Plant and Soil Science

Journal homepage: https://agriculturalscience.com.ua/en
*Plant and Soil Science, 14(2), 57-69*
Received: 28.12.2022 Revised: 28.03.2023 Accepted: 26.04.2023

UDC 631.8 DOI: 10.31548/plant2.2023.57

**Iryna Smetanska***
Doctor of Agricultural Sciences, Professor
University of Applied Sciences Weihenstephan-Triesdorf
91746, 16 Markgrafenstrasse Str., Weidenbach, Germany
https://orcid.org/0000-0003-2298-2888

**Mamdouh Diaa**
Master of Biological Science, Assistant Professor
University of Applied Sciences Weihenstephan-Triesdorf
91746, 16 Markgrafenstrasse Str., Weidenbach, Germany
https://orcid.org/0000-0003-1379-8894

**Gruda Nazim**
Doctor of Agricultural Sciences, Professor
Humboldt University of Berlin
10117, 6 Unter den Linden Str., Berlin, Germany
https://orcid.org/0000-0002-2663-1594

**Mykola Patyka**
Doctor of Agricultural Sciences, Professor
National University of Life and Environmental Sciences
03041, 15 Heroyiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-2506-8699

**Oksana Tonkha**
Doctor of Agricultural Sciences, Professor
National University of Life and Environmental Sciences
03041, 15 Heroyiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-0677-5494

# Impact of nutrient supply on growth and synthesis of metabolites of *in vitro* shoot cultures of *S. rebaudiana*

**Abstract.** *Stevia rebaudiana* plants produce sweet tasting compounds, steviosides, which are 300 times sweeter as sugar and can be used as natural sweeteners. Moreover, stevia leaves contain high amount of antioxidants. The use of plant compounds as components of functional nutrition and nutraceuticals is extremely relevant today. The purpose of this study was to investigate the

**Suggested Citation**:
Smetanska, I., Diaa, M., Nazim, G., Patyka, M., & Tonkha, O. (2023). Impact of nutrient supply on growth and synthesis of metabolites of *in vitro* shoot cultures of *S. rebaudiana*. *Plant and Soil Science,* 14(2), 57-69. doi: 10.31548/plant2.2023.57.

*Corresponding author

effect of increased nitrogen and saccharose supply on growth and accumulation of steviosides and anthocyanins as well as antioxidative activity in fast-growing shoot cultures of *S. rebaudiana.* The morphological changes of the leaves, the accumulation of steviosides and anthocyanins, and the antioxidant properties of the extracts were determined. It was shown that during three weeks of *in vitro* cultivation of shoots on MS medium with double concentration of nitrogen, the biomass increased by 71.0%, after application of double concentration of saccharose – by 133%, and after increasing nitrogen in combination with saccharose supply – by 162.0% compared to the control. Sucrose stimulated the accumulation of biomass. Anti-oxidative potential after nitrogen application was 2.4 times higher than the control, and with saccharose supply it increased 2.7 times. Shoots treated with increased nitrogen and saccharose concentration contained 50.7 and 57.8 mg/g$^{-1}$ steviosides, respectively. However, the combination of nitrogen and saccharose led to accumulation of 73.4 1 mg/g$^{-1}$ steviosides. Shoots grown on MS medium culture had 26.0 mg/g$^{-1}$ of steviosides. The content of anthocyanins was 1.7 times greater under added nitrogen supply, and 2.3 times greater after the application of nitrogen and saccharose. Optimum cultivation media developed individually for each *in vitro* culture increase the production of valuable plant secondary metabolites up to 3 times

**Keywords:** plant biomass; cultivation; nutrient medium; secondary metabolites; steviosides; anthocyanins; antioxidative activity

## INTRODUCTION

Many health issues and complications – from cardiovascular diseases, bowel, and dental issues to cancer – have been associated with obesity and excessive consumption of free sugars. According to WHO recommendations, consumers must reduce daily free sugars intake to less than 10% of their total calorie intake (WHO Guideline, 2023). As consumers become more aware of the health benefits of low-calorie foods, the market for these products is expected to grow rapidly (Mordor Intelligence, 2022). Artificial sweeteners are the predominant sugar substitutes in the market to produce low-energy products. In addition, due to the COVID-19 pandemic, people started to show more interest in natural non-caloric sweeteners, such as stevia (Emergen Research, 2022). *Stevia rebaudiana* plant, commonly known as stevia, has been used by South Americans for thousands of years.

Steviol glycosides are sweetening compounds from the leaves of the *S. rebaudiana* plant. However, some steviosides are characterized with bitter aftertaste (Nguyen & Tan, 2021). The technological advantage of steviosides is the low-cost extraction technology which reduces the production costs (Al-Taweel *et al.,* 2021). Steviosides are not only non-caloric sweeteners of natural origin but also are suitable to use in the food industry due to their technological characteristics. The attributes of being heat and pH stable, water and alcohol soluble provides a technological advantage (Raspe & Silvio, 2022).

*S. rebaudiana* is the sweetest species of the genus *Stevia* which involves about 150 species of herbs. They belong to the *Asteraceae* family also known as daisy or sunflower family (Nguyen *et al.,* 2019). *S. rebaudiana* is an herbaceous, semi-bushy, perennial shrub which grows in humid environments at an average temperature of 24°C. The plant is native to Paraguay and some parts of Brazil. For at least 1,500 years, stevia leaves have been used in the cuisine of Guarani tribes on a daily basis. Dr. Bertoni rediscovered this plant by the end of 19$^{th}$ century.

Steviol glycosides are mainly found in plants, including stevia. The molecular structure of steviol glycosides is described as a sugar molecule bound to a non-carbohydrate moiety. The chemical names of individual sweet tasting substances from stevia plants depend on the type of sugar they contain, e.g., glucosides (glucose), pentosides (pentose), fructosides (fructose), etc. The substances can be converted into sugar and non-sugar compounds, the so-called aglycones, by hydrolytic cleavage (Jawad *et al.,* 2022). Steviol occurs in the plant as steviol glycosides, which are sweet compounds.

Moreover, stevia plants hold a high content of polyphenols, including anthocyanins. Polyphenols are the secondary metabolites that

can act as free radical scavengers. However, the seeds of this plant have extremely low germination rate (Shevchenko *et al.,* 2010). A promising method of obtaining *S. rebaudiana* biomass with a high and stable content of steviosides and phenolic compounds, free from plant protection agents, from mycotoxins that can be synthesized during unfavourable plant storage conditions, is the application of *in vitro* techniques. Additionally, synthesis of metabolites in *in vitro* cultures can be regulated through nutrient supply. Due to the slow growth often encountered in plant cell cultures, inducing *in vitro* shoot culture of *S. rebaudiana* has been proposed.

The hypothesis of the study is that application of certain nutrients as nitrogen and saccharose can induce biomass accumulation of *in vitro* shoot culture and influence synthesis of valuable secondary metabolites. The purpose of this study was to identify the role of nitrogen and saccharose on growth and biochemical composition of *in vitro* shoot cultures of *S. rebaudiana*.

## LITERATURE REVIEW

Numerous protocols for micropropagation of *Stevia rebaudiana* have been established (Ramírez-Mosqueda *et al.,* 2016). Nonetheless, for the past few decades, the high expenses incurred in micropropagation compared to that seen in traditional field cultivation have been significant limiting factors (Amien *et al.,* 2022). The latest developments in plant *in vitro* techniques have led to a successful transition from the experimental stage to industrial-scale production. Cultivation of plant differentiated *in vitro* systems from last year is believed to be an effective method for generating valuable natural compounds (Hassanein, 2022). Numerous research studies have investigated the effect of nitrogen concentration in cultivation media on the growth and morphogenesis of tissue cultures. According to J. Wang *et al.* (2020), the concentration and chemical form of nitrogen were deemed as highly significant.

*In vitro* cultures typically consider nitrate as the most essential form of nitrogen. Yet, in many instances, the application of nitrate only as a nitrogen source has not resulted in success. Tissue cultures require ammonium as a source of nitrate ion, according to P. Olgunsoy *et al.* (2017) findings on the *Rosa sp.* callus culture cultivation. Research conducted by T. Wu *et al.* (2021) has demonstrated that cell cultures of *Nicotiana tabacum* can flourish on cultivation medium containing solely ammonium. However, the resulting accumulation of biomass was only half as much as that with a combination of ammonium and nitrate sources. Meanwhile, S. Alvarez & J.R. Motos (2023) noted that dicarboxylic acid must also be included to promote *in vitro* cultures' growth if the cultivation medium solely depends upon ammonium as a nitrogen source. M.M. El-Dawayati & Z.E. Zayed (2017) hypothesized that the suitable concentrations of ammonium and nitrate ions in culture media for *in vitro* cultures should be selected at 10:15 (825:1425 mg/L) of the MS culture. In experiments on hairy root cultures of *Psoralea corylifolia*, M. Zaheer *et al.* (2016) demonstrated that the uptake of ammonium and nitrate was dependent on the pH of the cultivation medium. Under acidic pH, nitrate was utilized more intensively, leading to an increase in the alkalinity of the cultivation medium. The uptake of ammonium led to a shift in the pH of the medium towards acidity, which further inhibited the uptake of ammonium by the cultures.

Plant *in vitro* cultures are mostly heterotrophic, meaning that they use monosaccharides or disaccharides as a carbon source. According to T. Fidemann *et al.* (2018), the concentration of saccharides in culture media essentially influences both the growth of biomass and the synthesis of secondary metabolites in *in vitro* cultures. To provide a source of carbohydrates, sucrose or glucose is usually added as a carbon source in concentrations ranging from 2 to 4%. Certain cultures require fructose or maltose as a source of sugar. The selection of the most efficient source of carbon and its concentration depends on the species of the plant, as well as the form of its *in vitro* cultivation and the objective of the cultivation, such as the synthesis of alkaloids or phenolic compounds. K. Ibrahim & H. Musbah (2018) discovered that *Coleus blumei* cell cultures, when subjected to a saccharose concentration of 2.5% in the cultivation medium, produced 0.8 $g/L^{-1}$ of rosmarinic acid, but after increasing the saccharose concentration to 7.5%, the yield of rosmarinic acid reached 3.3 $g/L^{-1}$.

Currently, many research groups worldwide are focusing on cultivating differentiated shoot

*in vitro* cultures. Shoot cultures are characterized by intensive growth and metabolite synthesis due to their tissue differentiation. According to M. Kikowska *et al*. (2020), the biomass accumulation in the *in vitro* shoot culture of *Eryngium alpinum* L. was 12.5 times higher than the biomass growth in callus cultures. A. Król *et al*. (2021) have also demonstrated the feasibility of effectively utilizing *in vitro* shoot cultures from various plants for producing phenolic compounds and antioxidants. *In vitro* shoot cultures are particularly valuable due to their capability of plant tissue differentiation, which contrasts with undifferentiated cell and root *in vitro* cultures, making them capable of producing metabolites that are not present in the latter. Equally important, the growth of shoot cultures is often more intensive when compared to other *in vitro* cultures and, in many instances, even compared to their native counterparts.

## MATERIALS AND METHODS

Introduction of *in vitro* shoot cultures. The surface of *S. rebaudiana* seeds was sterilised for 5 minutes using 70% ethanol, followed by 10% sodium hypochlorite for 10 minutes and rinsed three times with distilled water. The explants (leaf, nodal, and inter-nodal segments) were cut into small pieces of approximately 0.1 cm in length, one week after germination. The pieces were transferred to 250 mL Erlenmeyer flasks containing 50 mL of liquid MS medium and 0.4 mg/L kinetin. The cultures were grown at a temperature of 25°C on a rotary shaker (75 rpm), under illumination of 18:6 (3000 lx). An autoclave, a sterile cabinet, and an incubator (VWR, Germany) were utilized for the *in vitro* cultivation process. To prepare the material for chemical analysis, the following devices were used: ALPHA 1-4 lyophilic dryer (Christ, Germany), Memmert thermal cabinet, Eppendorf centrifuges (without and with cooling), vacuum concentrator Speed Vac SC110, ultrasonic bath, and ultrasonic homogenizer.

*Propagation medium*. The growth and development of stevia cultures were tested for their susceptibility to four different propagation medium compositions. A standard nutrient MS medium, containing 1.90 g/L$^{-1}$ $KNO_3$, 1.65 g/L$^{-1}$ $NH_4NO_3$, and 30 g/L$^{-1}$ saccharose, with 2% agar, was used as a control. The experiment tested modifications of the MS medium with double the concentration of nitrogen (3.80 g/L$^{-1}$ $KNO_3$ and 3.30 g/L$^{-1}$ $NH_4NO_3$), 60 g/L$^{-1}$ saccharose, and twice the concentration of nitrogen and saccharose (3.80 g/L$^{-1}$ $KNO_3$, 3.30 g/L$^{-1}$ $NH_4NO_3$, and 60 g/L$^{-1}$ saccharose). Cultivation media was autoclaved at 121°C. Vitamins were added after autoclaving, using microfiltration through sterile 0.25 µm disc membrane filters (Membrane Solution, USA).

The experiments were conducted as randomized controlled factorial trials using *S. rebaudiana* seeds. The measurement results were analysed using the ANOVA method – a statistical technique for variance analysis.

*Measurement of anthocyanins*. To the *S. rebaudiana*, 100 mg was added, and subsequently 750µl of $CH_3COOH$/EtOH was also introduced. For a period of 20 minutes at 85°C, the sample was placed on the block heater. Subsequently, the mixture was centrifuged for 5 minutes at 12,000 rpm and the supernatant was collected. The given extraction procedure was repeated three times, and all the supernatants were subsequently pooled together. To each sample, 50 µl 37% HCl was added and vibrated on the vortex for 30 seconds. λ=535 nm was used for photometric determination of anthocyanin.

*Analysis of steviosides*. Stevioside content measurements were made according to Y. Shevchenko *et al*. (2010), with some modifications. One gram of *S. rebaudiana* biomass was ground to a powder in a ball mill. Subsequently, 25 mL of distilled water was added. The samples were heated to 95°C in a microwave oven for 30 minutes, then cooled to 25°C and filtered using filter paper. This process was performed three times, and the extracts were combined in a graduated flask. The total volume was then adjusted to 100 mL using distilled water. To clean 0.5 ml of the extract, a solid-phase-extraction column was used. The extract was washed with 3 ml of methanol followed by 3 mL of distilled water. The final elution was achieved by adding a mixture of methanol and acetonitrile in a 50:50 (v/v) ratio to the SPE column using 1 mL of the said mixture. Subsequently, the eluted solution was analysed using HPLC, following the methodology of Y. Shevchenko *et al*. (2010).

*Determination of antioxidant activity*. The DPPH radical scavenging activity assay was conducted following A.A. Mohdaly *et al*. (2011) method. To obtain an absorbance value of 0.7±0.02 at 515 nm, 100 mL of methanol was mixed

with 6 mL of the stock solution to prepare DPPH solution ($6\times10^{-5}$ mol/L$^{-1}$). 0.02 g of the sample was mixed with 1 mL of distilled water and heated at 95°C for 15 minutes. Afterward, the sample was centrifuged at 12000 rpm for 5 minutes. 5 µL supernatant with 2 mL DPPH was vortexed for 30 s and allowed to react for 30 min. The absorbance at 515 nm was measured using the UV-Vis Spectrophotometer Jena-Specol 2100. The scavenging activity was calculated using the following formula:

$$DPPH\ (\%)=[(Abs.control - Abs.sample)/Abs.control] \times 100, \quad (1)$$

where Abs – is the absorbance at 515 nm.

The study complied with the requirements of the Convention on Biological Diversity (1992).

## RESULTS

*In vitro* shoot cultures of *S. rebaudiana* were established from seeds as described above. After germination, the shoots were transferred into a sterile medium and cultivated for 21 days. The development of shoots cultivated on the MS medium is presented in Figure 1.

The effect of nutrient composition in cultivation media on the dynamic biomass growth of sprout cultures was investigated.


**Figure 1.** *In vitro* shoot culture growth of *S. rebaudiana*

*In vitro* shoots of *S. rebaudiana* demonstrated a significant positive response to nitrogen and saccharose application in terms of biomass accumulation. The initial weight of the shoots grown on MS medium at the time of transformation was 20 g/flack$^{-1}$, and after three weeks of cultivation, it increased to 122.3 g/flack$^{-1}$ (see Fig. 2a).

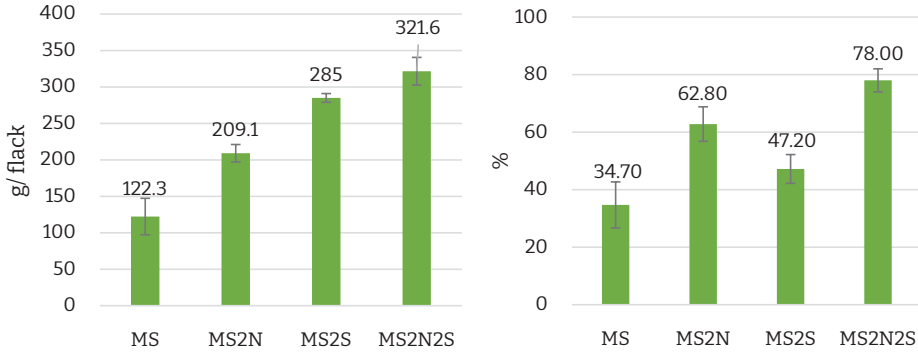


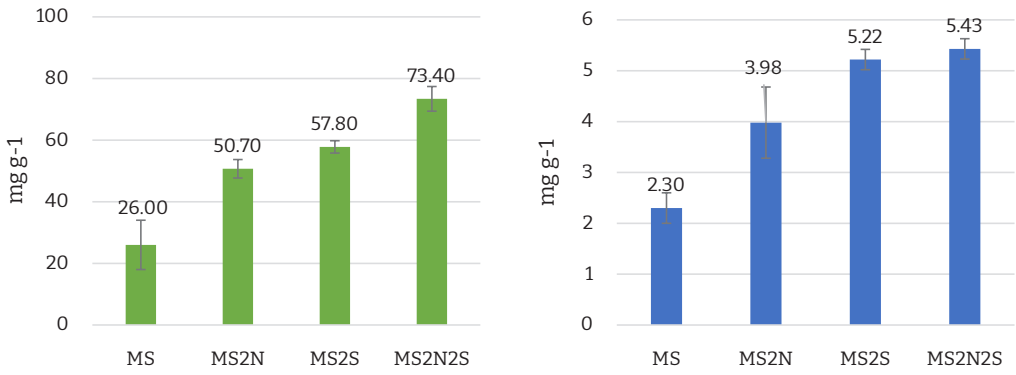

**Figure 2.** Biomass growth (a) and antioxidant activity (b) of *in vitro* cultures of *S. rebaudiana* on the 21st day of cultivation

**Note:** MS is the standard medium, MS2N is the MS medium with twice the concentration of nitrogen, MS2S is the MS medium with twice the concentration of saccharose, MS2NS is the MS medium with twice the concentration of nitrogen and saccharose

Raising the $KNO_3$ and $NH_4NO_3$ concentrations in the MS medium from 1.90 g/L$^{-1}$ and 1.65 g/L$^{-1}$ to 3.80 g/L$^{-1}$ and 3.30 g/L$^{-1}$, respectively, stimulated a significant accumulation of biomass in the shoot cultures. After 21 days of cultivation, the sprout biomass reached 209.1 g/flack$^{-1}$, a 71% increase compared to the control group. Raising the saccharose concentration in the MS medium from 30 g/L$^{-1}$ to 60 g/L$^{-1}$ resulted in the formation of 285.0 g/flack$^{-1}$ of shoot biomass, which was 133% higher than the biomass in the control group. After applying additional nitrogen and carbohydrate sources, the fresh weight of the sprout cultures reached 321.6 g/flack$^{-1}$, which was 199.3 g more than the biomass of shoot cultures grown on MS medium. Four different media showed varying results concerning the growth parameters of the stevia sprout cultures. For the growth of *S. rebaudiana*, MS medium was the least suitable. This conclusion can be drawn from the results obtained 21 days after *in vitro* sprout cultivation, which are represented in Figures 1 and 2. These data showed statistically significant differences for all other experiment variants.

Y. Shevchenko *et al.* (2010) showed that the DPPH-free radical scavenging capacity of the shoot culture of stevia grown on the MS medium was 34.07% on the 21$^{st}$ day of cultivation (Fig. 1b). Increasing the nitrogen supply led to an enhancement of DPPH to 62.8%, which was 2.5 times higher than in the tissues of stevia cultivated on MS medium. The treatment with 60g/L$^{-1}$ of saccharose rather than 30g/L$^{-1}$ did not affect the DPPH significantly compared to the additional application of nitrogen. However, it captured 88% prevalence of the control plot. The combined modification of MS medium, with increased nitrogen and carbohydrate content, resulted in the formation of 78% DHHP activity. Afterward, it was concluded that it could be attributed to the higher concentrations of steviosides and anthocyanins present in the plant biomass.

The application of additional $KNO_3$ and $NH_4NO_3$ caused an accumulation of 50.70 mg of steviosides per 1 g of fresh weight of biomass in the shoots after 21 days of cultivation. This amount was 95% more than that in cultures grown on MS medium (Fig. 3a).




a) steviosides, mg g$^{-1}$

b) anthocyanins, mg g$^{-1}$

**Figure 3.** Content of steviosides and anthocyanins in *in vitro* cultures of *S. rebaudiana* on the 21$^{st}$ day of cultivation

**Note:** MS is the standard medium. MS2N is the MS medium with twice the concentration of nitrogen compared to the standard medium, MS2S is the MS medium with doubled saccharose concentration, MS2NS is the MS medium with doubled concentration of nitrogen and saccharose

The culture's stevioside content increased to 57.8 mg/g$^{-1}$ after a 21-day cultivation period, with the additional carbohydrate source resulting in a 7.1 mg/g$^{-1}$ increase compared to the nitrogen treatment. The plot with *in vitro* cultures supplied with additional nitrogen and saccharose had the highest stevioside content, exceeding the control by 2.8 times and reaching

73.40 mg/g$^{-1}$. The content was 2.8 times higher than the control, reaching 73.40 mg/g$^{-1}$.

The anthocyanin content in the shoot culture of *S. rebaudiana* reached 2.3 mg/g$^{-1}$ of fresh biomass after being cultivated for 21 days on MS medium. Synthesis of 3.98 mg/g$^{-1}$ of anthocyanins occurred after supplementing with a double concentration of $KNO_3$ and $NH_4NO_3$ compared to MS medium typical concentration. Application of 60 g/L$^{-1}$ saccharose, instead of 30 g/L$^{-1}$, caused a two-fold increase in anthocyanin quantities. The substance content of this plot reached 5.22 mg/g$^{-1}$, which was similar to that after the treatment of sprout culture with increased nitrogen and saccharose concentrations. The combination of nitrogen and saccharose did not have as significant an effect as the single application of saccharose alone.

MS is among the most commonly used media for *in vitro* plant cultures. Additionally, B5 medium (Gamborg), LS (Linsmaier & Skoog), Velicky and Martin, and Blaydes and Heller medium compositions are widely used. The selection of the cultivation medium depends on the requirements of the initial plant and cultivation form, such as callus, suspension, hairy root, or shoot cultures. The MS culture is commonly employed for *in vitro* cell cultures, whereas B5 is used for hairy root cultures. On the other hand, LS medium is known to promote intensive biomass production in many cases. M.H. Zenk *et al*. (1978) conducted several tests to produce the indole alkaloid serpentine using *Catharanthus roseus* suspension cell cultures. The alkaloid content in cell cultures was dependent on the nutrient medium's composition. It was discovered that LS medium was optimal for the production of alkaloids, whereas MS medium was optimum for cell growth.

J.J. Zhong (2001) demonstrated that the intensity of alkaloid synthesis in cell suspension cultures of *Holarrhena antidysenterica* varies depending on the form of nitrogen in the cultivation media. Adding nitrogen to the MS nutrient solution stimulated anthocyanin formation in cell suspensions of *Vitis vinifera*. D.J. Kim & H.N. Chang (1990) reported the increased production of shikonin in *Lithospermum erythrorhizon* cell cultures under the influence of increased nitrogen concentration. However, K. Sakamoto *et al*. (1994) reported that a reduced level of total nitrogen in the cultivation media improved the production of capsaicin in *Capsicum frutescens* and anthraquinones in *Morinda citrifolia*. It is possible to conclude that the response of *in vitro* culture to increased nitrogen supply may differ, depending on the physiological and biochemical status of native plants.

J.J. Zhong's (2001) research revealed that the accumulation of alkaloids in *Holarrhena antidysenterica* and shikonin in *Lithospermum erythrorhizon* suspension cell cultures were significantly affected by the carbon source. In 1980, Knobloch and Berlin examined the impact of saccharose concentration on the synthesis of indole alkaloids in *Catharanthus roseus* cell cultures, ranging between 4-12%. It was found that the optimal concentration of sucrose was 8%. Moreover, by increasing the sucrose concentration to 8%, the yield of benzophenanthridine alkaloids in *Eschscholtzia californica* cell suspension culture increased tenfold and reached 150 mg/L$^{-1}$ (Berlin *et al*., 1981). However, Do and Cormier (1990) found that greater amounts of sucrose may induce osmotic stress in cells of suspension cultures, resulting in reduced biomass accumulation, but also increased anthocyanin production in *Vitis vinifera* cell cultures.

N. Sae-Lee *et al*. (2014) added $NH_4NO_3$ in quantities of 0.5, 1.0, 5.0, and 10.0 mg/L to the MS medium as a nitrogen source to stimulate *Vitis vinifera* cv. Pok Dum cell culture growth and induce secondary metabolite synthesis. The optimum result was achieved using MS medium enriched with 0.5 mg/L$^{-1}$ $NH_4NO_3$. On the 14$^{th}$ day of the experiment, the biomass of the cell cultures was 27.8 g/L$^{-1}$, representing a 5.6-fold increase compared to the control group.

F. Akbari *et al*. (2018) showed an increase in shoot length, seedling dry weight, and leaf fresh weight of stevia when $NH_4NO_3$ was applied along with $KNO_3$.

In this experiment, increasing the concentration of $KNO_3$ and $NH_4NO_3$ in MS medium stimulated intensive biomass accumulation of shoot cultures. After a 21-day cultivation period, the biomass of sprouts was 71% higher than the biomass of shoots grown on the control plot on MS medium. The increase in saccharose concentration in MS medium elicited 2.3-fold growth stimulation of biomass. The combination of extra nitrogen and carbohydrates was found

to be the most effective. This methodology stimulated biomass accumulation, which exceeded the control plot by 2.6 times. The stevia sprout cultures' growth parameters varied across four distinct media. The MS medium was found to be the least suitable for the growth of *S. rebaudiana*. This conclusion was drawn based on the results obtained after 21 days of *in vitro* sprouts cultivation, as presented in Figures 1 and 2. These data show significant statistical differences when compared to all other experimental variants.

Evaluating the antioxidant potential is important for determining the stability of a product during storage, and this value has a direct relationship with the concentration of substances with antioxidative properties. Substances with antioxidative properties can reduce the number of free radicals in foods and are therefore important components of a healthy diet (Nshimiyimana & He, 2010). Plant secondary metabolites, including polyphenolics such as anthocyanins, are known for their high antioxidant potential.

Prior research into the antioxidant potential of phenolic compounds has shown that their mechanism of action involves directly capturing free radicals. Furthermore, free radicals (ROO·) oxidise phenolic compounds and inactivate them, causing the fragmentation of less reactive substances (Smetanska *et al*., 2021). The phenolic compound radical (O·) formed during the reaction is stable. The unpaired electron can delocalise within the aromatic ring and can cause dimerisation, dismutation, recombination with other radicals, oxidation of quinones, producing semiquinones. Semiquinones can react with other radicals, forming stable quinones (Mohdaly *et al*., 2010). Antioxidants can prevent or inhibit substrate oxidation, thus eliminating or decreasing the oxidative damage caused to target molecules (Fig. 4).

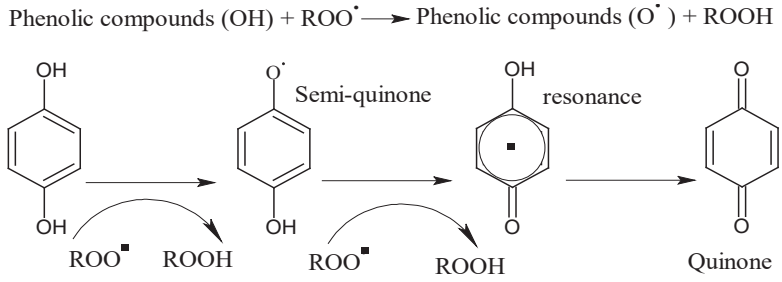



**Figure 4.** Mechanism of neutralization of reactive radicals by phenolic compounds
**Source:** A. Mohdaly *et al.* (2010)

Y. Shevchenko *et al.* (2010) demonstrated that the DPPH of the shoot culture of stevia increased by 150% after the introduction of additional nitrogen supply. However, the application of saccharose caused a slight increase in DPPH compared to the control. On the other hand, the combined application of additional nitrogen and saccharose positively influenced the increase of DPPH in plant tissues. There has been a recent rise in demand for industrial biotechnologies and natural antioxidants, aimed at replacing synthetic analogues. Free radicals are responsible for lipid peroxidation, which in turn can lead to many chronic illnesses in humans, including cancer and cardiovascular disorders; thus, they play a crucial role in their development.

N. Sharma *et al.* (2015) also reported that using a nutrient MS medium with twice the concentration of $NH_4NO_3$ and 4.5% sucrose increased the growth of *S. rebaudiana* cell cultures and the synthesis of steviol glycosides. Yet, the steviol glycoside amount in cell cultures was 2.99 mg/g$^{-1}$, whereas in the leaves of the original plants, it was 40.0 mg/g$^{-1}$.

These studies indicate that nitrogen positively affects the content of steviosides and anthocyanins, while sucrose has an even stronger impact. The concentration of steviosides in *in vitro* culture was 2.7 times higher after the treatment with nitrogen and sugar as in MS-cultivated *in vitro S. rebaudiana*. Anthocyanins are secondary metabolites found in plants that respond to stressors from unfavourable biotic and abiotic conditions, such as a lack or an excess of minerals. According to N. Sae-Lee *et al.* (2014), there was an increase in the accumulation of

anthocyanins in grape (*Vitis vinifera* L.) cell suspension cultures with increased nitrogen supply.

The effect of various concentrations of ammonium nitrogen on anthocyanin synthesis was investigated in *in vitro* cell cultures of *Rosa hybrida* by P. Olgunsoy *et al.* (2017). The induction of rapidly growing callus was observed during cultivation on MS solid medium with 4.0 mg/L$^{-1}$ 2,4-dichlorophenoxyacetic acid, but without additional nitrogen supply. According to J.J. Zhong (2001), raising the nitrogen content of the nutrient medium augments the anthocyanin composition in suspension cultures of *Vitis vinifera*. T.P. Magangana *et al.* (2018) demonstrated that the availability of nitrogen directly affects steviol concentration in microplants. Microplants subjected to a medium containing half the phosphate concentration were associated with the highest levels of stevioside (740 mg g/L).

Suspension cultures of *Nicotiana tabacum* have been found to accumulate more nicotine when exposed to increased saccharose concentration, resulting in osmotic stress (Shibasaki *et al.*, 2007). *Aralia cordata* cell suspension cultures showed decreased anthocyanin synthesis with a higher concentration of sucrose at 5%, whereas a concentration of 3% sucrose promoted anthocyanin accumulation. The osmotic pressure was also increased by adding mannitol to the medium, and it was discovered that after the addition of 3 g/L$^{-1}$ of this substance, the level of anthocyanins accumulated in the *Vitis vinifera* culture was elevated by up to 1.5 times, reaching 55 µg/cell$^{-1}$ (Dai *et al.*, 2014). An additional boost in anthocyanin content was observed in the callus cultures of *Euphorbia milli* grown in MS medium plus 7% sucrose. This indicates that, in numerous instances, growth-promoting environments are distinct from those required for synthesising secondary metabolites in cultures.

## CONCLUSIONS

It was established that various breeding environments had diverse effects on *in vitro* development of stevia culture. The four chosen media demonstrated distinct outcomes in terms of the growth parameters and synthetic potential of stevia cultures.

The MS medium was the least suitable for *S. rebaudiana* growth. The biomass increased twice as much as the control plot, after nitrogen and sucrose content was increased. The best medium for obtaining steviosides was MS with twice the nitrogen concentration. The stevioside content exceeded the control by 182.0%. However, this environment was less suitable for accumulating biomass. Anthocyanin accumulation increased by 1.5-fold with an additional nitrogen supply and 2.0-fold was associated with an increased sucrose concentration. This could be due to the role of carbohydrates in the anthocyanin biosynthetic pathway.

The findings strongly suggest that it is possible to modify the nutrient solution according to various tasks, such as increasing the biomass, antioxidants or certain valuable metabolites like steviosides and anthocyanins. In this particular case, the MS medium with a doubled nitrogen and sucrose supply can be used for high biomass production of stevia hoot culture and for obtaining antioxidants. To increase biomass for stevioside production, it is advantageous to increase nitrogen supply and possibly extend the cultivation period. For anthocyanin production, increasing the saccharose content in the cultivation media would be meaningful.

Achieving the optimal nutritional environment using MS is an important step in the innovative biotechnology of obtaining valuable secondary metabolites from plants, considering the biological features of each *in vitro* culture.

## ACKNOWLEDGEMENTS

None.

## CONFLICT OF INTEREST

The author of this study declares no conflict of interest.

## REFERENCES

[1] Akbari, F., Arminian, A., Kahrizi, D., Fazeli, A., & Ghaheri, M. (2018). Effect of nitrogen sources on gene expression of Stevia rebaudiana (Bertoni) under *in vitro* conditions. *Cellular and Molecular Biology*, 64(2), 11-16. doi: 10.14715/cmb/2018.64.2.3.

[2] Al-Taweel, S., Azzam, C., Khaled, K., & Abdel-Aziz, R. (2021). Improvement of stevia (*Stevia rebaudiana* Bertoni) and steviol glycoside through traditional breeding and biotechnological approaches. *Ssabrao Journal of Breeding and Genetics*, 53, 88-111.

[3] Alvarez, S., & Motos, J.R. (2023). *Advances in plant physiology of abiotic stresses*. Retrieved from https://www.mdpi.com/books/book/6646-advances-in-plant-physiology-of-abiotic-stresses.
[4] Amien, S., Maulana, H., Ruswandi, D., & Nurjanah, S. (2022). Stevia (*Stevia rebaudiana* b.) genotypes assessment for leaf yield stability through genotype by environment interactions, ammi, and gge biplot analyses. *SABRAO Journal of Breeding and Genetics*, 54, 767-779. doi: 10.54910/sabrao2022.54.4.8.
[5] Berlin, J., Kukoschke, K.G., & Knobloch, K.H. (1981). Selection of tobacco cell lines with high yields of cinnamoyl putrescines. *Planta Medica*, 42(6), 173-180. doi: 10.1055/s-2007-971622.
[6] Convention on Biological Diversity. (1992). Retrieved from https://zakon.rada.gov.ua/laws/show/995_030#Text.
[7] Dai, Zh., Meddar, M., Renaud, Ch., Merlin, I., Hilbert, Gh., Delrot, S., & Gomès, E. (2014). Long-term *in vitro* culture of grape berries and its application to assess the effects of sugar supply on anthocyanin accumulation. *Journal of Experimental Botany*, 65(16), 4565-4577. doi: 10.1093/jxb/ert489.
[8] El-Dawayati, M.M., & Zayed, Z.E. (2017). Controlling hyperhydricity in date palm *in vitro* culture by reduced concentration of nitrate nutrients. In *Date palm biotechnology protocols* (pp. 175-183). New York: Humana Press. doi: 10.1007/978-1-4939-7156-5_15.
[9] Emergen Research. (2022). Retrieved from https://www.emergenresearch.com/industry-report/stevia-market.
[10] Fidemann, T., Pereira, G, Heluy, T., Gallego, R., Bertão, M., Silva, R., & Fernández, E. (2018). Handling culture medium composition for optimizing plant cell suspension culture in shake flasks. *Plant Cell, Tissue and Organ Culture*, 133, 137-146. doi: 10.1007/s11240-017-1368-3.
[11] Hassanein, A. (2022). *Application of tissue culture techniques to improve the productivity of medicinal secondary products from medicinal plants*. Retrieved from https://www.intechopen.com/online-first/82048.
[12] Ibrahim, K., & Musbah, H. (2018). Increasing poly phenols in *Coleus blumei* at the cellular and intact plant levels using PEG stress. *Research Journal of Pharmacy and Technology,* 11(1), article number 321. doi: 10.5958/0974-360X.2018.00059.8.
[13] Jawad, M., Rizwan, B., Jawad, M., Khalid, F., & Ishaq, A. (2022). A nutraceutical and therapeutic potentials of stevia rebaudiana bertoni: Nutraceutical and therapeutic potentials of stevia. *Pakistan BioMedical Journal,* 5(1), 10-22. doi: 10.54393/pbmj.v5i1.143.
[14] Kikowska, M., Thiem, B., Szopa, A., & Ekiert, H. (2022). Accumulation of valuable secondary metabolites: Phenolic acids and flavonoids in different *in vitro* systems of shoot cultures of the endangered plant species *Eryngium alpinum* L. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 141, 381-391. doi: 10.1007/s11240-020-01795-5.
[15] Kim, D.J., & Chang, H.N. (1990). Enhanced shikonin production from *Lithospermum erythrorhizon* by *in situ* extraction and calcium alginate immobilization. *Biotechnology and Bioengineering*, 36(5), 460-466. doi: 10.1002/bit.260360505.
[16] Knobloch, K.H., & Berlin, J. (1980). Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* L. *Naturforschung*, 35(7), 551-556. doi: 10.1515/znc-1980-7-805.
[17] Król, A., Kokotkiewicz, A., Szopa, A., Ekiert, H., & Łuczkiewicz, M. (2021). Bioreactor-grown shoot cultures for the secondary metabolite production. In *Plant cell and tissue differentiation and secondary metabolites. Reference Series in Phytochemistry* (pp. 187-247). Springer: Cham. doi: 10.1007/978-3-030-30185-9_34.
[18] Magangana, T.P., Stander, M.A., & Makunga, N.P. (2018). Effect of nitrogen and phosphate on *in vitro* growth and metabolite profiles of *Stevia rebaudiana* Bertoni (Asteraceae). *Plant Cell, Tissue and Organ Culture (PCTOC)*, 134, 141-151. doi: 10.1007/s11240-018-1407-8.
[19] Mohdaly, A., Smetanska, I., Ramadan, F.R., Sarhanb, M.A., & Mahmoud, A. (2011). Antioxidant potential of sesame (*Sesamum indicum*) cake extract in stabilization of sunflower and soybean oils. *Industrial Crops and Products,* 34(1), 952-959. doi: 10.1016/j.indcrop.2011.02.018.

[20] Mohdaly, A.A., Sarhan, M.A., Smetanska, I., & Mahmoud, A. (2010). Antioxidant properties of various solvent extracts of potato peel, sugar beet pulp and sesame cake. *Journal of the Science of Food and Agriculture,* 90(2), 218-226. doi: 10.1002/jsfa.3796.
[21] Mordor Intelligence. (2022). Retrieved from https://www.mordorintelligence.com/industry-reports/stevia-market.
[22] Nguyen, T., Seo, C., Kwak, S.H., Kim, J., Kang, H.K., Kim, S.B., & Kim, D. (2019). Enzymatic production of steviol glucosides using β-glucosidase and their applications. In *Enzymes in Food Biotechnology* (pp. 405-418). Cambridge: Academic Press. doi: 10.1016/B978-0-12-813280-7.00023-2.
[23] Nguyen, Y., & Tan, Q.L.P. (2021). Chemical composition of dried *Stevia rebaudiana* Bertoni leaves and effect of ultrasound-assisted extraction on total steviosides content in extract. *Herba Polonica*, 67(1), 1-7. doi: 10.2478/hepo-2021-0003.
[24] Nshimiyimana, D.S., & He, Q. (2010). Radical scavenging capacity of Rwandan CTC tea polyphenols extracted using microwave assisted extraction. *Pakistan Journal of Nutrition,* 9(6), 589-593. doi: 10.3923/pjn.2010.589.593.
[25] Olgunsoy, P., Ulusoy, S., & Akçay, U. (2017). Metabolite production and antibacterial activities of callus cultures from *Rosa damascena* Mill. petals. *Marmara Pharmaceutical Journal,* 21(3), 590-597. doi: 10.12991/marupj.319331.
[26] Ramírez-Mosqueda, M., Iglesias, A.L., Ramírez-Madero, G., & Hernández-Rincón, E.U. (2016). Micropropagation of *Stevia rebaudiana* Bert. in temporary immersion systems and evaluation of genetic fidelity. *South African Journal of Botany,* 106, 238-243. doi: 10.1016/j.sajb.2016.07.015.
[27] Raspe, D.S., & Silvio, C.C. (2022). Compounds from *Stevia rebaudiana* Bertoni leaves: An overview of non-conventional extraction methods and challenges. *Food Bioscience*, 46, article number 101593. doi: 10.1016/j.fbio.2022.101593.
[28] Sae-Lee, N., Kerdchoechuen, O., & Laohakunjit, N. (2014). Enhancement of phenolics, resveratrol and antioxidant activity by nitrogen enrichment in cell suspension culture of *Vitis vinifera. Molecules*, 19(6), 7901-7912. doi: 10.3390/molecules19067901.
[29] Sakamoto, K., Iida, K., Sawamura, K., Hajiro, K., Asada, Y., Yoshikawa, T., & Furuya, T. (1994). Anthocyanin production in cultured cells of *Aralia cordata* Thunb. *Plant Cell, Tissue and Organ Culture*, 36, 21-26. doi: 10.1007/BF00048311.
[30] Sharma, N., Gauchan, D., Dhakal, A., Luitel, A., Shakya, S., & Shakya, R. (2015). Establishment of regenerative callus, cell suspension system and molecular characterization of *Stevia rebaudiana* Bertoni for the production of stevioside in *in vitro*. *International Journal for Research in Applied Science & Engineering Technology*, 3, 133-144.
[31] Shevchenko, Y., Wendt, A., & Smetanska I. (2010). Sprout culture of *Stevia rebaudiana Bertoni*. In *Stevia science, no fiction* (pp. 5-26). Euprint Heverlee: Leuven.
[32] Shibasaki, N., Hirose, K., Yonemoto, T., & Tadaki, T. (2007). Suspension culture of *Nicotiana tabacum* cells in a rotarydrum bioreactor. *Journal of Chemical Technology and Biotechnology*, 53(4), 359-363. doi: 10.1002/jctb.280530407.
[33] Smetanska, I., Tonkha, O., Patyka, T., Hunaefi, D., Mamdouh, D., Patyka., M., Bukin, A., Mushtruk, M., Slobodyanyuk, N., & Omelian, A. (2021). The influence of yeast extract and jasmonic acid on phenolic acids content of *in vitro* hairy root cultures of Orthosiphon aristatus. *Slovak Journal of Food Sciences*, 15, 1-8. doi: 10.5219/1508.
[34] Wang, J., Zhao, H., Wang, Y., Lau, H., Zhou, W., Chen, C., & Tan, S. (2020). A review of stevia as a potential healthcare product: Up-to-date functional characteristics, administrative standards and engineering techniques. *Trends in Food Science & Technology*, 103, 264-281. doi: 10.1016/j.tifs.2020.07.023.
[35] WHO Guideline. (2023). Retrieved from https://www.who.int/publications/who-guidelines.
[36] Wu, T., Kerbler. S., Fernie, A., & Zhang, Y. (2021). Plant cell cultures as heterologous bio-factories for secondary metabolite production. *Plant Communications*, 2(5), article number 100235. doi: 10.1016/j.xplc.2021.100235.

[37] Zaheer, M., Vudem, D., & Giri, Ch. (2016). Enhanced daidzin production from jasmonic and acetyl salicylic acid elicited hairy root cultures of *Psoralea corylifolia* L. (Fabaceae). *Natural product research*, 30(13), 1542-1547. doi: 10.1080/14786419.2015.1054823.
[38] Zenk, M.H. (1978). The impact of plant cell cultures on industry. In *Frontiers of plant tissue culture* (pp. 1-14). Calgary: The International Association of Plant Tissue Culture.
[39] Zhong, J.J. (2001). Biochemical engineering of the production of plant-specific secondary metabolites by cell suspension cultures. *Advances in Biochemical Engineering/Biotechnology*, 72, 1-26. doi: 10.1007/3-540-45302-4_1.

**Ірина Миколаївна Сметанська**

Доктор сільськогосподарських наук, професор
Університет прикладних наук Вайенштефана-Трісдорфа
91746, вул. Маркграфенштрассе, 16, м. Вайденбах, Німеччина
https://orcid.org/0000-0003-2298-2888

**Мамдох Діаа**

Магістр біологічних наук, доцент
Університет прикладних наук Вайенштефана-Трісдорфа
91746, вул. Маркграфенштрассе, 16, м. Вайденбах, Німеччина
https://orcid.org/0000-0003-1379-8894

**Груда Назім**

Доктор сільськогосподарських наук, професор
Берлінський університет імені Гумбольдта,
10117, вул. Унтер-ден-Лінден, 6, м. Берлін, Німеччина
https://orcid.org/0000-0002-2663-1594

**Микола Володимирович Патика**

Доктор сільськогосподарських наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-2506-8699

**Оксана Леонідівна Тонха**

Доктор сільськогосподарських наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-0677-5494

## Вплив поживних речовин на ріст і синтез метаболітів у культурах пагонів *in vitro S. rebaudiana*

**Анотація.** Рослини стевії ребаудіани виробляють солодкі на смак сполуки – стевіозиди, які в 300 разів солодші за цукор і можуть використовуватися як натуральні підсолоджувачі. Крім того, листя стевії містить велику кількість антиоксидантів. Використання рослинних сполук як компонентів функціонального харчування та нутрицевтиків є надзвичайно актуальним на сьогоднішній день. Метою даної роботи було дослідити вплив підвищеного азотного живлення та сахарози на ріст і накопичення стевіозидів та антоціанів, а також антиоксидантну активність у швидкорослих культурах пагонів *S. rebaudiana*. Визначено морфологічні зміни листків, накопичення стевіозидів і антоціанів та антиоксидантні

властивості екстрактів. Показано, що протягом трьох тижнів культивування пагонів *in vitro* на середовищі MS з подвійною концентрацією азоту біомаса збільшилася на 71,0 %, після застосування подвійної концентрації сахарози – на 133 %, а після збільшення азоту в поєднанні з подачею сахарози – на 162,0 % порівняно з контролем. Сахароза стимулювала накопичення біомаси. Антиоксидантний потенціал після внесення азоту був у 2,4 рази вищим за контроль, а при внесенні сахарози – у 2,7 рази. Пагони, оброблені підвищеною концентрацією азоту та сахарози, містили 50,7 та 57,8 мг/г$^{-1}$ стевіозидів відповідно. Однак поєднання азоту та сахарози призводило до накопичення 73,4$^{-1}$ мг/г$^{-1}$ стевіозидів. Пагони, вирощені на середовищі MS, містили 26,0 мг/г$^{-1}$ стевіозидів. Вміст антоціанів був в 1,7 рази більшим за умови внесення азоту та в 2,3 рази більшим за умови внесення азоту та сахарози. Оптимальні живильні середовища, розроблені індивідуально для кожної культури *in vitro*, збільшують продукцію цінних вторинних метаболітів рослин до 3 разів

**Ключові слова:** рослинна біомаса; культивування; поживне середовище; вторинні метаболіти; стевіозиди; антоціани; антиоксидантна активність