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Optimizing nitrogen fertilization for enhanced antioxidant and phytochemical profiles in *Stevia rebaudiana* Bertoni

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Abstract

Stevia rebaudiana Bertoni, along with its primary sweet constituents, stevioside and rebaudioside A, offers sweetness that is 250–300 times greater than that of sucrose while contributing negligible caloric content which makes this an important plant to cultivate. This research examined defining the upper limits of nitrogen (N) uptake and optimizing its use efficiency. Experiments were conducted in Bihar, India, to evaluate the effects of different dosages N concentrations on *Stevia rebaudiana*. The treatments are control and plants treated with N at 200kgN/ha. The study measured various antioxidant and phytochemical assay of leaves (segregated into upper node, middle node, and lower node), stem, root. It is concluded that 200kgN/ha is the optimal concentration for enhancing stevia physiological health.

Key words : *Stevia rebaudiana*, nitrogen, urea, antioxidant activities, phyrochemical activity.

Stevia rebaudiana Bertoni, a perpetual herb from the Eupatorieae tribe belongs to Asteraceae family). Due to their low-calorie sweetening properties[12], 200–300 times sweeter than sucrose and offer significant nutritional and pharmacological advantages it is important commercial medicinal plant. Additionally, food-derived antioxidants, particularly phenolic, phytochemicals and vitamins, have gained increasing attention as these agents prevent oxidative damage through chemotherapy[10]. Generally, plants have a strong antioxidant defence system comprising both enzyme- and non-enzyme-based antioxidants in an effort to mitigate the oxidative damage that ROS cause. This system not only counters oxidative stress but also stimulates the production of secondary metabolites[17]. Flavonoid and total phenolic compound analyses are important methods for assessing antioxidant activity, as are 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP), which are non-enzymatic antioxidants[3,13,14,19]. Given the medicinal properties and growing use of *S. rebaudiana,* investigating its antioxidant properties is of significant importance[18]. The purpose of this study was to examine, for the

first time, how N affected the antioxidant and phytochemical characteristics of the whole Stevia plant, including the stem, roots, and various leaf positions (upper, middle, and lower nodes).

Material :

DPPH (2, 2-diphenyl-1-picrylhydrazyl), Ferrous sulphate ($FeSO_4$), Potassium Ferricyanide, Trichloroacetic acid, ferric chloride ($FeCl_3$), Gallic acid, Folin–Ciocalteu (FC) reagent, sodium carbonate (Na_2CO_3), 2,4,6-Tris-(2-pyridyl)-s-triazine (TPTZ), Quercetin, Aluminum chloride ($AlCl_3$), Hydrochloric acid (HCl), Urea ($CO(NH_2)_2$)were acquired from, SRL Pvt. Ltd., Thermo Fisher Scientific Ltd. (Waltham, MA, USA) HiMedia Laboratories, India and Sigma-Aldrich (St. Louis, MO, USA).

Experimental conditions, plant material and N treatments :

To investigate how nitrogen affects the yield of SG in *S. rebaudiana,* the trials were carried out at the field of study in April 2024 for the growing season of the Department of Biotechnology, Central University of South Bihar (CUSB), Gaya, Bihar, India (24.891689 N latitude; 84.860611 E longitude). The SA-178 variety of *S. rebaudiana* Bertoni saplings, sourced from Jamuna Biotech Farm in Pune, India, were two weeks old at the start of the experiment. The saplings were first kept in a greenhouse after being planted into a peat-moss vessel to choose well-established plantlets. The plants were relocated to an outdoor space in April 2024. The Various N treatments were used to stevia plants: N0 (no N fertilization) and 200kgN/ha of N. These N levels were based on previous studies that defined the nutritional needs of growing stevia under open field. N, as urea, was applied in days during the vegetative growth phase. After 20 days, harvesting was done, the antioxidant and phytochemical study were monitored 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).

Methanol extraction for estimation of antioxidant activities :

S. rebaudiana the preparation of leaf extracts began with letting the leaves dry and then grinding them into a fine powder. 10ml of this powder, which comes from plants grown in different amounts of N, were dissolved in 1ml of methanol for every sample. After 5min of vortexing, the mixture was subjected to 30min of sonication. After that, the mixture was centrifuged for 15 minutes at 10,000 rpm to obtain the supernatant, which was then used for assays of antioxidants as per Javed *et al*.,[8]. Prior to additional analysis, the extract was filtered using 0.22 µm filters. The response parameters evaluated included total phenolic content (TPC; µg GAE/ml DW), total flavonoid content (TFC; µg QE/ml DW), total antioxidant activity (TAA; assessed via DPPH radical scavenging activity), and Ferric Reducing Antioxidant Power (FRAP; µg Fe^{2+} /ml DW).

Diphenyl-1-picrylhydrazyl (DPPH) :

The solvent and DPPH solution alone served as the control. The extract was combined with 1.5 ml of a 0.1mM DPPH solution after 30 min of dark incubation period at room temperature, the sample absorbance at 517 nm was determined[5,9].

Ferric reducing antioxidant power :

To determine the FRAP assay, a fresh Acetate buffer (0.300M, pH 3.6), 1.25 ml of ferric chloride (0.02M), and 1.25 ml of TPTZ (0.04M) were combined to create the FRAP solution. Subsequently, 1.5 ml of the resultant solution was combined with 200 µl of the extracted sample extract, and the entire mixture was incubated at 37°C for 30 minutes. Following that, the absorbance at 593 nm was calculated using FRAP values expressed as µg Fe^{2+}/ml [4].

Phytochemical assay -

Total phenolic content :

The extract's TPC was measured by combining 0.5 ml of the extract with 2.5 ml of 7.5% sodium carbonate and a 10-fold diluted Folin-Ciocalteu (FC) reagent. After 30 minutes of incubation at 25°C, the absorbance measured at 765 nm using a spectrophotometer. To establish a standard curve, gallic acid at concentrations that vary from 10 to 100 µg/ml was substituted for the extract. TPC was expressed as ug GAE/ml DW based on the standard curve[9,16].

Total Flavonoid content) :

TFC of stevia plant samples was assessed following the method outlined by Tavarini *et al*.,[19]. Next, 100 µL of potassium acetate (1M), 100 µL of aluminium chloride (10%), and 1.60 ml of distilled water was added to the 200ul representative extract. The final mix was then incubated for thirty minutes at room temperature. Using a spectrophotometer, every sample's absorbance was measured at 645 nm. Quercetin equivalents (µg QE/ml) were utilised to express the results, with QE serving as the standard.

Antioxidant activities :

Current antioxidant assays not only give a thorough representation of the overall antioxidant capacity of a plant extract, but they also ought to take into account both lipophilic and hydrophilic capacities and differentiate between the processes of transfer of hydrogen atoms (radical quenching) and transfer of electrons (radical reduction). Therefore, a combination of assays measuring these individual capacities is necessary to thoroughly assess a sample's ROS scavenging ability. To address this, both the (FRAP) and (DPPH) assays were conducted (Figures 1 and 2). The interaction of N levels significantly influenced the antioxidant activity determined by both the DPPH and FRAP assays. Notably, the highest antioxidant potential, and as a result, the highest concentration of bioactive compounds were found in the treatments receiving 200 kg N/ ha, including LnL (61% and 94.7 mmol Fe^{2+} µg/ml DW), MnL (59.3% and 85.9 mmol Fe^{2+} µg/ml DW), UnL (58.800 kg N/ha and 83.9 mmol Fe^{2+} µg/ml DW), S (60 kg N/ha and 53.2 mmol Fe^{2+} µg/ml DW), and R (57.0 kg N/ha and 51.6 mmol Fe^{2+} µg/ml DW), compared to the control treatments, LnL (60 kg N/ha and 92.2 mmol Fe^{2+} µg/ml DW), MnL (58.400 kg N/ha and 80.2 mmol Fe^{2+} µg/ml DW), UnL (56.1% and 78 mmol Fe^{2+} µg/ml DW), S (58.9% and 43.5 mmol Fe^{2+} µg/ml DW), and R (54.80 kg N/ha and 33.4 mmol Fe^{2+} µg/ml DW) for FRAP and DPPH, respectively. This approach allows for a more comprehensive evaluation of the antioxidant capacity of plant samples, providing valuable insights into the effects of N levels on the production of bioactive compounds with antioxidant properties.

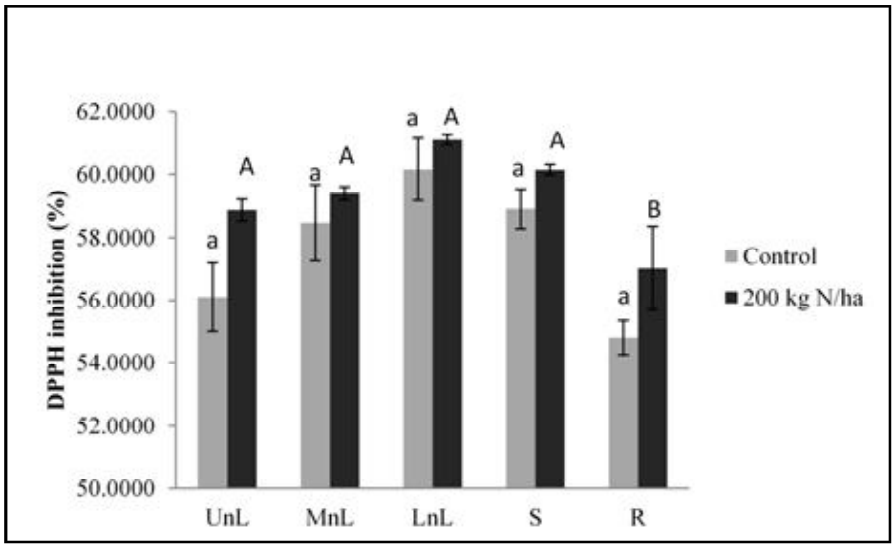

Figure 1: Percent (%) of scavenging activity, 2,2-Diphenyl-1-picrylhydrazyl (DPPH)at different parts of plant in *S rebaudiana* subjected to controls and 200 kg N/ha. One way ANOVA Values represent mean ± standard error, followed by same letters are not significantly different according toTukey's post hoc test (5%), conducted at $P < 0.05$, were utilised for statistical analyses to determine significant variations in stevia treatment and control conditions

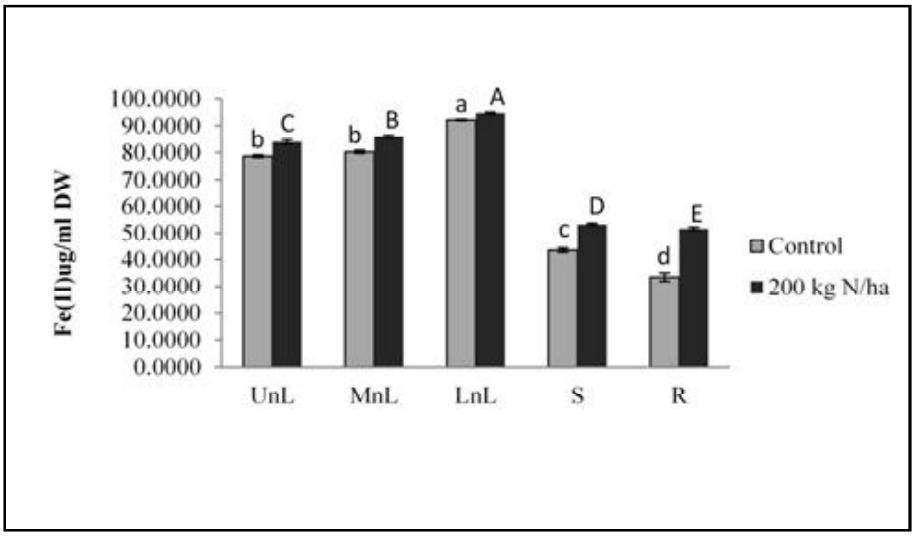

Figure 2. Ferric reducing antioxidant power (FRAP) µgFe(II)/ml of D/W at different parts of plant in *S rebaudiana* subjected to controls and 200 kg N/ha.

Phytochemical accumulation :

As with antioxidant assays, N rate had a substantial impact on total phenols and total flavonoids (Figures 3 and 4). Interestingly, the distribution of other nutrients among plant organs showed variability. After a 20-day treatment period, the average levels of phenols and flavonoids across different N treatments were notably higher in plants treated with 200 kg N/ha compared to those treated with lower N concentrations (LnL: 79 µg GAE/DW and 42.2 µg QE/DW; MnL: 48.2 GAE/DW and 35.72 µg QE/DW; UnL: 28.2 GAE/DW and 18.32 µg QE/DW; S: 5.6 µg GAE/DW and 9.82 µg QE/DW; R: 4.4 µg GAE/DW and 6.8 µg QE/DW). This suggests that the application of 200 kg N/ha positively influenced the accumulation of these secondary metabolites, particularly in the lower nodes, possibly due to the larger leaf size associated with this treatment. Furthermore, when comparing the N0-untreated control plants with those treated with 200 kg N/ha, it was evident that the untreated plants exhibited significantly lower phenolic and flavonoid contents (LnL: 50.5 µg GAE/DW and 34.7 µg QE/DW; MnL: 45.8 µg GAE/DW and 32.9 µg QE/DW; UnL: 27.4 µg GAE/DW and 12.8 µg QE/DW; S: 4.8 µg GAE/DW and 8.4 µg QE/DW; R: 3.3 µg GAE/DW and 5.6 µg QE/DW). This indicates that nitrogen supplementation, particularly at the 200 kg N/ha level, significantly enhanced the accumulation of these important bioactive compounds in all plant organs, with a pronounced effect in the lower nodes.

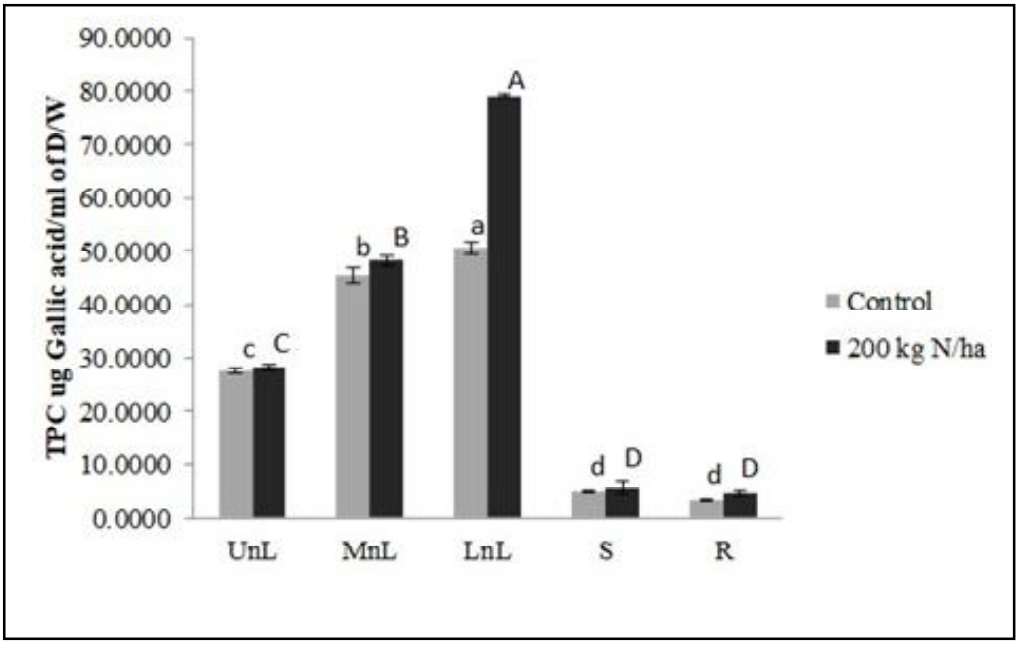

Figure 3. Total phenolic content (TPC) µg GAE/ml of DW at different parts of plant in *S. rebaudiana* subjected to controls and 200 kg N/ha .

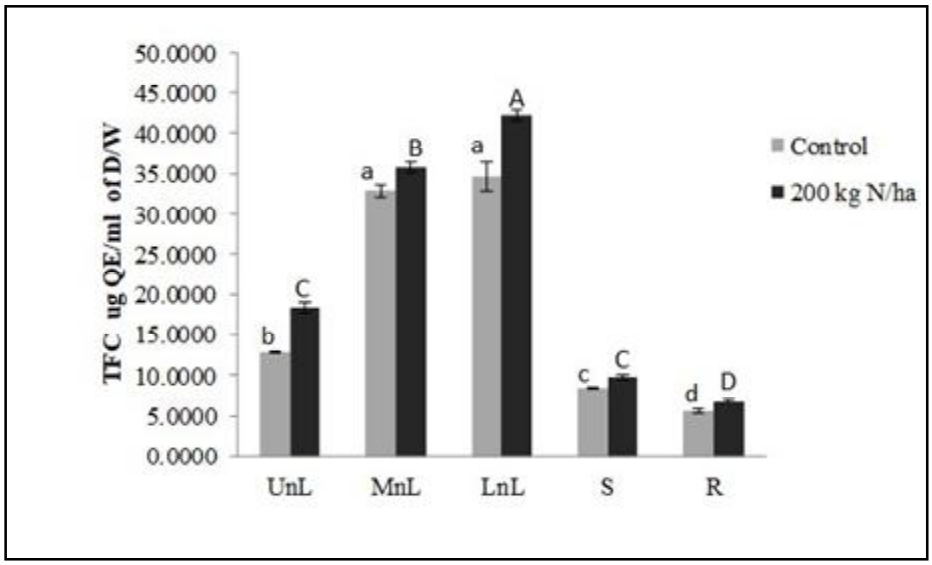

Figure 4. Total Flavnoid content (TFC) µg QE /ml of dry weight at different parts of plant in *S. rebaudiana* subjected to control and 200 kg N/ha N. Quercetin (QE).

Effect of N on Antioxidant assay :

The antioxidant activity, evaluated through the radical scavenging rate towards DPPH, demonstrated that an application of 200 kg N/ha significantly enhanced this protective function, while lower dosages were ineffective in this regard. This finding aligns with previous research on *Cymbopogon citratus*, where crucial oil yield and citrus acid content that were highest was achieved 150 days post-transplant at a N concentration of 200 kg/ha. Correspondingly, both DPPH and FRAP assays indicated peak antioxidant activity at this N level. Ahmad *et al*.,[1] similarly reported increased antioxidant activities in sorghum leaves with rising N doses, specifically at 150 kg N/ha and 3 kg N/ha. In another study, N fertilizer rates were found to significantly influence the antioxidant activity in Mas cotek, when compared to stems and roots, showed greater antioxidant activity in leaves across all treatments. Notably, Sheikh *et al*.,[17] observed that the 100 kg N/ha treatment revealed the highest DPPH antioxidant capacity in all plant parts. It is worth noting that plants tend to accumulate higher levels of phytochemicals and antioxidant compounds under mild stress conditions, such as those induced by lower fertilization rates during development[11]. This phenomenon suggests that moderate nutrient stress might stimulate the process by which plants respond adaptively by producing bioactive substances.

Effect of N on Phytochemical content :

Within this study, TPC and TFC increased up to the N200 concentration, with no further increase in phytochemical concentrations beyond this point. This observation aligns with findings from other studies. For instance, Bukhori *et al*.,[6] reported a significant decrease

in TPC values in Gynura procumbens under different N treatments, following a descending order of N60 > N90. This phenomenon can be attributed to the fact that low N supply enhances phenolic content, as sustained low N levels continuously stimulate phenolic accumulation in *Kacip Fatimah*. Over extended periods, low N availability has been shown to upregulate the phenolic biosynthetic pathway, reported that reside in content of TPC.[2]. Similarly, Bukhori *et al*.,[6] observed a significant decrease in TFC values in Gynura procumbens with different N treatments, following the descending order of N60 > N90, while the control condition showed increased TFC. This outcome can be explained by the role of N availability in modulating phytochemical responses in plants. For example, Galieni *et al*.,[7] found that Lack of N causes flavonoid and flavonol content in lettuce plants. Furthermore, Pal *et al*.,[15] demonstrated that N deficiency triggers an increase in these compounds, highlighting the intricate relationship between N supply and phytochemical synthesis. Thus, the findings of this study underscore the significant impact of N availability on the estimation of phenolic and flavonoid compounds in stevia plants.

The results demonstrate that optimal concentration of urea for promoting stevia plant growth and survival was determined to be 200 kg N/ha. 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 exvitro setting and under specific soil conditions.

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Conflict of research: None

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