: The way assimilation products (such as carbohydrates, proteins and other nutrients produced during photosynthesis) are distributed among the various plant organs, illustrates how each organ integrates and grows[35]. Within our research, we analyzed the cumulative fresh biomass yield of various organs of *Stevia* plants after 20 days of N fertilizer treatment based on survival concentration (Figure 1). The N fertilizer levels, particularly at 200 kg N/ha highest fresh biomass yield (LnL-9.5 g) and dry yield (LnL-3.29 g) were achieved and significantly influenced these parameters compared to the control treatment yielding significantly lower fresh biomass (LnL-9.0 g) and dry leaf yield (LnL-3.15 g) due to fewer branches and leaves per plant.

The biomass yield and nutritional value of *Stevia* were considerably enhanced by N fertilizers [35]. In our experiment, as compared to the control, the biomass yield (leaf, stem and root) was highest at a N fertilizer rate of 200 kg/ha. In support of this, similar experiments in Malaysia by Rosnani et al[26] demonstrated applying 250 kg/ha of N increased fresh and dry yield (leaf and stem only). Additionally, they reported a lower biomass yield under absolute control conditions.

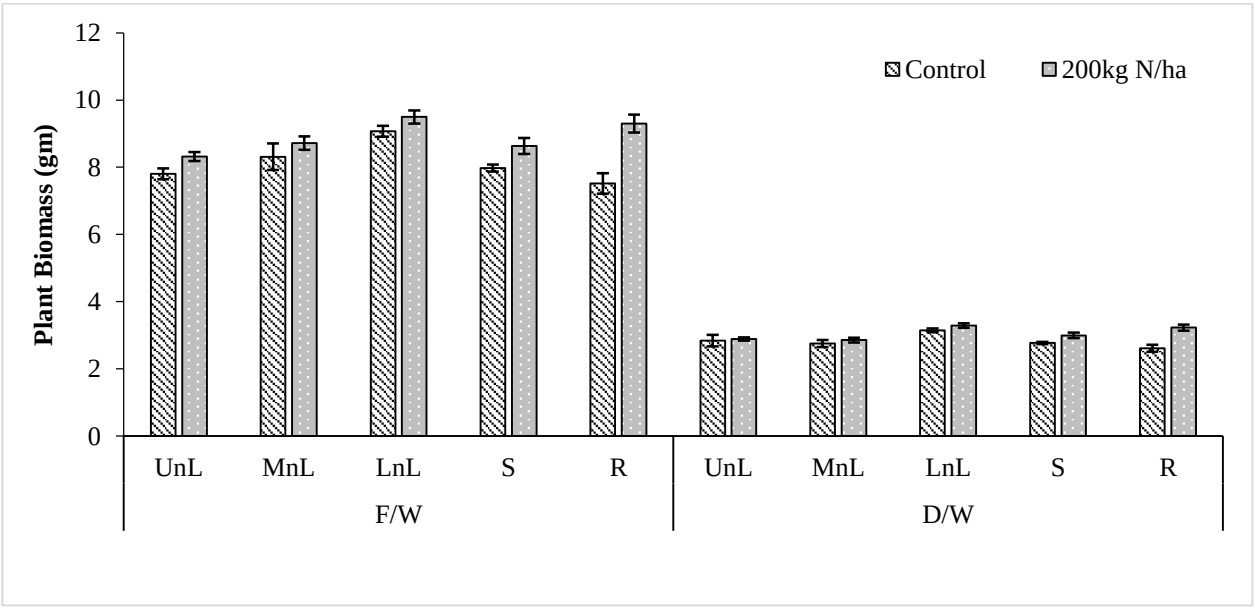

Figure 1: A -Fresh weight (FW) and B- Dry weight (DW) in *Stevia* plant under control and 200 kg N/ha. Upper node leaf(UnL), Middle node leaf(MnL), Lower node leaf(LnL), Stem(S), Root(R), control(C) and 200 kg N/ha. Two-tailed Student's t-test, P < 0.05. Data are shown as mean±SEM.

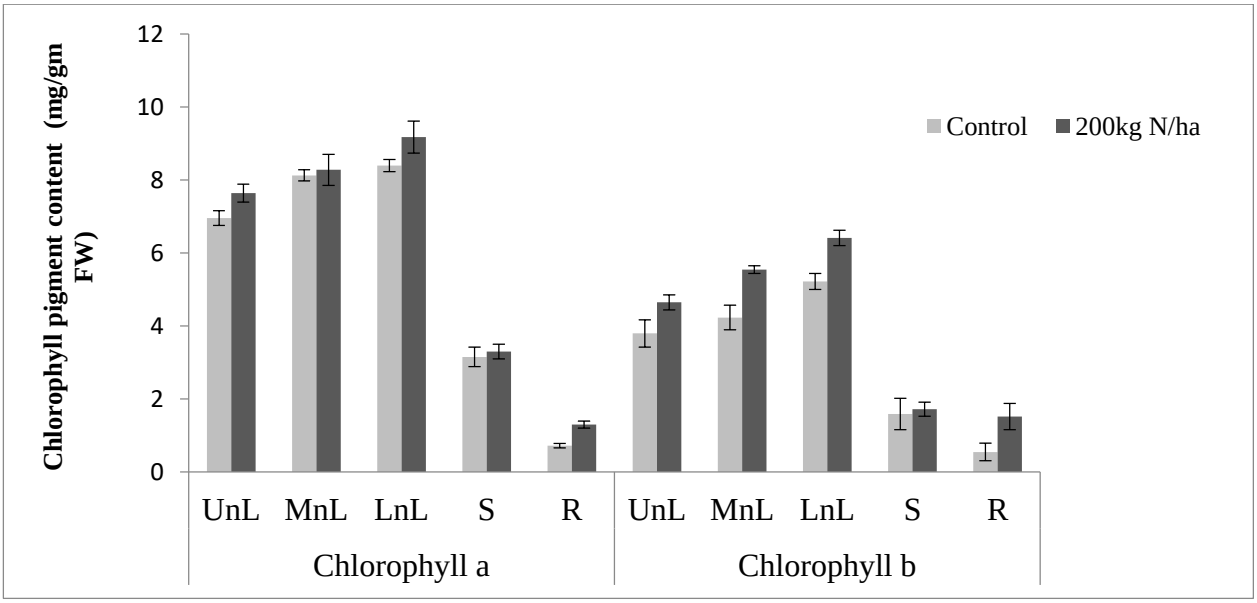

Figure 2: Chlorophyll pigment(mg/gm) FW of *Stevia* plant under control and 200 kg N/ha. Upper node leaf (UnL), Middle node leaf (MnL), Lower node leaf (LnL), Stem (S), Root (R). Two-tailed Student's t-test, P < 0.05. Data are shown as mean±SEM.

Ucar et al [33] also found with a dose of 200 kg N/ha, resulting in the maximum dry leaf yield. Fertilizers are absorbed by plants up to a certain point, after which there is no increase in biomass yield or plant productivity[1].

Conversely, Aladakatti et al[3] reported that the 300 kg N/ha and 400 kg N/ha N doses produced the highest dry leaf yield. In contrast to the other N levels and the control, plants produced at 60 kg N/ha made the maximum yield of dry leaves [25]. Here, the fertilizer doses have an impact on the final yield *Stevia* biomass. This could be because too much fertilizer inhibits plant growth and yield and it also depends on the cultivation method. Therefore, uptake of nutrients does not always result in higher biomass yield or higher plant productivity.

Chlorophyll pigment: The effect of different N treatments on chlorophyll pigments extracted from various plant organs was investigated and is presented in figure 2. After a 20-day period following the treatments, the maximum amounts of chl a and b were recorded with the application of 200kg N/ha (LnL-9.1 and 6.4 mg g^{-1} FW), followed by MnL (8.2 and 5.5 mg g^{-1} FW), UnL (7.6 and 4.6 mg g^{-1} FW), S (3.3 and 1.7 mg g^{-1} FW) and the lowest in R (1.3 and 1.5 mg g^{-1} FW). Conversely, a significant reduction was noted in the control group after 20 days, with chl a and b contents of 8.3 and 5.2 mg g^{-1} FW for LnL, 8.1 and 4.2 mg g^{-1} FW for MnL, 6.9 and 3.8 mg g^{-1} FW for UnL, 3.1 and 1.5 mg g^{-1} FW for S and the lowest in R at 0.7 and 0.5 mg g^{-1} FW respectively.

The amount of chlorophyll in the leaves of plants serves as a vital indicator of chloroplast development, photosynthetic capacity and overall plant vigor[22]. In this study, it has been observed that the concentration of Chl a is typically higher than that of Chl b, consistent with the general trend in most plant species[10]. The results are consistent with those of Maurya et al[21], who reported that the highest chlorophyll pigment levels in *Stevia* and biochemical parameters were achieved with the 200 kg N/ha treatment. Conversely, lower N fertilizer doses led to a significant decline in chlorophyll content.

Interestingly, Sniegowska et al[29] reported that the highest concentration of Chl b was obtained with 100 kg N/ha treatment, while lower N levels corresponded with the lowest concentrations. These differences can be attributed to the varying impacts of N fertilizer and the amount of sunlight received by the *Stevia* plants, with sunlight playing a more critical role in chlorophyll accumulation[29].

Carotenoid estimation: The application of 200 kg N/ha indicated no significant changes in carotenoid content in LnL recording the highest value of 1.3 mg g^{-1} FW followed by MnL and UnL with values of 1.1 and 0.8 mg g^{-1} FW respectively (Figure 3). S and R showed significant changes with values of 0.2 and 0.1 mg g^{-1} FW, in contrast to the control group. Conversely, other N fertilizer dosages were found to be ineffective in enhancing carotenoid levels. This trend was consistent across the first and second harvests of *Stevia* leaves, where N fertilizer concentrations between 50-200kg N/ha led to a marked increase in carotenoids, while lower concentrations resulted in decreased accumulation[6].

Similarly, Maurya et al [21] also reported the highest carotenoid accumulation in *Stevia* at the 200 kg N/ha dosage. Moreover, throughout the development of a plant, light intensity significantly impacts the concentration of carotenoids in the *Stevia* leaves. This relationship was highlighted in the study by Simlat et al[28] who demonstrated the effect of light intensity on carotenoid levels in *Stevia* plants.

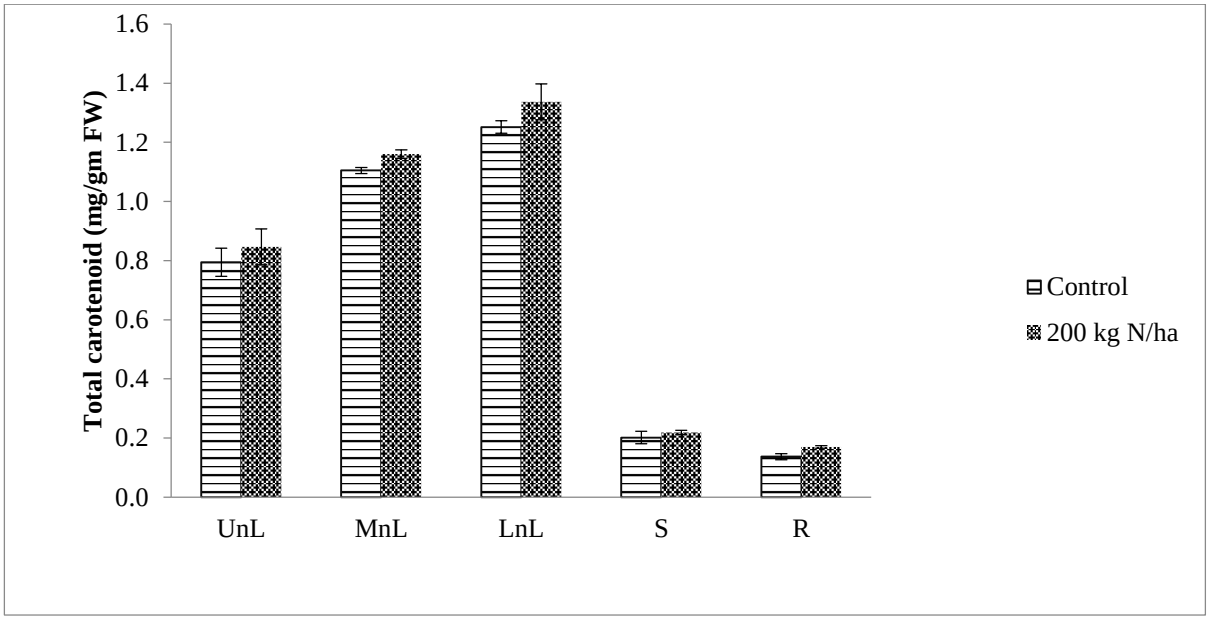

Figure 3: Total Carotenoid concentration of *Stevia* plant under control and 200 kg N/ha condition. Two-tailed Student's t-test, P < 0.05. Data are shown as mean±SEM.

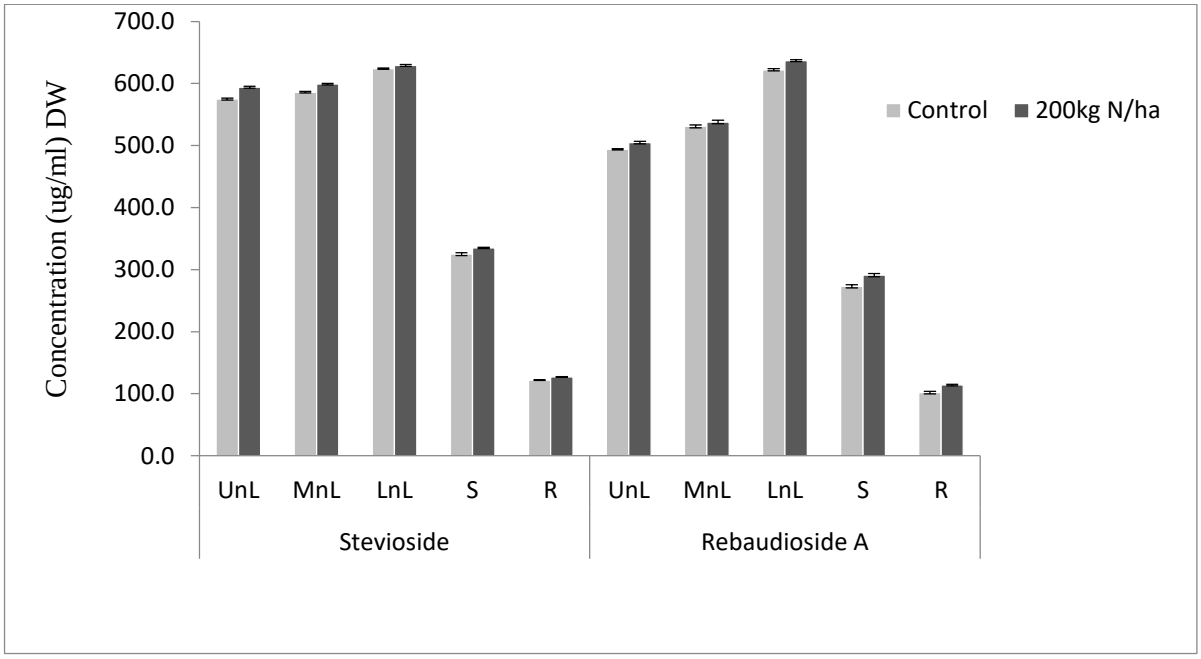

Figure 4: Effects of Nitrogen on: (A) Stevioside concentration(µg/ml) (B) Reb A (Rebaudioside A) concentration (µg /ml) of DW of different parts of plant. Two-tailed Student's t-test, P < 0.05. Data are shown as mean±SEM.

Stevioside and Rebaudioside A accumulation: The most well-known and prevalent SG in *Stevia* are stevioside and Reb A [17]. Figure 4 showed that in the control group, the leaves exhibited the highest Stevioside content (624 µg/ml DW for LnL, 586 µg/ml DW for MnL and 575 µg/ml DW for UnL leaves), whereas the stem and roots showed the lowest concentrations (325 µg/ml DW and 122 µg/ml DW, respectively). At 200 kg N/ha, noticeable variations were found between the leaves (629 µg/ml DW for LnL, 599 µg/ml DW for MnL and 594 µg/ml DW LnL), with a decrease in concentration noted in the stem (335 µg/ml DW) and roots (127 µg/ml DW) compared to the control. Similarly, for Reb A content, plants treated with 200 kg N/ha exhibited the highest levels in leaves (637 µg/ml DW for LnL, 538 µg/ml DW for MnL and 505 µg/mL UnL) followed by the stem (291 µg/ml DW) and root (114 µg/ml DW).

In comparison, slightly lower amounts of Reb A were found in the leaves under control conditions (622 µg/ml DW for LnL, 531 µg/ml DW for MnL and 494 µg/ml DW LnL respectively), with the lowest content observed in the stem (273 µg/ml DW) and root (102 µg/ml DW). SG is mostly biosynthesized in leaves under natural circumstances and transported to other plant organs[8,15]. Depending on the cultivation conditions and genotype, the stevioside content can range from 5 to 22% and Reb A from 22 to 61.6%[17].

Numerous elements influence the content of these metabolites in an *in vitro* setting, particularly biotic and abiotic elicitors [9].

On the other hand, not much is known about how N fertilizer affects SG biosynthesis in the stem, root and leaves of *Stevia* (UnL, MnL and LnL). According to our findings, *Stevia* leaves (UnL) were more concentrated than stems and roots because higher N rates (up to 200 kg N/ha) increased *Stevia* growth, which was followed by a slight decrease in these traits. This suggests that higher N rates were ineffective within the soil's medium used for *Stevia* harvesting. Our results are similar to those of Ingraha et al[13], who found that applying 250 kgN/ha of nitrogen fertilizer at each harvest significantly increased the Stevioside content in plants. Additionally, Buyuk et al[9] reported that the plants treated with mineral fertilizers (urea, 220 kg N/ha) possessed the most content as determined in the first (5.58%) and second (4.25%) harvests.

However, N levels had no effect on the leaf's major steviol glycosides, stevioside and Reb A [33]. This study included both the stem and the root whereas the SG yield above was significantly lower because it only recorded the leaf yield. Additionally, 100 and 150 kg N/ha were found to have significantly higher stevioside concentrations compared to other samples in *Stevia* plants cultivated in Poland, according to Sniegowska et al [29]. It is essential to recognize that plants can only absorb a limited amount of nutrients; above this threshold, there is no relationship between nutrient uptake and increased biomass output or plant productivity. It is essential to recognize that plants can only absorb a limited amount of nutrients; above this threshold, there is no relationship between nutrient uptake and increased biomass output or plant productivity[1].

Conclusion

This study comprehensively examined the effects of N fertilizer treatments on the growth, biomass, pigment content and SG accumulation in potted *Stevia* plants. Among the various N levels tested, 200 kg N/ha was found to significantly enhance plant growth, biomass yield, chlorophyll and carotenoid contents, as well as SG (stevioside and Reb A) accumulation, particularly in the leaves. The study highlighted a dose-dependent response to nitrogen, with higher N concentrations (above 200 kg N/ha) negatively impacting plant growth, especially plant height and biomass yield, due to potential nutrient imbalances and toxicity.

Conversely, moderate N concentrations, particularly 200 kg N/ha, were optimal for promoting healthy growth and maximizing the production of bioactive compounds in *Stevia*. It should be highlighted that further study is required to determine how *Stevia* growth generally responds to nitrogen rates because these results were obtained in an *ex vitro* setting and under specific soil conditions. Subsequent studies might explore further to understand the correct and most beneficial concentrations of urea between 0 kg N/ha and 200 kg N/ha. This comprehensive assessment offers novel perspectives on the function of N fertlizer in enhancing the bioactive compounds and overall health benefits of *Stevia*.

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