CULTIVAR-SPECIFIC RESPONSES OF *STEVIA REBAUDIANA* TO MULTI-WALLED CARBON NANOTUBES: EFFECTS ON GROWTH, STEVIOL GLYCOSIDE PRODUCTION, AND GENE EXPRESSION

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Stevia rebaudiana Bertoni, commonly known as stevia, is a perennial herbaceous plant cultivated for its natural sweetening properties derived from high-potency natural sweeteners known as steviol glycosides. The major steviol glycosides found in stevia leaves are stevioside and rebaudioside A, which serve as non-caloric sugar substitutes in various food and beverage products (Brandle and Telmer, 2007). With the increasing demand for low-calorie sweeteners in the food industry, there is a growing interest in optimizing stevia cultivation and enhancing the yields of steviol glycosides.

Tissue culture techniques provide a versatile platform for studying and optimizing *Stevia rebaudiana*, a plant valued for its natural sweetening compounds. This *in vitro* approach allows precise control over the growth environment, enabling investigations of stevia's biology and physiology, which are difficult in field-based studies (Rokosa and Kulpa 2020 and Ghose *et al*., 2022). By establishing stevia cultures in the lab, researchers can closely monitor the plant's responses to various stimulus and optimize the production of steviol glycosides, the primary sweet compounds. Tissue culture techniques offer promising solutions for the mass propagation and biomass production of stevia. These methods, which apply *in vitro* propagation, genetic engineering, suspension cultures, callus cul-

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ture, adventitious root culture, and elicitation, provide efficient and scalable approaches for generating large quantities of high-yielding stevia plant material (Miladinova *et al.,* 2022). By leveraging these advanced techniques, researchers can enhance the yield and quality of stevia's valuable metabolites, which contributes to the growing global demand for natural, low-calorie sweeteners, while promoting sustainable agriculture.

The investigation of nanoparticle-like multi-walled carbon nanotubes (MWCNTs) on stevia growth and steviol glycoside yields is of particular interest because the potential impacts of MWCNTs on plant physiology are influenced by factors such as plant species, cultivar, and growth conditions (Anjum *et al.,* 2019). The study of MWCNTs on stevia growth and steviol glycoside yields can lead to the development of nanotechnology-based protocols to enhance stevia propagation and increase steviol glycoside yields, contributing to the growing global demand for natural, low-calorie sweeteners, while promoting sustainable agricultural practices (Adesanya and Fayemi 2023).

High-performance liquid chromatography (HPLC) has been extensively used for the analysis and quantification of stevioside and rebaudioside A in *Stevia rebaudiana.* Various studies have focused on developing and validating high-performance liquid chromatography (HPLC) methods for the accurate determination of these sweet compounds in stevia leaves. The HPLC method allows for the separation and quantification of stevioside and rebaudioside A, providing a reliable and sensitive technique for analyzing these glycosides in *Stevia rebaudiana* (Aranda-González *et al.,* 2015).

The expression of *UGT85C2* and *UGT74G1* genes in *Stevia rebaudiana* has been studied to understand the regulation of glycosides in this plant. These genes are involved in the biosynthesis pathways of stevioside and rebaudioside A, which are the primary sweet compounds found in stevia leaves. The expression levels of these genes were measured using RT-PCR, and it was found that they play a crucial role in the production of stevioside and rebaudioside A (Abdelsalam *et al.*, 2019 and Wu *et al.*, 2020). *UGT85C2* and *UGT74G1* are two of the main genes involved in the biosynthesis pathway of stevioside and rebaudioside A. *UGT85C2* is responsible for the conversion of steviol to steviol-13-O-glucoside, whereas *UGT74G1* is responsible for the conversion of steviol-13-O-glucoside to stevioside (Behroozi *et al.,* 2017 and Hashem *et al.,* 2021).

The expression levels of these genes were influenced by various factors, including water and nutrient availability and different irrigation conditions (Hajihashemi and Geuns, 2016). The relative expression levels of the *UGT74G1, UGT76G1, UGT85C2*, and *KS* genes involved in the synthesis of steviol glycoside were detected using semi-quantitative RT-PCR. The results showed that the ex-

pression levels of these genes were significantly downregulated under polyethylene glycol treatment, indicating that drought stress affects the biosynthesis of steviol glycosides in *Stevia rebaudiana* (Ghorbani *et al.,* 2017).

This study aimed to assess the effects of MWCNT treatment on *in vitro* growth parameters and the expression of key biosynthetic genes involved in steviol glycoside biosynthesis in the three cultivars of *S. rebaudiana*: (High sugar, ShouA3-2, and Levan).

MATERIALS AND METHODS

Plant materials and growth conditions

Three *Stevia rebaudiana* cultivars (High Sugar, ShouA3-2, and Levan) were obtained from the Sugar Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt. Seedlings were propagated by established protocols for tissue culture conditions, as outlined by Abdel Hamid *et al.* (2018) and Hashem *et al.* (2021). Disinfected explants were cultured in glass jars containing basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), with regular subculturing to maintain a continuous supply of plant materials.

Multiwalled carbon nanotube (MWCNT) characterization

The MWCNTs used in this study were powder-based carbon material with 94.5% carbon purity. The dimensions were verified through Transmission Electron Microscopy (TEM) using the specifications supplied by Nanofab Technology Co., located in Giza, Egypt. The structural characteristics of MWCNTs were examined using transmission electron microscopy (TEM) and the average length of MWCNTs was found to be longer than 1 µm with an outer diameter of 25°± 5 nm and inner diameter of 10 ± 5 nm (Fig. 1). This result was consistent with the nanometer size range of MWCNTs, which were thought to affect plant growth and development through their unique physicochemical properties.

Application of carbon nanotubes in *in vitro* cultures

Axillary buds of *Stevia rebaudiana* (≈2 cm with two leaves) were isolated from the shoots and cultured in an MS medium that was supplemented with different concentrations of MWCNTs (1, 1.5, and 2 mg/L) and dissolved in 1 mL of Aqua regia. Two controls were also included; one was without MWCNTs or Aqua regia and the second consisted of only Aqua regia (Table 1). After 21 days, the plantlets were transferred to a mixture of peat moss and perlite at a volumetric ratio of 2:1. Afterwards, they were acclimatized in a greenhouse with controlled conditions (temperature: 25 ± 2°C; relative humidity: 65 ± 5%; photoperiod: 16h light and 8h darkness). The solutions were reapplied with MWCNT treatments every 5 days.

Morphological characteristics

Six morphological traits (number of shoots, shoots length, number of leaves, number of roots, roots length, and shoots diameter) were recorded as a means of nine plants after 21 days of treatment.

Analysis of steviol glycosides using HPLC

• Sample preparation

Dried leaves (1 g per cultivar) were ground with a Tissue Lyser II (Qiagen) and extracted according to Nishiyamla *et al.* (1992). Standards, samples, and mobile phase components were degassed and filtered through a 0.45 µm membrane filter (Millipore) to remove particulates (Snyder *et al.*, 2010).

• HPLC analysis

The chromatographic analysis was carried out with the help of Thermo Ultimate 3000 HPLC system and equipped with a DAD-3000 diode array detector and processed with Cromelion7 software. Separation was performed on a Thermohypersil C18 column (2.5 × 30 cm) that was maintained at 25°C with a mobile phase of distilled water and acetonitrile (65:35) at 1.0 mL/min. The column temperature was maintained at 35°C, and 10 µL of each sample was injected. Compounds were identified at 210 nm by comparing retention times and UV spectra with those of standards.

Real-Time Quantitative PCR (RT-qPCR) analysis

RNA extraction and cDNA synthesis

Genomic DNA was removed by DNase I treatment (Thermo Fisher Scientific, USA) after RNA extraction (Wilfinger *et al.*, 1997). The Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used to synthesize cDNA.

Primer design and RT-qPCR

Primers for *UGT74G1*, *UGT85C2* genes, and the *β-actin*-housekeeping gene were designed in Primer2 Online Software and indicated with OLIGO 5 analyzer software as shown in detail in Table (2). RT-qPCR was conducted on a QuantStudio 5 Dx Real-Time PCR System (Thermo Fisher Scientific, USA) in a 25 µL reaction using Maxima SYBR Green qPCR Master Mix. The thermal cycling consisted of an initial denaturation cycle (95°C for 10 min), followed by 40 cycles of denaturation (95°C for 30 s), an annealing cycle (primer-specific temperature for 15 s), and an extension cycle (72°C for 30 s). The $2^{\wedge-\Delta\Delta Ct}$ method was used to calculate relative gene expression (Rao *et al.*, 2013).

Statistical analysis

Data were analyzed using SPSS Version 22.0 and expressed as mean ± standard error (S.E.). Comparisons were made using one-way ANOVA and LSD post-hoc tests with probability values set

at $P < 0.05$ (Snedecor and Cochran, 1980). SPSS and Microsoft Excel programs were used to create graphs.

RESULTS AND DISCUSSION

Multi-walled carbon nanotubes (MWCNTs) can be safely used as an additive to enhance plant growth and development and enhance secondary metabolite production in plant tissue culture. In this study, three *Stevia rebaudiana* cultivars (High Sugar, ShouA3-2, and Levan) were utilized with MWCNTs to induce morphogenic responses and affect shoots, roots, and leaves development. The biosynthesis of steviol glycosides was investigated. Furthermore, the molecular mechanisms exerted by MWCNTs on the *UGT74G1* and *UGT85C2* genes during the steviol glycoside pathway were revealed by gene expression analysis. This provided an insight into the potential of MWCNT to optimize *in vitro* propagation and second metabolite production in the three cultivars of *Stevia rebaudiana*.

Morphogenic responses of stevia using multi-walled carbon nanotubes (MWCNTs)

The morphogenic responses of stevia plants in tissue culture experiments were significantly enhanced by multi-walled carbon nanotubes (MWCNTs). The three *in vitro*-grown stevia cultivars (High sugar, ShouA3-2, and Levan) explants under different treatments appeared in Fig. (2). The explants were subjected to the control, Aqua regia treatment, and MWCNTs treatments T1, T2, and T3 as shown in Table (1). As evident from the qualitative assessment in Fig. (2), the explants treated with MWCNTs exhibited notable differences in growth compared to the control and Aqua regia treatments after 21 days of culture. The MWCNTs-treated explants displayed enhanced shoots growth characterized by an increase in both shoots number and length, which suggested a stimulatory effect of MWCNTs on shoots development in stevia plants, indicating their potential influence on plant growth and morphogenesis.

The unique nanoscale tubular structure and physicochemical properties of MWCNTs, such as their ability to penetrate plant cell walls, have been attributed to their potential to enhance growth and development in plant tissue cultures especially T1 and T2 in High Sugar, T1 and T3 in ShouA3-2, and T1 in Levan cultivars compared with the control (Fig. 2). MWCNTs are believed to improve nutrient absorption, trigger beneficial signaling pathways, and modulate gene expression patterns, ultimately leading to a positive impact on plant morphogenic pathways. These findings agreed with the study conducted by Ramezan *et al.* (2022) who reported similar enhancements in stevia growth and metabolism following carbon nanotube treatment. The

ability of MWCNTs to significantly enhance the growth and morphological traits response of stevia plants opens new avenues for optimizing cultivation practices and maximizing the yield of valuable compounds like steviol glycosides.

The application of multi-walled carbon nanotubes (MWCNTs) has emerged as a promising approach for enhancing growth parameters in plant tissue cultures. The results in Table (3) and Fig. (3) demonstrate the significant impacts of MWCNTs on *Stevia rebaudiana* micropropagation for some of the following traits:

Number of shoots/plants means

A non-significant difference in the means of the number of shoots/plants trait among the three cultivars was observed under all treatments. However, T1 with the Levan cultivar producing the highest number of shoots mean (3.33 ± 1.33) per explants compared to the control, which confirmed that MWCNTs led to a remarkable increase in shoots proliferation (Table 3 and Fig.3).

Length of shoots means

Treatment T1 resulted in a significant increase in shoots length means for all the three cultivars compared with the control. The greatest shoot elongation under T1 was exhibited by the ShouA3-2 (14.33 ± 1.35 cm) and Levan (13.07 ± 2.26 cm) cultivars, which were significantly higher than that of the High sugar cultivar (6.07 ± 0.71 cm) (Table 3 and Fig. 3). ShouA3-2 gave the highest length of shoots under the three treatments (T1, T2, and T3) compared to the other two cultivars. Additionally, MWCNTs exposure (T1 and T2) stimulated a substantial 80% increase (1.8-fold) in shoot length across the tested cultivars.

The number of roots means

This trait did not show significant differences among cultivars. However, the Levan cultivar had the highest significant means for the number of roots under the T1 treatment (8.33 ± 1.66) and T3 (7.0 ± 1.52) compared with the other treatments and cultivars (Table 3 and Fig. 3). Moreover, under AR and T3, significant differences were observed but not conclusively validated due to experimental variability (Table 3).

The number of leaves means

The number of leaves means increased ($p \leq 0.05$) in the Levan cultivar under T1 treatment compared with the control, suggesting a positive effect of this treatment on the number of leaves means, T2 also showed significant differences among the three cultivars (Table 3 and Fig. 3).

The length of roots means

The High sugar and Shoua3-2 cultivars gave significant differences from Levan. The High- sugar cultivar exhibited significantly the highest roots length under T3 treatment (3.73 cm) compared with the control, at the same time, the ShouA3-

2 cultivar also exhibited high roots length means under T1 (2.43 cm) compared with the control conditions (1.67 cm) (Table 3 and Fig.3).

Shoots diameter means

No significant differences were found among the cultivars in the means of shoots diameter among the three cultivars under all treatments (Table 3 and Fig. 3). However, the High sugar cultivar gave the highest shoot diameter mean in the T2 treatment (0.073).

The ShouA3-2 cultivar displayed the optimal tissue culture responses under most treatments means, producing elongated shoots (14.33 ± 1.35 cm, p=0.134 NS), high roots number (7.00 ± 1.53, p=0.090 NS), and increased leaves production as shown in Table (3) and Fig. (3). Treatment T1 containing specific plant growth regulators was the most effective across cultivars for enhancing shoots proliferation and elongation, with significant increases observed particularly in Levan (3.33 ± 1.33, p=0.452 NS) for shoot number and in ShouA3-2 (14.33 ± 1.35 cm, p=0.134 NS) and Levan (13.07 ± 2.26 cm, p=0.009*) for shoot length compared to their respective controls (Table 3 and (Fig.3).

The positive effects of MWCNTs on shoots and roots growth metrics underscore their potential to increase micropropagation efficiency and productivity in plant tissue culture systems. Further optimization of the MWCNTs dosage and incorporation into media formulations can help to maximize the capacity of this nanotechnology for improving *in vitro* plant propagation. Moreover, the beneficial impacts of MWCNTs were retained during the acclimatization of plantlets to greenhouse conditions, with sustained enhancement of growth parameters, including shoots length, leaves number, and plant biomass traits, compared to non-treated controls (Fig.4)

These results are confirmed by Kumar *et al.* (2018) who demonstrated the synergistic role of zinc (Zn) and multi-walled carbon nanotubes (MWCNTs) synthesized as a nanocomposite for enhancing the growth of onion seeds in arid conditions. They highlighted the potential of this nanocomposite as a smart distributor of micronutrients. Our study aligns with the findings of other research, also on the role of zinc and MWCNTs in plant growth. For example, the study of Al-Shaheen *et al.* (2021) showed the beneficial effects of nano-fertilizer and amino acid treatments on stevia growth and phytochemical content, which supports the beneficial effects of MWCNTs on stevia growth and development.

Effect of MWCNT treatments on stevioside content (HPLC)

The application of multi-walled carbon nanotubes (MWCNTs) for *in vitro* cultures of *Stevia rebaudiana* showed varying effects on the production of stevi-

ol glycosides, particularly stevioside and rebaudioside A, across the three studied cultivars.

HPLC analysis of steviol glycosides in *Stevia rebaudiana* cultivars

High-performance liquid chromatography (HPLC) technique was used to detect the production of steviol glycosides (stevioside and rebaudioside A) in the three studied cultivars, as shown in Fig. (5).

High Sugar cultivar

In the High Sugar cultivar (Fig. 5A), the AR treatment (aqua regia solvent) significantly increased the content of both stevioside (2625.60 ppm) and rebaudioside A (1971.34 ppm) compared to the control. The T1, T2, and T3 treatments (1 mg/L, 1.5 mg/L, and 2 mg/L of MWCNTs, respectively) showed mixed results. T1 and T2 treatments decreased stevioside and rebaudioside A levels, especially T2 treatment, which showed a high reduction in rebaudioside A (337.22 ppm). Interestingly, T3 treatment resulted in a higher rebaudioside A content (1650.63 ppm) than the control (1467.3 ppm). These cultivar-specific responses to MWCNTs have been reported in various plant species, highlighting the need for careful application and monitoring of concentrations to optimize desired metabolite production (Ramezani *et al.,* 2020).

ShouA 3-2 cultivar

In the ShouA 3-2 cultivar (Fig. 5B), the T1 treatment was the most effective in increasing stevioside content (1681.86 ppm), while the T2 treatment reduced it (156.82 ppm). These results support studies indicating that MWCNTs can enhance shoot proliferation and secondary metabolite production traits of *in vitro* cultures (Elgeabeily *et al.,* 2020). For rebaudioside A, the AR treatment decreased content (1630.50 ppm), while T1 and T2 treatments showed significant reductions. T2 treatment showed the most dramatic effect (23.87 ppm). However, T3 treatment slightly increased rebaudioside A (2088.05 ppm) compared to the control. The mixed results highlighted the complex interactions between MWCNTs and plant metabolism, which can vary among studied cultivars (Ghose *et al.,* 2022).

Levan cultivar

The Levan cultivar exhibited a distinct response to the different treatments applied in this study (Fig. 5C). Notably, the AR treatment significantly decreased the stevioside content t (1095.45 ppm) and rebaudioside A to (529.57 ppm) compared to the control. Similarly, the T1 and T3 treatments also resulted in decreasing levels of both stevioside and rebaudioside A, though not as dramatically as the AR treatment. Interestingly, the T2 treatment yielded a high stevioside value of 2788.194 ppm, which was significantly higher than the control (2436.3 ppm). This result suggested that the Levan culti-

var had a unique ability to utilize MWCNTs in a way that enhanced its secondary metabolite production, specifically stevioside, under certain conditions. In contrast, the consistent decrease in rebaudioside A across all treatments indicated that MWCNTs may exert a negative influence on this metabolite in the Levan cultivar, which contrasted with the effects observed in the other two stevia cultivars.

This result aligned with the findings of Salem (2020) in micropropagation studies, where repeated subcultures were essential for shoot multiplication but often led to vitrification. These findings collectively underscore the complex interactions among MWCNTs, anti-ethylene agents, and metabolite production in *Stevia rebaudiana*, emphasizing the need for cultivar-specific approaches to maximize desired outcomes. These results underscore the importance of cultivar-specific responses when applying MWCNTs in tissue culture systems and highlight the need for tailored approaches to optimize growth conditions and treatment strategies for different Stevia cultivars to maximize the yield of desired steviol glycosides.

Gene expression

In this study, analysis of the expression for key genes in the biosynthesis pathway of stevioside and rebaudioside A (*UGT85C2* and *UGT74G1*) revealed that the glycosides were significantly different in the treated plants than those in the control plants in Stevia leaves. Thus, the expression levels of the two genes were changed dramatically after treatment with various concentrations of MWCNTs. The total RNA was extracted from the leaves of the Levan cultivar as an example. All samples had an optimum OD 260/280 ratio ≥ 1.9. RNA integrity was evaluated by denaturing agarose gel electrophoresis. Denaturing agarose gel electrophoresis was used to confirm the integrity of the isolated RNA by observing 28S and 18S rRNA bands. The results showed the intact and visibility of the two bands, indicating a high RNA quality (Fig. 6).

Differential gene expression patterns in stevia cultivars reveal complex interactions with multi-walled carbon nanotubes (MWCNTs)

- **The relative expression level of the *UGT74G1* gene among the three Stevia cultivars treated with MWCNT**

When comparing the relative expression levels of the *UGT74G1* gene across the three stevia cultivars (High Sugar, ShouA3-2, and Levan) in (Fig. 7), it was observed that they showed minimal and non-significant changes in gene expression in response to MWCNT treatments. The untreated control groups showed baseline expression levels that differed among the three cultivars, with High Sugar having the highest baseline (1.06), followed by ShouA3-2 (1.027), and Levan showing the lowest (0.698). The AR groups (0.5 ml Aqua regia/l) showed slight variations from their re-

spective controls, but these changes were not statistically significant. Among the MWCNT treatments, no consistent dose-dependent pattern was observed across the cultivars. In the High Sugar cultivar, T1 (1 mg CNT + 0.5 ml Aqua regia/l) showed the highest expression (1.07), while in ShouA3-2, T3 (2 mg CNT + 0.5 ml Aqua regia/l) had the highest value (1.066). In the Levan cultivar, T2 (1.5 mg CNT + 0.5 ml Aqua regia/l) exhibited the highest expression (0.717). The variations observed among the three cultivars were non-significant. However, Levan was the least expressed for gene *UGT74G1,* which confirmed the presence of cultivar-specific role in studying this gene expression.

- **The relative expression level of the *UGT85C2* gene among the three stevia cultivars treated with different concentrations of MWCNT**

When comparing the relative expression levels of the *UGT85C2* gene across the three stevia cultivars (High Sugar, ShouA3-2, and Levan) treated with different concentrations of MWCNT, all cultivars showed only minor, non-significant variations in gene expression (Fig.8).

In the High Sugar cultivar, expression levels ranged from 1.12 (control) to 1.22 (AR treatment), with MWCNT treatments (T1, T2, T3) showing intermediate values (1.19, 1.13, and 1.15, respectively). No clear dose-dependent pattern was observed, as the highest expression was found in the AR group rather than any MWCNT treatment.

For the ShouA3-2 cultivar, expression levels varied between 1.16 (T2) and 1.31 (T1), with the control group showing relatively high expression (1.27). Like High Sugar, no dose-dependent response was evident, as the lowest MWCNT concentration (T1) yielded the highest expression.

The Levan cultivar exhibited the lowest overall expression levels among the three cultivars (ranging from 0.569 to 0.629). Expression tended to decrease slightly with increasing MWCNT concentration, with T3 showing the lowest value (0.569) compared to the control (0.614) and AR treatment (0.629).

According to the previous interpretations, there were no significant differences in this gene expression among the three cultivars. However, Levan was the least expressed for gene *UGT85C2,* which confirmed the presence of cultivar-specific role in studying this gene expression.

These findings revealed notable alterations in the expression of *UGT74G1* and *UGT85C2* genes in response to MWCNT treatments, exhibiting concentration-dependent effects that varied among the different stevia cultivars. The observed cultivar-specific modulation of steviol glycoside biosynthesis in response to MWCNTs aligns with previous studies demonstrating the ability of nanomaterials to influence plant gene expression. These

results were consistent with those of Ramezan *et al.* (2022), who reported enhancements in stevia growth and metabolism following carbon nanotube treatment. Similarly, Al-Shaheen *et al.* (2021) showed positive effects of nano-fertilizer and amino acid treatments on stevia growth and phytochemical content.

The cultivar-specific responses in gene expression observed in this study were also in line with findings from other plant species. Zhuzhukin *et al.* (2023) documented the upregulation of biosynthetic genes in response to nanomaterial treatments in branch, while Wang *et al.* (2016) and Lala (2021) observed variety-specific responses to nanomaterial treatments in different plant species.

However, it is important to note that there was conflicting evidence regarding the effects of carbon nanomaterials on plant gene expression and secondary metabolite biosynthesis. Cisneros *et al.* (2023) reported only a moderate decrease in the level of *AtTOR* gene expression in *Arabidopsis thaliana* treated with multi-walled CNTs, suggested limited disruption of the *TOR* signaling pathway. Additionally, Rezaei *et al.* (2019) found no significant differences in the growth parameters or secondary metabolite production in *Melissa officinalis* plants treated with MWCNTs.

The results suggest that while *UGT74G1* and *UGT85C2* are involved in steviol glycoside biosynthesis, the regulation of stevioside and rebaudioside A production in response to MWCNT treatments is likely to involve complex mechanisms beyond transcriptional control of these specific genes. Post-transcriptional regulation, enzyme activity, substrate availability, or the influence of other genes in the biosynthetic pathway may play significant roles in determining the final metabolite content.

The discrepancy between gene expression and metabolite accumulation highlights the complexity of plant secondary metabolism and suggests that MWCNTs may influence steviol glycoside production through multiple pathways, potentially including effects on enzyme activity, metabolite transport, or other regulatory mechanisms not captured by these two gene expression analyses alone.

The relative stability of both *UGT74G1* and *UGT85C2* expressions across stevia cultivars suggests that steviol glycoside biosynthesis in these cultivars may be regulated by mechanisms that are not readily influenced by MWCNTs at the tested concentrations. These findings underscore the complexity of the interactions between carbon nanomaterials and plant physiology, influenced by factors such as plant species, cultivar, nanomaterial properties, and experimental conditions.

Conclusion: This study highlights MWCNTs as effective nano-tools for en-

hancing *Stevia rebaudiana* growth and steviol glycoside production *in vitro*. MWCNTs significantly improved shoot proliferation, root development, and leaf growth, with cultivar-specific optima (e.g., ShouA3-2 under T1: 14.33 cm shoots). Steviol glycoside content varied by cultivar and treatment, notably with Levan exhibiting elevated stevioside (2788 ppm) under T2. Gene expression (*UGT74G1, UGT85C2*) showed non-significant modulation under different treatments, underscoring genetic variability over MWCNT-driven changes. Results emphasize the need for tailored MWCNT to balance growth promotion and metabolite yields. This advances nanotechnology's role in sustainable agriculture, enabling optimized phytochemical production in stevia and related species. Future research should focus on refining MWCNT concentrations, exploring synergistic effects with growth regulators, and elucidating molecular mechanisms underlying cultivar-specific responses. This study advances the integration of nanotechnology in plant biotechnology, providing a foundation for sustainable agricultural practices aimed at maximizing the production of high-value phytochemicals in *Stevia rebaudiana* and other medicinal plants.

SUMMARY

This study investigated the effects of multi-walled carbon nanotubes (MWCNTs) on growth, steviol glycoside production, and gene expression in three *Stevia rebaudiana* cultivars: High Sugar, ShouA3-2, and Levan. The research aimed at understanding how MWCNTs impact these important aspects of stevia cultivation, potentially informing future agricultural practices and steviol glycoside production methods. MWCNTs non-significantly affect shoots number and shoots diameter means in the three cultivars, and showed significant differences for shoots length means across all cultivars and number of roots in Levan under T3 and number of leaves in all cultivars under T2 when applied to *in vitro* cultures at 1, 1.5, and 2 mg/L MWCNTs concentrations. HPLC analysis revealed cultivar-specific responses in steviol glycoside content. The High Sugar cultivar showed increased stevioside (2625.60 ppm) and rebaudioside A (1971.34 ppm) content with Aqua regia treatment. The ShouA3-2 cultivar exhibited maximum stevioside content (1681.86 ppm) at 1 mg/L MWCNTs, while the Levan cultivar produced the highest stevioside (2788.194 ppm) at 1.5 mg/L MWCNTs. Gene expression analysis of *UGT74G1* and *UGT85C2* demonstrated cultivar-specific modulation in response to MWCNTs. The two cultivars, High Sugar and ShouA3-2, showed higher expression than Levan. The study reveals that the effects of MWCNTs on *Stevia rebaudiana* are cultivar-specific, particularly regarding steviol glycoside production and gene expression. The relationship between gene expression and steviol glycoside content under MWCNT treatment is complex and not always directly correlated, highlighting the intricate nature of secondary metabolite biosynthesis in *Stevia rebaudi-*

ana. The relative stability of both *UGT74G1* and *UGT85C2* expression across stevia cultivars suggests that steviol glycoside biosynthesis in these cultivars may be regulated by mechanisms that are not readily influenced by MWCNTs at the tested concentrations.

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Table (1): Different concentrations of MWCNT and Aqua regia treatments.

Treatments	Aqua regia concentrations	MWCNT concentration
-ve Control	0	0
Aqua regia	1 ml	0
T1	1 ml	1 mg / l
T2	1 ml	1.5 mg/l
T3	1 ml	2 mg/ l

Table (2): Sequences of the gene-specific primer pairs used for RT-PCR analysis of MWCNT-treated *S. rebaudiana* plants.

Gene	Primer's name	Sequence 5′ → 3′	Annealing Temp. (°C)	Product size (bp)	Reference
UDP-glycosyltransferase 74G1 (*UGT74G1*)	*UGT74G1*-F	TCCTGGATTTCCAGTGCTTC	56	80	**(Ramezani, *et al.*, 2020)**
	UGT74G1-R	GAGACCAAGGGCTCTGTATTTG			
UDP-glycosyltransferase 85C2 (*UGT85C2*)	*UGT85C2*-F	CAAGAGTTGATGGGAGAAGGAG	56	137	
	UGT85C2-R	AGCACGGTGATTTCCTTGAC			
β-Actin housekeeping	Actin -F	TCGAACACGGTATTGTCAGC	56	143	
	Actin -R	CTTTTCTCTGTTCGCCTTGG			

Table (3): The effect of MWCNT treatments on growth parameters.

Parameter	Cultivar	-ve Control	T1	T2	T3	p-value
Shoots/Plant	High Sugar	1.33 ± 0.67	2.00 ± 0.00	1.67 ± 0.33	1.78 ± 0.15	0.682 (NS)
	ShouA3-2	1.33 ± 0.33	1.67 ± 0.33	2.00 ± 0.00	1.67 ± 0.17	0.382 (NS)
	Levan	2.00 ± 0.00	**3.33 ± 1.33***	2.00 ± 0.00	1.67 ± 0.17	0.452 (NS)
Shoot Length (cm)	High Sugar	2.00 ± 0.12	**6.07 ± 0.71***	3.27 ± 0.50	**6.57 ± 0.23***	**0.001***
	ShouA3-2	7.47 ± 2.80	**14.33 ± 1.35***	**12.40 ± 2.49***	**10.00 ± 0.51***	0.134 (NS)
	Levan	5.07 ± 0.93	**13.07 ± 2.26***	2.73 ± 1.71	6.47 ± 1.69	**0.009***
Roots/Plant	High Sugar	0.00 ± 0.00	1.00 ± 1.00	0.00 ± 0.00	**1.83 ± 0.17***	0.211 (NS)
	ShouA3-2	3.00 ± 1.16	5.33 ± 1.20	7.00 ± 1.53	5.00 ± 0.58	0.090 (NS)
	Levan	4.00 ± 0.58	**8.33 ± 1.67***	3.33 ± 2.85	**7.00 ± 1.53***	0.220 (NS)
Leaves/Plant	Levan	10.67 ± 1.76	**17.00 ± 2.52***	**14.33 ± 0.33***	9.67 ± 0.88	**0.031***

NS = non-significant. * = Significant at $p \leq 0.05$* = Significant at $p \leq 0.05$.

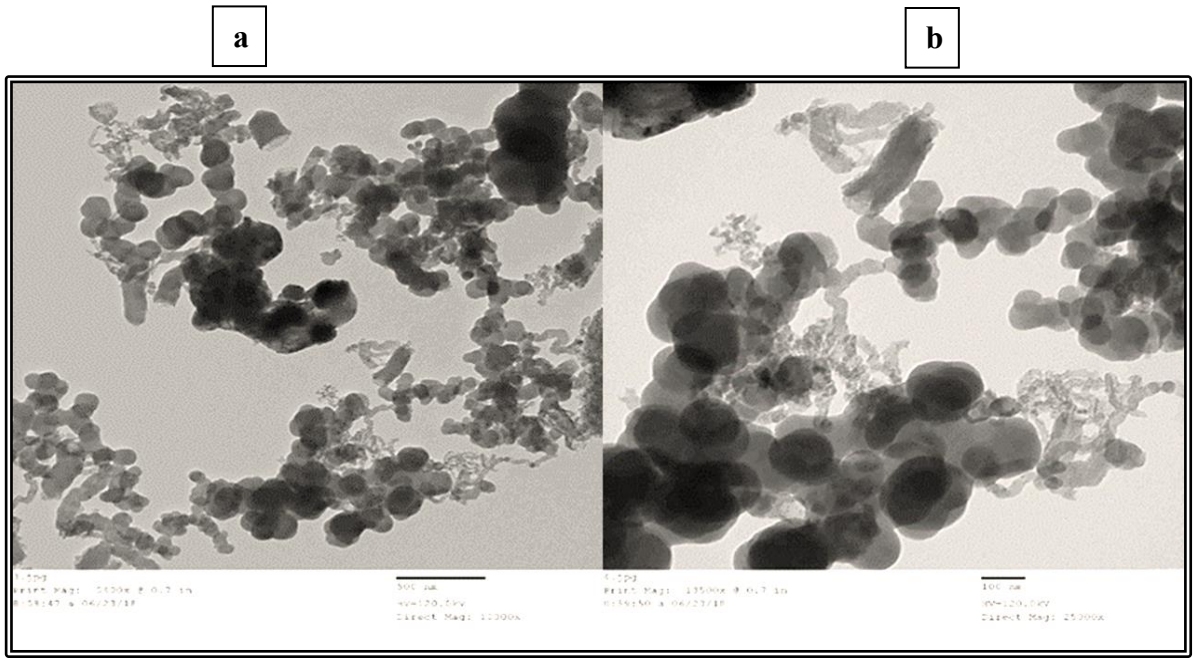

Fig. (1): Multi-walled carbon nanotubes (MWCNTs) were observed by transmission electron microscopy (TEM) using different magnifications. (a) Black bar = 500 nm. (b) Black bar = 100 nm.

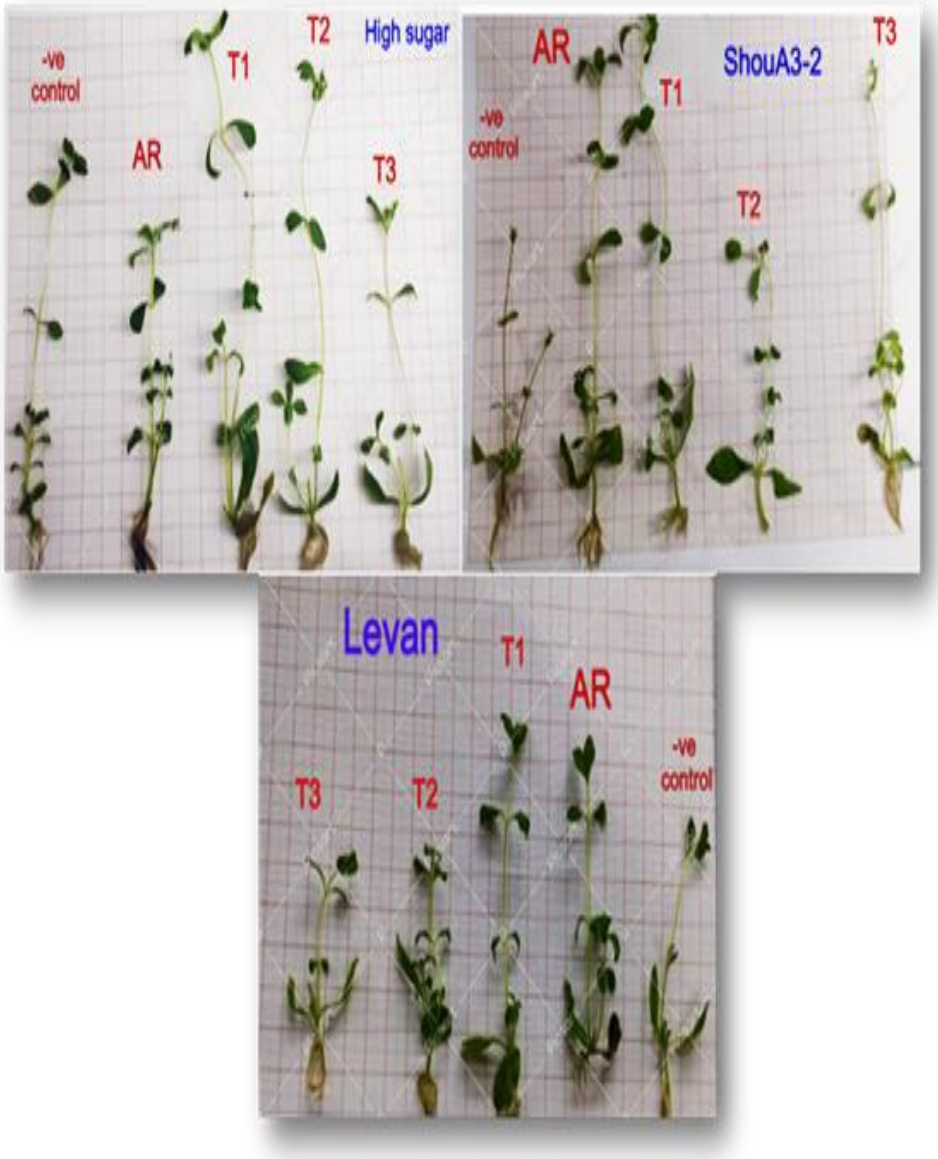

Fig. (2):The effect of MWCNTs on three Stevia cultivars (High sugar, ShouA3-2, and Levan). The treatments are -ve control (0) AR (1ml Aqua regia) T1(1mg/L MWCNT+ 1ml Aqua regia) T2(1.5mg/L MWCNT+ 1ml Aqua regia) T3(2mg/L MWCNT+ 1ml Aqua regia).

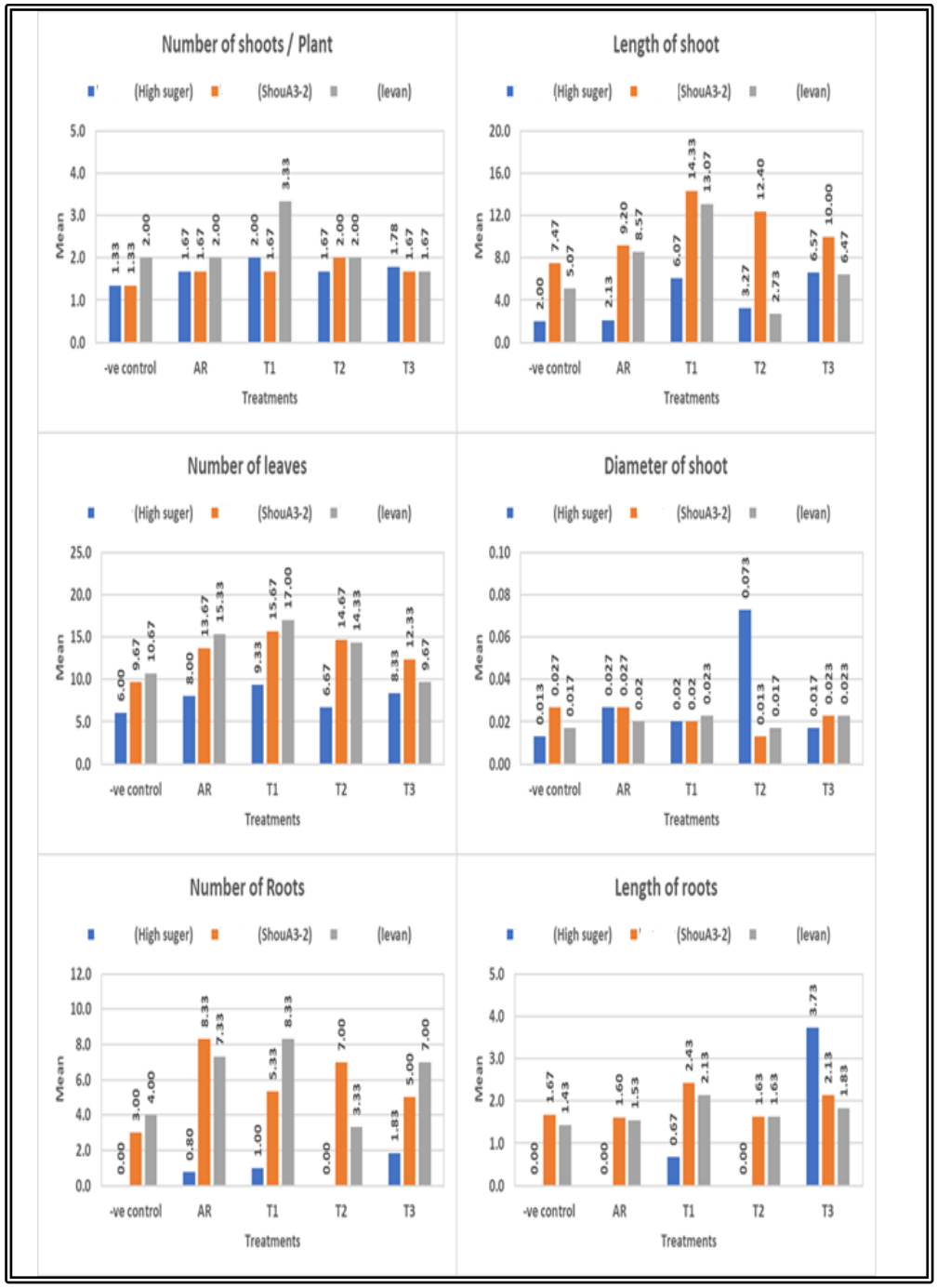

Fig. (3): Dendrogram for the means of the six morphological characters in the three studied stevia cultivars under the different tested treatments. The cultivars were cultured

in a free hormone MS medium supplemented with varying concentrations of MWCNTs.

Fig. (4): The three cultivars after acclimatization in the greenhouse, **A**: ShouA3-2, High sugar, and Levan. **B**: ShouA3-2 shows superior traits under T1 treatment after acclimatization.

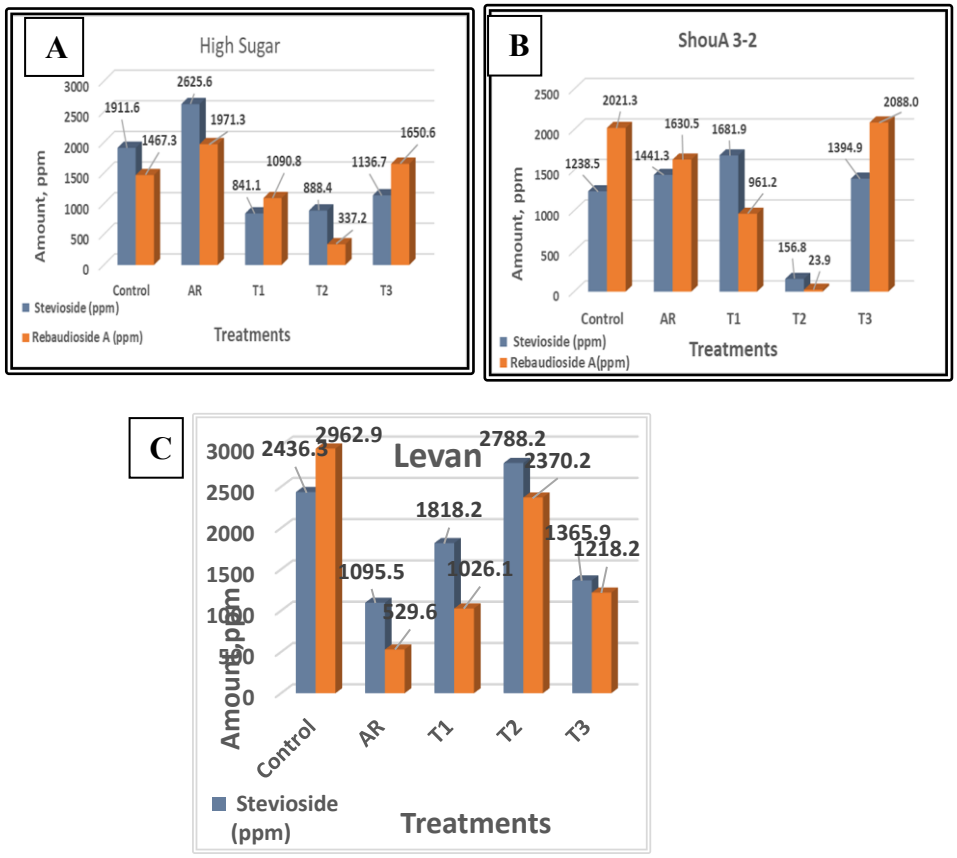

Fig. (5): Dendrogram for HPLC analysis of steviol glycosides in *Stevia rebaudiana* (A) High Sugar, (B) ShouA3-2, and (C) Levan cultivars.

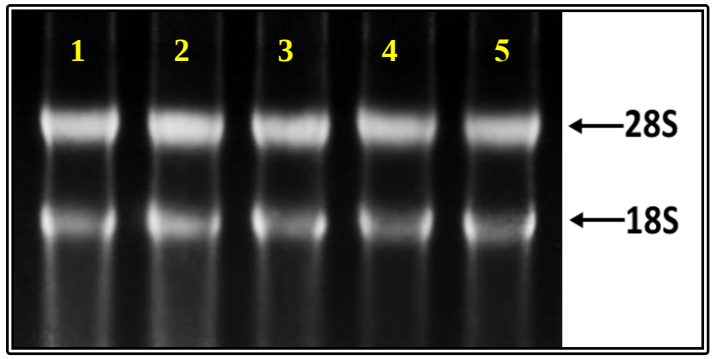

Fig. (6): Agarose gel electrophoresis results of RNA isolates from the samples (1= -control, 2= AR, 3= T1, 4= T2, and 5= T3). The arrows indicate the location of 28S and 18S rRNA fragments.

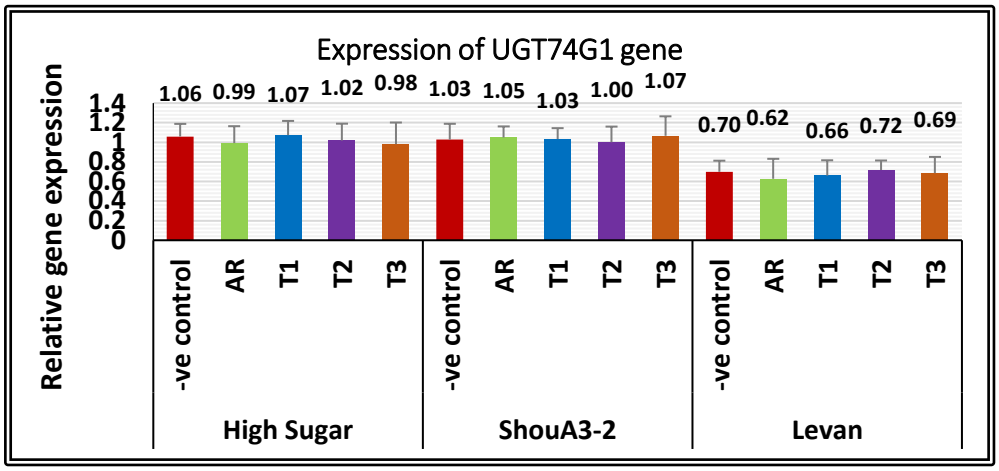

Fig. (7): Dendrogram for the relative expression level of the *UGT74G1* gene in the three stevia cultivars (High Sugar, ShouA3-2, and Levan) was treated with different concentrations of carbon nanotubes (CNT). -ve control: untreated group, AR: 1ml Aqua regia/L, T1: 1 mg CNT+1 ml Aqua regia/L, T2: 1.5 mg CNT+ 1 ml Aqua regia/L, and T3: 2 mg CNT+ 1 ml Aqua regia/L. Mean values with different superscript letters differed significantly ($p < .05$).

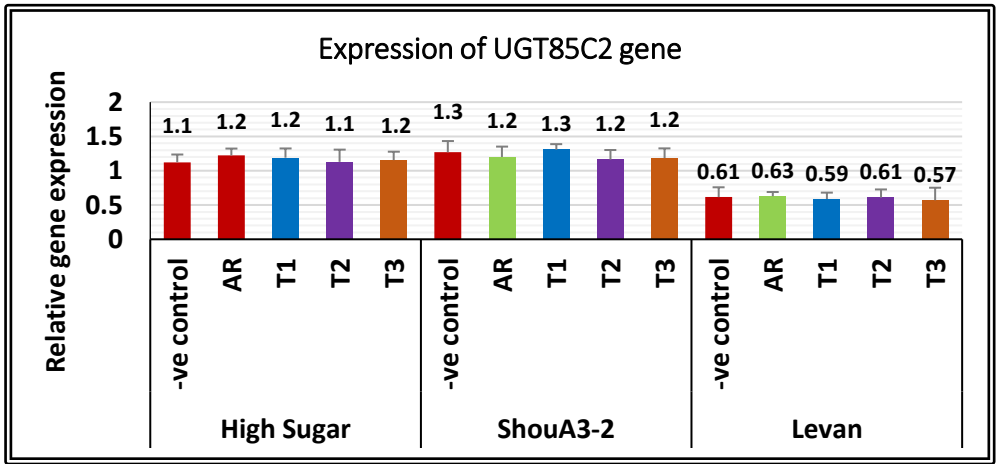

Fig. (8): Dendrogram for the relative expression level of the UGT85C2 gene in the three stevia cultivars (High Sugar, ShouA3-2, and Levan) were treated with different concentrations of carbon nanotubes (CNT). -ve control: untreated group, AR: 1 ml Aqua regia/L, T1: 1 mg CNT+1 ml Aqua regia/L, T2: 1.5 mg CNT+1 ml Aqua regia/L, and T3: 2 mg CNT+ 1 ml Aqua regia /L. Mean values with different superscript letters differed significantly ($p < .05$).