Plant Archives Volume 20 No. 1, 2020 pp. 1715-1720 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF SOME GROWTH REGULATORS ON GLYCOSIDES LEAF CONTENT OF *STEVIA REBAUDIANA BERTONI IN VITRO*

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Abstract

This research was conducted to study the effect of some plant growth regulators on *in vitro* shoots multiplication of *Stevia* plant and estimation of stevoides content of leaves to compare the production of this substances in the leaves of *in vitro* and in vivo plants. Plant tissue propagation experiments included on the experience, the impact of BAP and IBA in the cultures multiplication, while in the rooting stage included experiments on the effect of NAA and IBA concentrations., Stevioside, Rebaudioside and were detected sugary in the textile and seed plants of *Stevia* leaves using HPLC device. Results showed the effect of interaction, it was noticed that the effect was significant in the number of branches formed and gave the interaction between 1.0 $mg.l^{-1}$ BAP with 0.3 $mg.l^{-1}$ IBA the highest number of branches formed (3.10 branch) which differed significantly from the treatment of the interaction between treatment of 0.1 $mg.l^{-1}$ IBA and BAP-free, which gave the lowest average number of branches was 1.667. The results indicate the superiority of the acclimatized tissue plant in its content of the compound Stevioside and amounted to 115.4 $\mu g.gm^{-1}$ it was significantly superior to laboratory tissue plant (75.5 $\mu g.gm^{-1}$) and seed seedlings (3 9.6). The acclimatized tissue plant was superior in Rebaudioside A and reached 175.2, and laboratory tissue plant 172.7, while the seed seedlings gave the lowest value of the content of this compound was 55.3.

Key words: Growth Regulators, glycosides leaf, *Stevia*.

Introduction

The sugar leaf *Stevia rebaudiana* Bertoni belongs to the compositae family. Native to parts of Brazil and Paraguay native to parts of Brazil and Paraguay than 300 times the sweetening of ordinary sugar used in food, It does not contain any calories in its leaves and is therefore ideal for sweetening foods used by diabetics. It is also used in diet and fitness systems, and it is a perennial herbaceous plant. Stevioside is present in their leaf which is responsible for its sweet taste (Mizutani and Tanaka 2002). South America, especially Paraguay and Brazil, is native to Stevia, There are more than 280 species. Stevia leaves are a source of binary turbine glucosides such as Steviolbioside, Rebaudioside: and Stevioside Dulcoside (Starratt *et al.*, 2002). Stevia is a herbaceous plant that is classified as a perennial plant in tropical or subtropical areas where it stays in the ground for up to seven years. In cold areas, however, the crop is annual plant harvested once before the onset of winter. Stevia is sexually propagated with seeds. The seed multiplication method has many problems, including low germination rate due to the existence of self-incompatibility between plants which result in decreases of seed vitality, so germination does not exceed 20% at best. In addition of, the seeds are small in size and not suitable for direct sowing in field where most of them are damaged, and significant genetic differences between plants caused by seeds due to genetic segregation that occurs during cross-pollination, this leads to variations in the shape of plants and the different concentration and percentage of sweet materials from one plant to another (Miyagawa *et al.*, 1986). Another way of propagation in a way rooting plants start from the age of one year in the formation of 3-5 root parts, each of which gives the whole plant. However, a disadvantage of this method is the small number of plants produced. To overcome these problems, the researchers attempted to propagate through tissue culture, in which vegetative tissue is taken from selected mothers with distinctive productivity, both in terms of vegetative growth or the concentration of sweet materials (Hassanen and Khalil 2013). Khan et al. (2011) confirmed the superiority of Stevioside in stevia leaves among all common stevolic cyclosides extracted by HPLC. Singh and Dwivedi (2014) found that the use of TDZ at a concentration of 0.01 mg / l gave the highest average number of branches of 11 branches / plant part, and the highest percentage of Stevioside in stevia leaves was 236.9 ± 0.46%. The highest number of roots was 11.0 root when 1.0 mg / l IBA and 50 mg / l activated coal were added to MS medium. Abdul Razak *et al*. (2014) were able to obtain the highest number of branches with 7.8 branches / plant part by adding 0.5 mg / L BAP and 0.25 mg / L Kin, and highest root count was 30.12 root when 1.0 mg / l IBA was added to the media of the stevia branch radicles.

Hassanen and Khalil (2013) noted that the addition of 2.0 mg / L BAP to the MS medium for shoot apex and stevia nodules resulted in the highest growth rate. The length of the branches was 1.92 cm, and the highest number of roots was 7.3 roots when adding Alar at a concentration of 0.5 mg / l with IBA at a concentration of 1.0 mg / l. Given the importance of sugar leaf plant from the medical and economic point of view, the aim of this study were:

1. Study the effect of growth regulators on stevia propagation *in.vitro*
2. Evaluation of plant tissue culture efficiency in plant content of Stevioside and Rebaudioside compounds

Materials and Methods

Preparation of plant parts

15-20 cm vegetative branches were taken from the stevia seedlings and the leaves and roots were removed from them, and the ends of the branches and nodes were separated at 0.5 cm long and were cleaned with running water and liquid soap, then sterilized with sodium hypochlorite solution NaOCl at concentrations of 1, 2, 3% for 15 minutes with addition of 20 drops of Tween and wash with sterile distilled water three times.

Initiation stage

Plant parts were planted on the MS media (Murashige and Skoog, 1962) equipped with NAA concentrations of 0.0,0.1, 0.2, and 0.3 mg / l and different concentrations 0.0, 0.1, 0.2, and 0.3 mg/l BAP. Cultures were incubated at 25 ° C under 1000 lux and for a period of 16/8 light / dark for four weeks.

Shoot multiplication stage

The resulting branches from initiation stage were transferred to the MS media equipped with BAP,and the effect of different concentrations of 0.0, 0.5, 1.0 and 1.5 mg/l was studied, with concentration of 0.0,0.1, 0.2, and 0.3 mg/l of IBA. Cultures were incubated at 25°C under 1000 lux and for a period of 16/8 light / dark for four weeks, Then the measurements were taken.

Rooting stage

The plantlets produced from the propagated vegetative phase was transferred to the rooting medium equipped with 0.0, 0.5, 1.0 mg / l of IBA and NAA). Cultures were incubated at 25°C under 1000 lux and for a period of 16/8 light / dark for 4-6weeks, Then the measurements were taken.

Determination of Glycosides

Extraction and estimation of Glycosides**:** Stevioside and Rebaudioside were estimated according to Hurum and Rohrer Method (2011) with some minor adjustments using the HPLC

Characters studied

1. Average number of branches formed
2. The average number of leaves
3. Average length of vegetative branch
4. Percentage of rooting
5. The number of roots
6. Determination of Glycosides (Stevioside and Rebaudioside)

Experimental Design and statistical Analysis

The trial data were analyzed as factorial experiments and ten replications per treatment using Complete Randomized Design (CRD). Means were compared with the least significant difference test (LSD) at a probability level of 0.05 (El-Sahooki and Waheeb, 1990).

Results and Discussion

Effect of BAP and IBA concentrations and their interaction on the average number of branches formed by stevia multiplication after four weeks of *in vitro* transplantation

The results shown in Fig. 1 indicate that the addition of BAP to the media of multiplication of stevia branches *in vitro* resulted in a significant increase in the average number of branches formed up to the concentration of 1.0 mg. l^{-1}, which gave the highest rate of the number of branches formed 2.708 branches compared to the average number of control treatment, which gave the lowest rate of 1.875 branches. As for the effect of the auxin (IBA) on the multiplication of stevia branches, there is also the same significant effect on the average number of branches. It increased significantly and concentration of 0.3 mg.l^{-1} gave the 2.692 branch compared to the control treatment (without auxin) that gave 2.0922 branches. As for the effect of interaction, it was noticed that the effect was significant in the number of branches formed and gave the interaction between 1.0 mg.l^{-1} BAP with 0.3 mg.l^{-1} IBA the highest number of branches formed (3.10 branch) which differed significantly from the treatment of the interaction between treatment of 0.1 mg.l^{-1} IBA and BAP-free, which gave the lowest average number of branches was 1.667.

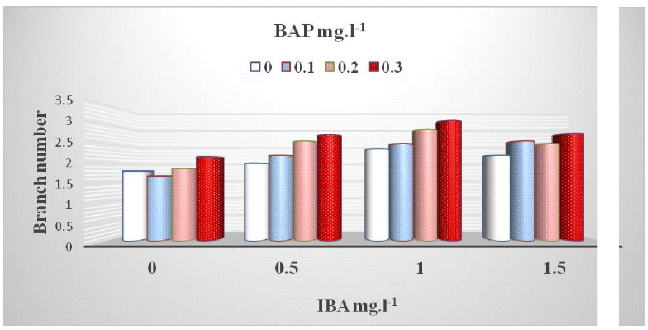

Fig. 1: Effect of BAP and IBA concentrations and their interaction on the average number of branches formed of stevia branch multiplication after four weeks of *in vitro* transplantation.

Effect of BAP and IBA concentrations and their interaction on the average length of branches (cm) formed of multiplication of stevia branches after four weeks of *in vitro* transplantation

The results showed that the addition of different concentrations of BAP led to a significant reduction in the length of the branches of Stevia significantly *in vitro* (Fig. 2), Control treatment (without BAP) gave the highest branch length of 7.615 cm, The length was then significantly reduced by increasing BAP concentrations down to the 1.5 mg.l^{-1} concentration which gave the lowest average length reached 2.098 cm. As for the effect of Auxin (IBA), although the addition of 0.1 mg.l^{-1} led to an increase in the average length of 4.702 cm, then decreased significantly after increasing the concentrations added to the media to reach length of 3.520 cm at a concentration of 0.3 mg. l^{-1} IBA. As for the interaction between BAP and IBA, the effect was significant in reducing the average length of the branches. The interaction between 1.5 mg.l^{-1} BAP and 0.3 mg.l^{-1} IBA gave the lowest branch length average of 1.323. Whereas, the highest average length was 8.093 cm in the BAP-free media containing 0.3 mg.l^{-1} IBA.

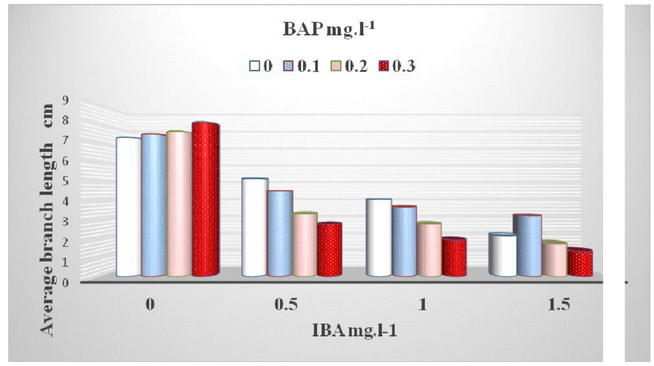

Fig. 2: Effect of BAP and IBA concentrations and their interaction on the average branch length formed of multiplication of stevia branches after four weeks of *in vitro* transplantation.

Effect of BAP and IBA concentrations and their interaction on the number of leaves.branch^{-1} formed of multiplication of stevia branches after four weeks of *in vitro* transplantation

The results showed that the addition of BAP to the media of multiplication of stevia branches *in vitro* resulted in a significant decrease in the number of leaves formed as the concentration gave 5.1 mg. (Fig. 3)

The addition of BAP to the medium of multiplication of stevia branches outside in vivo resulted in a significant decrease in the number of leaves formed as the concentration of 1.5 mg.l^{-1} gave the lowest leaf number per branch (12.120 leaf.branch^{-1}), while the control treatment (without BAP) gave the highest number of leaves, which gave 24.290 leaf.branch^{-1}. As for the effect of the Auxin (IBA) on the average number of leaves for stevia, it is noted that the addition of 0.1 mg.l^{-1} IBA resulted in a non-significant increase in the average number of leaves, which reached 17.790 leaves. Then the number of leaves decreased significantly by increasing the concentration of IBA added to the media to reach 16.305 leaves at the concentration 3.0 mg.l^{-1}.

As for the effect of interaction between BAP and IBA, it was observed that it was significant in reducing the average number of leaves, and the interaction between 1.5 mg.l^{-1} BAP and control treatment IBA the lowest leaf number (10.310 leaf.branch^{-1}), Which differed significantly from the control treatment of BAP and treatment of 0.1 mg.l^{-1} IBA, which gave the highest rate of the number of leaves reached 27.460 leaf.branch^{-1}.

Rooting Stage

Effect of NAA on rooting percentage and root number of Stevia branch four weeks after *In vitro* transplantation

The results indicate that the effect of different

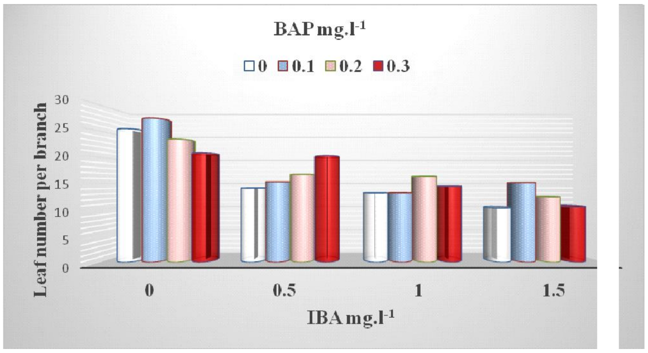

Fig. 3: Effect of BAP and IBA concentrations and their interaction on the number of leaves per branch formed of multiplication of stevia branches after four weeks of *in vitro* transplantation.

concentrations of NAA on the percentage of rooting in stevia branches *in vitro* , that the effect was not significant but the treatment of 0.50 mg.l^{-1} gave the highest rooting percentage of 90%, while the control treatment (without NAA) gave the lowest rooting percentage of 50% (Figures 4 and 5). The mean number of roots indicates that the addition of different concentrations of NAA had a significant effect in increasing the number of roots.

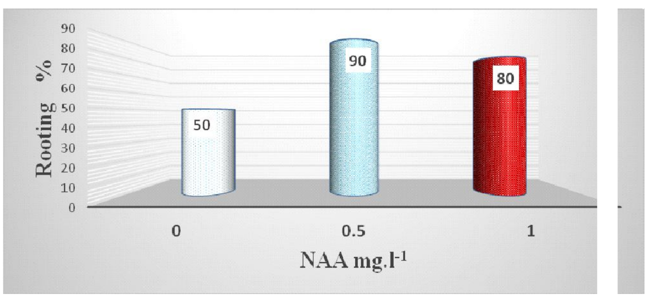

Fig. 4: Effect of the Auxin concentration NAA on stevia rooting percent after four weeks of *in vitro* transplantation.

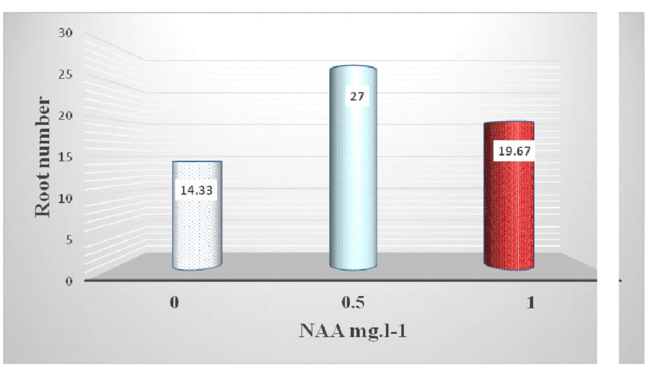

Fig. 5: Effect of the Auxin concentration NAA on stevia root count after four weeks of in vivo transplantation.

The concentration of 0.5 mg.l-1 gave the highest root number (27.0 root) and then decreased with increase concentration, the concentration of 1.0 mg.l^{-1} gave the lowest number of roots 19.67 root

Effect of IBA on Rooting Percentage and Root number of Stevia Branch after 4 weeks of *in vitro* transplantation

The results show the effect of different concentrations of IBA in the percentage of rooting of *in vitro* transplantation of stevia branches that there was no significant difference between treatments (Fig. 6). The treatment of 0.5 mg.l^{-1} gave the highest percentage of rooting 80%,, while the control treatment (without Auxin) gave the lowest rooting percentage of 50%. As for the results of the average number of roots in Figure 7 it is noted that its effect was significant in increasing the number of roots, the concentration of 0.5 mg.l^{-1} gave the highest number of roots was 22.0 root.branch^{-1} and the control treatment gave 13.33 root.branch^{-1}.

These results show the role of NAA in rooting the stevia branches in vivo, which gave the highest rate of rooting rate and the highest number of roots formed on each plant and for all studied concentrations compared to IBA.

These findings are consistent with the finding of many researchers who pointed to the importance of NAA in rooting in vivo stevia, including Ali *et al*., (2010). They found that the MS media with added Auxin concentration of 1.0 mg.l^{-1} NAA was the best for rooting the branches resulting from the stage of multiplication as it gave 9 6% rooting percentage and the number of roots reached 7.0 roots. Tiwari *et al.*, (2013) were rooted on the optimal rooting media of 0.01 mg.l^{-1} NAA which gave 11.18 root. It also agrees with Chotikadachanarong and Dheeranupattana (2013), which obtained the best rooting of a stevia branch by transferring the branches individually to the MS-media equipped with 0.1 mg.l-1 ý NAA, this treatment gave the highest number of roots (11.18 root) and a length of 0.78 cm. Other researchers obtained the best rooting.with other Auxins such as IBA (Abd Alhady, 2011 and Abd EL Motaleb *et al.*, 2013).

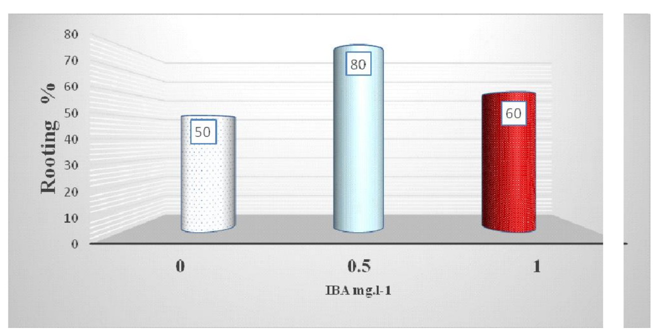

Fig. 6: Effect of IBA concentration on rooting percentage of stevia branches after four weeks of *in vitro* transplantation.

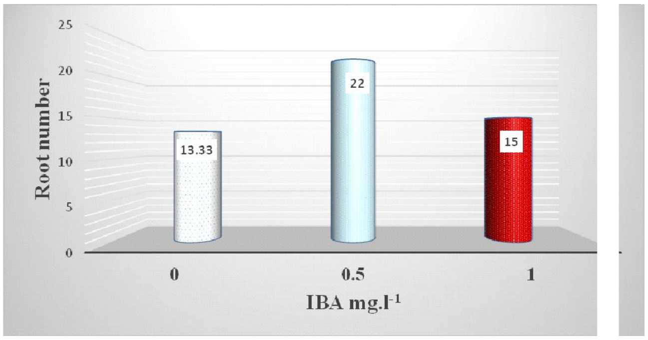

Fig. 7: Effect of IBA concentration on root number of stevia branches after four weeks of *in vitro* transplantation.

Results of extraction and estimation of Glycosides

The results shown in Figs. 8 and 9 indicate the superiority of the acclimatized tissue plant in its content of the compound Stevioside and amounted to 115.4 it was significantly superior to laboratory tissue plant (75.5) and seed seedlings (3 9.6). The acclimatized tissue plant was superior in Rebaudioside A and reached 175.2, and laboratory tissue plant 172.7, while the seed seedlings gave the lowest value of the content of this compound was 55.3. The superiority of tissue plants in their content of secondary substances in general and stevioles in particular can be attributed to the growth of these plants in a dietary media with essential nutrients and vitamins in general and plant growth regulators in particular. These compounds are produced through a series of biosynthesis of essential amino acids, which are the primers or raw materials for their production and growth regulators play an important role in the enzymatic activity of cells and their production of theses acids (Verpoort and Alfermann, 2000). These growth regulators also play a key role in increasing gene expression for coding to build enzymes involved in the biosynthesis of steviol in stevia leaves. These results are consistent with the findings of Sivaram and Mukundan (2003), which observed the superiority of tissue plants in the content of sweet glucosides (Rebaudioside A) and gave 4.9% while seed plants gave only 3.6%.

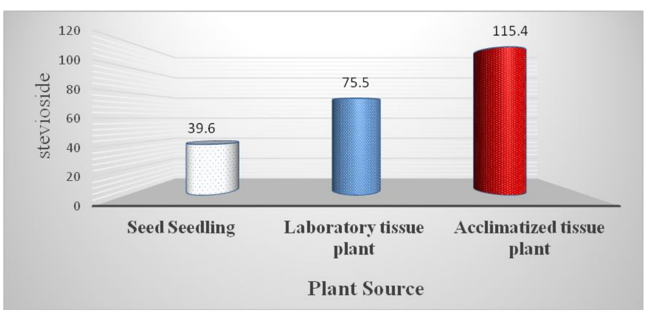

Fig. 8: Concentrations of Natural Stevioside Composites Extracted from Stevia, laboratory acclimatized tissue and Seed Leaves.

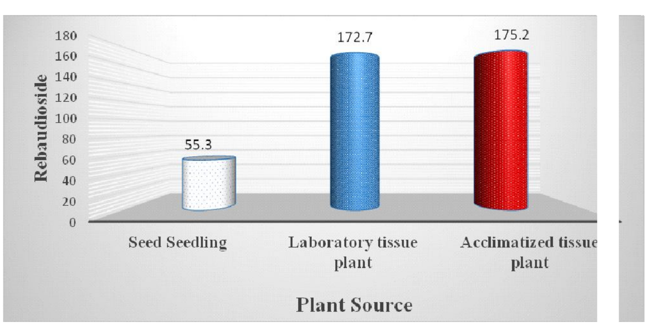

Fig. 9: Concentrations of Natural Rebaudioside Extracted from Stevia, laboratory acclimatized tissue and Seed Leaves.

They also what Singh and Dwivedi (2014) concluded when they made the same previous comparison using HPLC, found that the percentage of Stevioside in stevia seed plants was 7.017±0.058 (mean±standard deviation) while it increased to 9.236± 0.046 % in the leaves of tissue plants.

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