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Impact of Various Plant Growth Regulators on Induction *Stevia Rebaudiana* Shoots *in Vitro*

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Annotation: The study was carried out to establish and develop the protocol of micropropagation and overcome low germination rates of stevia seeds, Focusing on the role various growth regulators on induction of stevia plant shoot in vitro. The experiments involved adding different concentrations of Auxin Dichloro phenoxy aceticAcid (2.4-D) and Cytokinins Benzyle adenine (BA) and (TDZ) Thidiazuron to MS medium to determine their effect on the leaves and shoots number, shoot length and dry weight of the shoot. The results showed that interactions of 2 mg. L-1 2,4-D with 0.4 mg L-1 TDZ achieved highest average shoot length of 7.400 cm compare with the rest of the treatments. Also date appeared that the explants which grown in media containing 1mg L-1 2,4-D and 4 mg. L-1 BA achieved highest average number of leaves reaching 9.249 leaves and the number of shoots reaching 3.727 shoots compared with other treatments, which was reflected in the increase in the shoots dry weight formed, also same treatment gave an average dry weight 0.160 gm,. From this, we conclude that the multiplication and growth of the explant of the stevia was better when the MS nutrient medium was included with auxin 2,4-D and cytokinin BA.

Keywords: Plant growth regulators, stevia, tissue culture protocol, Invitro.

Introduction

Stevia rebaudiana Bertoni It is also called sugar bush is a perennial herbaceous plant the forests of Brazil and Mexico are original habitat in the original home of the stevia plant (38, 21 and 1). There are 230 species of the genus Stevia, the most common of which are S.ovata, S.salicifollia, and S.eupataria, but the sweetest species is S. rebaudiana 12 (31) In the late 1990s, the demand for this plant increased due to its medicinal and nutritional benefits. The plant has various properties, such as being antibacterial, antifungal, and antiviral, and is used to treat heart disease, as a diuretic, and as a hypoglycemic agent in blood (11). The Stevia plant is propagated either sexually by seeds or vegetatively by stem cuttings, The seed propagation method presents many problems, the huge problem in propagation of Stevia is low germination rate, which is, besides existence self- incompatibility in the species, these obstacles in general produces fertilization failure (in addition to the low germination rate Because the seeds in Stevia are very small, they do not provide the same genetic composition and sweetness as the leaves (22 and 37). Propagating through the stem cutting requires great efforts so The number of plants producing it is very limited. Based on the above, and to overcome all these problems, researchers have made attempts to harness plant tissue culture technology of propagation (44, 25, 26 and 30). growth regulators control growth and development plant and are compounds that are produced naturally or artificially and its play principle role in stimulants, inhibitors, or retardants, its added on some stages of plant growth to achieved development in other stages (6). So it's added in low concentrations, absorbed from plant tissues, and then transported to sites where they are bound Receptor, and then a secondary signaling system is activated to stimulate or inhibit cell activity. adding concentration and type of growth regulators to the media play essential role in determining the desired aim of cultivation Tissues, in vitro micropropagation (5 and 7). Auxins and cytokinins have an important role in the induction and multiplication of vegetative shoots, Auxins are important growth regulators used in plant tissue culture A group of indolic acids with an unsaturated cyclic nucleus, or auxins may be derivatives of these Acids, have high molecular weight, are added in minimum concentrations to produce a significant effect on the explant. The apical meristems, lateral shoots, and fresh leaves are the very important centers of its construction (24). Such as Indole acetic acid (IAA) and manufactured Such as (NAA) Naphthalene acetic acid, (IBA) Indole Butric acid and (2,4-D) Dichlorophenoxy acetic acid (18), and they work in stimulating the enzymes responsible for Building and decomposing the cell wall, and then influencing the mechanical properties of the cell wall and stimulating the softness of the cell wall By breaking the bonds of the cell wall and restoring them under the influence of swelling pressure, which contributes to increasing the cell volume Its expansion, cell elongation, and organ development or formation (40). In the same direction, cytokinins play a major role in tissue culture, as they encourage, also researchers (2 and 3) concluded that adding TDZ at low concentrations resulted in an increase in the number of leaves and shoots, exactly the opposite at high concentrations. Cell division and differentiation, stimulates the growth of axillary buds, breaks the apical dominance, and inhibits root formation. And it is used Several types, such as Zeatin, Kinetin, 2ip, and BA, in addition to TDZ. Due to the importance of cytokinins, and Auxiens many studies have been conducted to determine their effect on explant *invitro* micropropagated. (42) demonstrated the success of propagating stevia plants using MS medium It contains different concentrations of BA and IAA, where interaction treatment (1 mg L^{-1} BA and 0.1 mg L^{-1} NAA) got maximum shoots number per plant. So (28) added different concentrations of growth regulators BA and NAA in the propagation of stevia plants *invitro*, the interaction treatment (1 mg L^{-1} of BA with 0.1 mg L^{-1} of IAA) gave positive results of shoots number per plant. (45) was clear that medium prepared with 3mg. L^{-1} BA gave optimum shoot number and its length, leaves number, moreover fresh weight, (13) found there is a significant increasing of shoot number 8.79 and shoot length 2.81cm of *stevia rebandiana* invitro propagated from internode with

addition BA 3ppm. (17) was observed adding BAP, TDZ and Kin gave Positive results to increase shoot formation and leaf number of stevia. (16) tested adding different type of Auxine (NAA, IBA) and cytokinenes (TDZ, Kin and BA) on invitro propagation of stevia the results proved there was significant difference between the treatments where TDZ with IBA achieved maximum value of length and shoot number and compared with other type of growth regulator. (34) found that adding BA at 3 mg L^{-1} with NAA 0.3 mg L^{-1} gave significantly superior of length and number of shoots of stevia plant *invitro* propagated. (41) they proved that Auxines and cytokinens have positive role in increase shoot and leaf number and shoot length. The aim of research to Overcoming obstacles in propagating stevia plants in the field due to the low germination rate of its seeds and select a program to propagate it In vitro So test different concentrations of Auxin and cytokinen and their effect on the vegetative multiplication of stevia plants.

Material and methods

The research was conducted in the lab. of the Date palm Research Unit - College of Agricultural Engineering Sciences / Baghdad University on Stevia plant, variety Spanti.

Preparation of plant parts: Green branches of 17-20 cm in length were eradicated from Stevia plantlet, the leaves and roots were detached, and they were cut into 1 cm stem nodes and washed with running tap water for 20 minutes and next off with liquid soap for 2 minutes. After that, they were washed with sterile water to remove the liquid soap residue and soaked in 70% ethanol alcohol for 3 minutes and washed with sterile distilled water 6 times to remove the alcohol residue. After that, they were sterilized by soaking them in a 3% sodium hypochlorite solution (NaOCl) with continuous shaking for 15 minutes, adding drops of Tween 20 to break the surface tension. Then, they washed with sterilized distilled water three times and transferred to MS culture medium (26).

Initiation stage: Stem nodes were grown on MS medium (33) containing vitamins (mgL^{-1}) 0.1 Thiamin, 0.5 Nicotinic acid, 0.5 Pyridoxine, 2.0 Glycine,100 Inositol with 30 gm L-1 sucrose, Agar 7 gm L-1, NAA and Kin at a concentration of 0.3 mgL-1. MS was used supplied by the USA company (Caisson), with a weight of 4.41 grams per liter, so at 25°C explants were incubated further more light intensity for 4 weeks at (1000 lux) and light period 16/8 light/dark. (Fig. 17 and 18).

Bud proliferation stage: the resulting shoots from the initiation stage were transferred to MS medium with a length of 1 cm added to the following treatments (mgL-1): Auxins 2,4-D-dichlorophenoxyacetic acid (0, 1 and 2 and cytokinins benzyl adenine BA (0, 2 and 4) and TDZ thidiazuron (0, 0.2 and 0.4) and their reactions with ten replications. The cultures were incubated at 25°C under 1000 lux for 16/8 light/dark cycle for four weeks and then measurements were taken.

Characteristics studied

1- Average shoots length

2- Average leaves number

3- Average shoots number

4- mean dry weight

The experimental was desighned as factorial experiment with eight replicates with using completely randomized design (CRD) and the means were compared according to the least significant difference test at 0.05 probability level (9).

Result and discussion

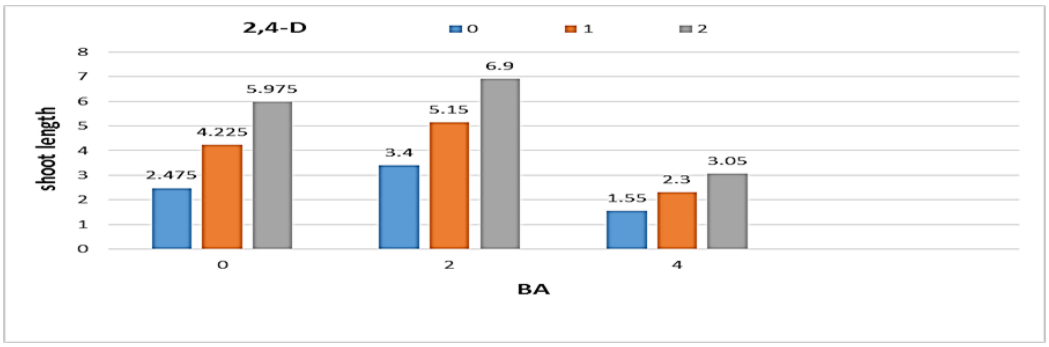

Fig 1. Influence of DiChlorophenoxy acetic acid (2,4-D) and Benzyleadenine (BA) on shoot length cm

The results of fig 1 indicate that there is a significant effect of adding 2,4-D to the growing medium, as the concentration of 2 mg.L-1 gave the highest average shoot length of 5.97cm, while this average decreased to 2.475 cm at the concentration of 0 mg.L-1. The results of fig 2 show that increasing BA concentration to 4 mg.L-1 led to a decrease in shoot length to 2,300 cm, while a concentration of 2 mg.L-1 gave the highest average of 5,150 cm. The results of the same Fig, indicate that there is a significant interaction between the study treatments in the effect on shoot length. The 2 mg.L-1 2.4-D and 2 mg.L-1 BA treatment achieved the highest average of 6.900 cm, while the 0 mg.L-1 2.4-D and 4 mg. L-1 BA treatment achieved the lowest average of 1.550 cm.

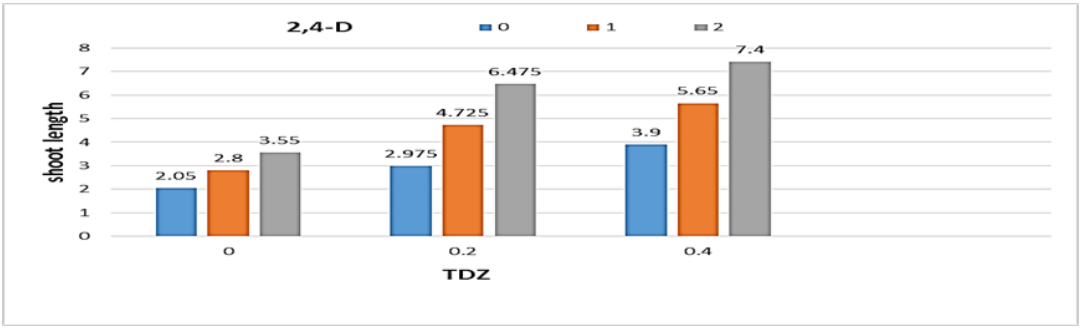

Fig2. Influence of DiChloroPhenoxyacetic acid (2.4-D) and (TDZ) Thidiazuron on shoot length cm.

The results of Fig 2 indicate that increasing the concentration of 2,4-D to 2 mg.L-1 led to an increase in the average shoot length reached to 5.808 cm, while this average decreased to 2.975 cm at the concentration of 0 mg.L-1. also from the same Fig, find adding TDZ to media achieved positive results of same characteristic where 0.4mg.L-1 gave maximum value 5.650 cm compared with lowest value 2.8 cm for the concentration 0 mg.L-1. concerning the interaction treatment of 2,4-D with TDZ achieved Positive results where treatment 2mg.L-1 2,4-D and 0.4mg.L-1 TDZ gave highest average shoot length 7.400 cm compared with lowest value reached to 2.050cm for the treatment 0 mg.L-1 2,4-D and 0 mg.L-1TDZ.

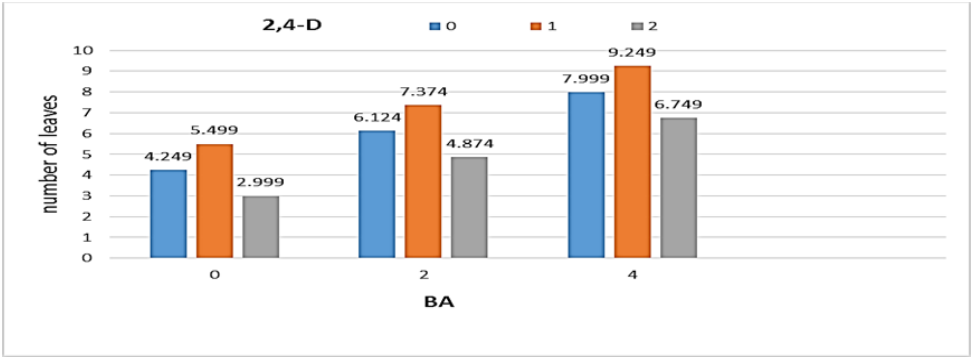

Fig3. Influence of DiChloroPhenoxyacetic acid 2.4-D and Benzyle Adenine (BA) on leaves number

The results in Fig 3. indicate that adding different concentrations of NAA to the culture medium led to a significant increase in the number of leaves formed from the culture of stem nodes, as the 0.6 mg L-1 NAA treatment gave the highest average of 6.192 leaves, while the 0 mg L-1 treatment gave the lowest average of 5.358 leaves. The results of Fig 7 showed a significant effect of TDZ on the number of leaves, as the concentration of 0.2 mg L-1 gave the highest number of leaves, 7.400 leaves, compared to the concentration of 0 mg L-1, which gave the lowest average number of leaves, 4.150 leaves. The effect of the interaction between NAA and TDZ was significant in increasing the number of leaves (Fig 3), as the concentration of 0.6 mg L-1 NAA and 0.2 mg L-1 TDZ gave the highest average of 8,650 leaves, while the interaction of the concentration of 0 mg L-1 for both NAA and TDZ gave the lowest average of 2,900 leaves.

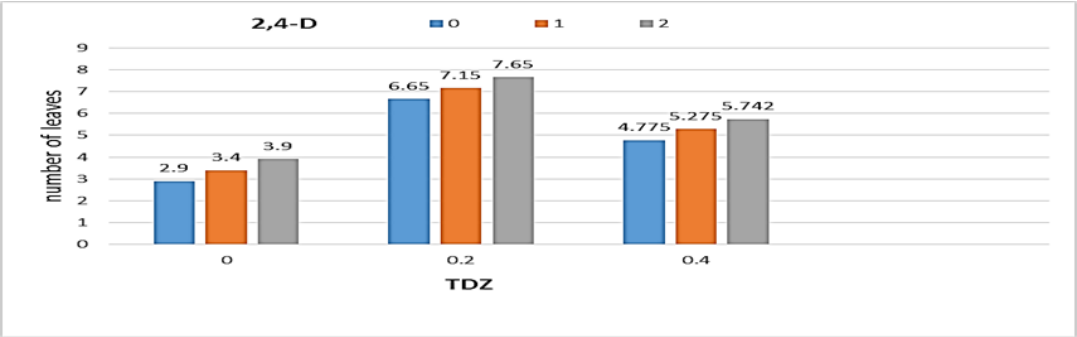

Fig 4. Influence of DichloroPhenoxyacetic acid (2.4-D) and (TDZ) Thidiazuron on leaves number.

The results of Fig 4. showed significant differences between the concentrations of 2,4-D to affect the number of leaves trait of the stevia plant, as the 2 mg L-1 treatment gave the highest average of 5.764 leaves, compared to the 0 mg L-1 treatment, which gave the lowest average of 4.775 leaves. The results of the same Fig.4 showed that TDZ concentrations gave positive results in the average number of leaves, as the highest average number of leaves reached 7,400 leaves at a concentration 0.2mg.L-1 compared to a concentration of 0 mg L-1, which gave the lowest average of 3,650 leaves. The results showed no significant differences between the concentrations of 2,4-D and TDZ in affecting the number of leaves.

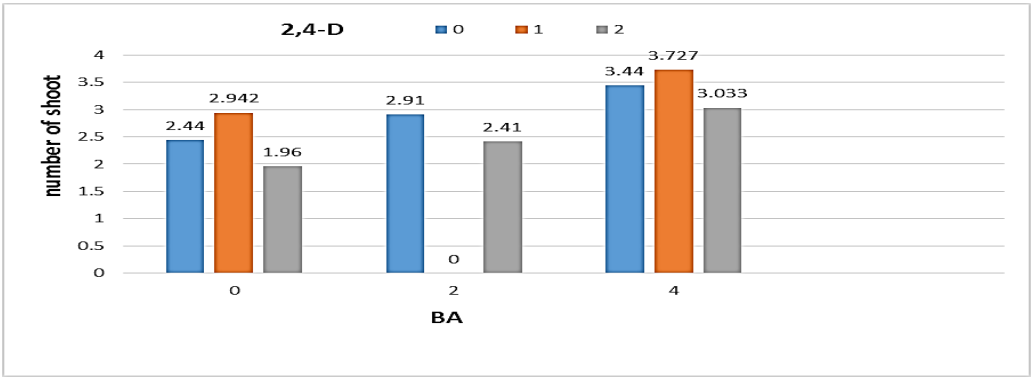

Fig 5. Influence of different concentrations of Dichlorophenoxyacetic acid (2.4-D) and Benzyle adenine (BA) on shoots number.

The results of Fig.5 indicate the average number of shoots increased to 3.353 shoot at a concentration of 1 mg. L-1 2,4-D, while this average decreased when the concentration increased to 2 mg. L-1 reaching 2.468 shoot. The results of the same Fig indicate an increase in the average number of shoots to 3.400 shoot with an increase in the BA concentration to 4 mg L-1, while this average decreased to 2.447shoot at a BA concentration of 0 mg L-1.The results of the Fig.10 indicate that the growth of explant in media containing 1 mg L-1 2,4-D and 4 mg L-1 BA gave the highest average number of shoots, reaching 3.727 shoot, while this average decreased to 1.960 shoot at a concentration of 2 mg L-1 2,4-D and 0 mg L-1 BA.

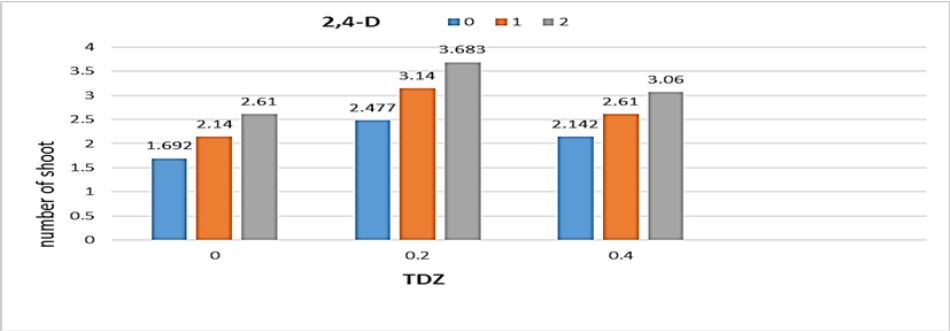

Fig 6. Influence of different concentrations of Dichlorophenoxyacetic acid (2.4-D) and (TDZ) Thidiazuron on shoots number.

Results of Figure 6. shows significant differences in 2,4-D concentrations in their effect on shoot number, where 2 mgL-1 produced the highest mean of 3.118 shoots, while 0 mgL-1 treatment produced the lowest mean of 2.103 shoots. The results of the same figure showed an increase in shoot number with 0.2 mgL-1 TDZ concentration where it produced a maximum mean of 3.100 shoots, while the 0 mgL-1 treatment produced a minimum mean of 2.147 shoots. Results Figure 6. shows that the growth of the sample in the medium containing 2 mgL-1 2,4-D and 0.2 mgL-1 TDZ gave the highest result in shoot number with 3.683 shoots, while the mean value decreased to 1.692 shoots when no growth regulators were added to the growth medium.

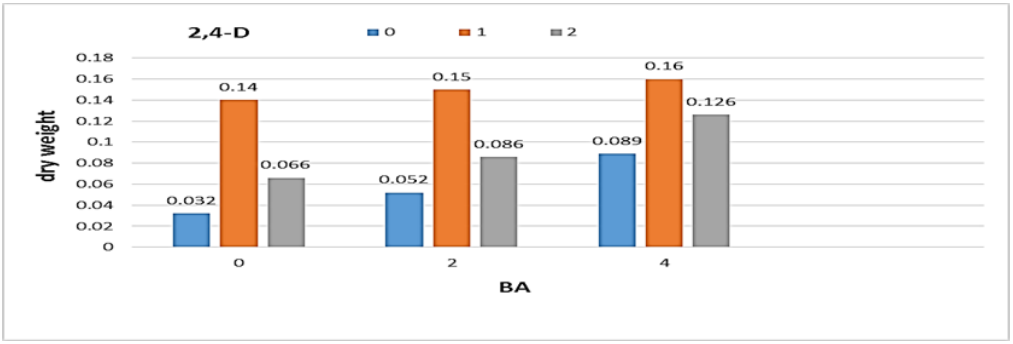

Fig7. Influence of Dichlorophenoxyacetic acid (2.4-D) and Benzyle adenine (BA) on dry weight gm.

The results in Figure 7. show that the media containing different concentrations of 2,4-D have a significant effect on the average dry weight, with the application of 1 mg L-1 significantly exceeding other concentrations, reaching 0.150 gm, and the average dry weight of decreases with the increase of concentration 2,4-D, while 0 mg L-1is the lowest and gave 0.058 gm as for the role of BA, its effect on the increase of average dry weight is positive, gave the concentration of 4 mg L-1 increases significantly, reaching 0.079 gm, while the average dry weight is 0.058 gm at concentration 0 mg L-1. The combined use of BA and 2,4-D had a significant effect on the average dry weight, the increased at 1 mg L-1 2, 4-D and 4 mg L-1 BA and reached 0.160 gm The comparative treatment without growth regulators gave a lower dry weight, reaching 0.032 gm.

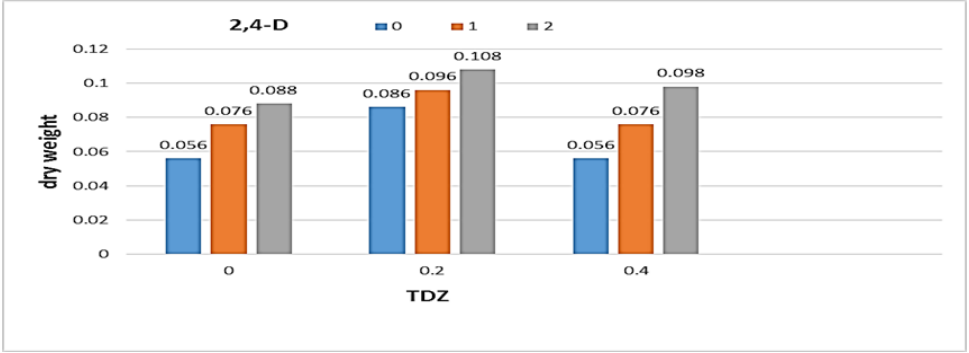

Fig 8. Influence of Dichlorophenoxyacetic acid (2.4-D) and (TDZ) Thidiazuron on Dry weight gm.

The data in Figure 8 show a significant effect of 2,4-D levels on the dry weight as the 2 mgL-1 treatment gave the highest mean value of 0.098 gm compared to the 0 mgL-1 treatment which gave the lowest mean value of 0.066 gm. The results of the same figure showed that TDZ concentrations gave positive results on the average dry weight as the highest value of average dry weight was achieved of 0.097 gm at 0.2 mgL-1 compared to the 0 mgL-1 concentration which gave the lowest mean value of 0.073 gm. The results of the interaction of 2,4-D and TDZ concentrations show that the addition of 2,4-D at 2 mgL-1 with TDZ at 0.2 mgL-1 resulted in a positive effect on the dry weight gave the highest average value of 0.108 gm compared to the addition of 2,4-D and TDZ at 0 mgL-1for both in the culture medium and obtained a below average value of 0.056 gm. This average value was not significantly different from the interaction treatment between the addition of 2,4-D at 0 mgL-1 and TDZ at 0.4 mgL-1 in the culture medium.

From previous studies on the effect of cytokines and auxin in determining the type of cell differentiation and organ formation outside the living organism, we conclude that high concentrations of cytokinins and low concentrations of auxins in the environment determine the formation of plant buds that grow inside the stem (4), while auxin stimulates genes that control the expression of cytokinin genes and causes gene expression to have a significant effect on biological activities such as chloroplast development, cell division, and nutrient metabolism, so the increase in the number of branches and leaves may be due to the action of cytokines (26). It has been reported (35) that 2,4-D promotes the destruction of the bonds that support the cell wall, which leads to cell expansion and increased metabolic accumulation, in addition to its effect on activating metabolic pathways responsible for mRNA synthesis, which produces amino acids with proteins, thus increasing the biomass of plant cells and thus the dry weight. These results are consistent with (14, 32, 18, 8) that auxins work to expand and divide cells by increasing the structure and activity of some enzymes responsible for increasing the flexibility of the cell wall and increasing its permeability, or they may affect the metabolism of nucleic acids and thus affect the structure of some important proteins involved in increasing the flexibility of the wall. From the results, we also see that increasing the concentration of cytokines caused doubling of vegetative growth and thus increasing the dry weight by stimulating cell division and interrupting apical dominance. It is believed that cytokines are part of the transfer RNA near the anticodon, which plays an important role in linking the transfer RNA to the messenger RNA during protein formation. Cytokines also work to attract nutrients to the sites of transfer RNA processing factories and increase the production of RNA, proteins and enzymes inside the cells (37 and 7). The dry weight of the obtained plant part increased with increasing the concentration of 2,4-D, BA and TDZ. BA also stimulated the flow of nutrients into the treated cells, activated cell division, inhibited protein degradation and stimulated photoenzymes, the effects of which were reflected in increasing cell volume and stimulating the division process, and led to increased formation of RNA, proteins and enzymes within the cells. The results showed that BA was the most effective in influencing the above-mentioned traits, which may be due to its molecular structure consisting of double bonds carrying a side chain, which makes it more stable and effective (19). Regarding the effect of TDZ, it is worth noting that increasing its concentration had a significant effect on the studied traits. TDZ is one of the phenylurea substitute compounds, known for its high efficiency, superior to the natural oleate produced in plants and most other types of cytokinins, and has an effective role in stimulating lateral buds, adventitious bud formation, and somatic embryo formation in the laboratory (22). Its mechanism of action is not yet known, but there are hypothesized mechanisms, the most important of which are activation of mRNA replication, amino acid synthesis, and increased permeability of cell membranes (28, 13, 36, 11, and 33).

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