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THE EFFECT OF GROWTH REGULATORS AND DIFFERENT CONCENTRATIONS OF SUCROSE IN CALLUS INDUCTION OF SUGAR LEAF PLANT *STEVIA REBAUDIANA* AND ITS CONTENT OF STEVOISIDE

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Abstract

The research was carried out at the tissue culture Laboratory of the College of Education for Pure Sciences, University of Diyala with the aim of induction of callus from leaves of *Stevia rebaudiana* Bertcni and estimate its content of Stevoiside compound in callus derived from plant leaves. The results showed that the best induction of callus was obtained from the plant leaves when planted on the MS medium supported with a concentration of 2.0 mg.L^{-1} of NAA. 100% compared to the control treatment which the induction rate was 0%. The highest average wet weight was 0.963 g.L^{-1} with (TDZ) and was 0.624 g.L^{-1} with (BA), The induced callus was characterized by in the presence of (TDZ) of its fragile texture, while the induced callus of the presence of (BA), was of a solid strength. The data showed presence of Stevoiside in the callus tissue culture supported with sucrose at a concentration of 30, 60 and 90 g.L^{-1} in terms of High Performance Liquid Chromatography readings compared to the standard sample. and It was present in the samples of tissue cultures growing on the MS medium supported with a concentration of 30, 60 and 90 g.L^{-1} of sucrose after 30 days reached to 661.72, 759.61 and 877.26 µg. ml^{-1}, respectively.

Key words: plant extracts, leaf; sucrose; *Stevia rebaudiana.*

Introduction

Sugar leaf plant *Stevia rebaudiana* is one of the plants of the Asteraceae family. There are 280 species that belong to the genus Stevia sugar leaf. The plant is known by several names as it is called sugar bush, meaning the sugar tree and the honey leaf plant (Ma and Yan, 2009). The herb is a newly discovered plant that is useful for human nutrition and health. The highlands of Brazil and Paraguay are the original habitat for plant growth, and then its cultivation has moved to several regions of the world including China, Thailand and Palestine, and this plant also grows in the tropical parts of North and South America (Suanarunsawat *et al.,* 2004). *Stevia rebaudiana* is a medicinal plant with good economic resource (Mubarak *et al.,* 2008). The plant is widely used commercially in the pharmaceutical and food industries (Mishra *et al.,* 2010). The leaves of the plant contain the sugar from Stevoiside and many cyclosides (Yoshida, 1986). The sugars in this plant are 450 to 450 times sweeter than raw sugar according to cultivated varieties (Gerami *et al.,* 2017). The leaves of the plant are described as having a sweet taste that stays for long hours in the mouth due to the distribution of sweet ingredients in the leaf blade, (Maiti and Purohit, 2008). Recent studies have proven that the leaves of sugar leaf plant contain a wide range of antioxidant compounds that help eliminate free radicals in the body, such as quercetin, kaempferol and other glucosides, which makes them complementary to ideal dietary meals, thus preventing the growth of cancer cells, as well as Antioxidants help prevent premature aging and prevent heart disease (Ma and Yan, 2009). Sugar leaf plant reproduces either vegetatively by stems or sexually by seeds, as the method of reproduction with seeds faces many problems, the most important of which is the low germination rate, due to the presence of a state of self-incompatibility that works on the failure of fertilization (Miyazaki and Wantenabe, 1974). As well as the difference in the genotype composition of the plants resulting from the seeds and

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accordingly the difference in their sweet substance content quantitatively and qualitatively due to the genetic heterogeneity of the seeds (Miyagawa *et al.,* 1986). To overcome this problem, the technology of plant tissue culture has been used in the large reproduction of sugar leaf in many countries of the world (Abdul Razak *et al.,* 2014). As tissue culture is one of the most important applications in the reproduction of medicinal plants using the technique of rapid breeding multiplication, it preserves the genetic and quality traits, and preserves the genetic origins of plant species threatened with extinction. Stevoiside is a crystalline odorless compound and is usually a white powder (Allam *et al.,* 2001). The chemical formula $C_3H_{60}O_{18}$ (Abdel-Rahman *et al.,* 2015). Stevoiside has a high sweetness that may reach 300-400 times that of sucrose, as well as that it is three times cheaper than sucrose (Lutsenko, 2016). It is chemically synthesized by three molecules of glucose which represent the glycine molecule in the cycloside compound and one stevol molecule which is diterpene carboxyl alcohol (Brandle and Telmer, 2007). Stevoiside is the highest cycloside compound in the leaves of sugar leaf, with a ratio of between 4-13% (w/w), followed by A rebaudioside at 2-4% (w/w). The rebaudioside C is ranked third, with a ratio of 1-2% (w/w) (Cacciola *et al.,* 2011).

Materials and Methods

The experiment was carried out in the tissue culture laboratory of the College of Education for Pure Sciences, University of Diyala and all tests were conducted under sterilization conditions. Sugar leaf was obtained from the Janat alnakhil Company in Baghdad.

Sterilization of the vegetable nodes to obtain sterile plants:

The nodes were separated from the plants growing in the green house, as the nodes were soaked in a washing powder solution for three minutes, which was washed with water for 20 minutes, to get rid of the suspended dirt soil, then sterilized the nodes inside the sterile planting table by immersing it with sodium hypochlorite solution. at concentration of 10% with continuous shaking for 15 minutes. The nodes were washed with sterile distilled water three times at a rate of five minutes each time in order to get rid of the effects of sterile material, the nodes were dried after sterilization by placing it on the filter papers in order to get rid of the residual water stuck in it. Next, sterile nodes were transplanted in to a solid MS medium with a concentration of 0.3 $g.L^{-1}$ Kin (Al-Obaidy, 2017). Part of the nodes were immersed vertically in 30 ml of the MS medium in 250 ml flask vials at a rate of three nodes. $Bottle^{-1}$ The samples were kept in a consecutive light and dark system at aerate of 16 hours of light and 8 hours of darkness and the brightness of 2000 lux.

Induction of leaves of the sugar-leaf plant to obtain callus.

To study the effect of growth regulators on induction of callus, the true leaves were separated from the multiplicative branches resulting from nodes cultivation at the age of four weeks and divided into pieces of 1.0 cm^2, Then the pieces were transferred to a flask of 100 ml containing 20 ml of solid MS medium and supported with growth regulators 0.0, 0.5, 1.0, 1.5 and 2.0 $mg.L^{-1}$ NAA interfered with 0.5 $mg.L^{-1}$ of BA or TDZ. The experiment was carried out with five replications per treatment. The samples were preserved in the aforementioned conditions. The samples were followed up until their shape changed and the callus was formed. The wet weight of the callus was determined from the calculation of the difference in the weight of the glass bottles and their contents before and after replanting the callus and determined the response rate. Observations were recorded on the color and texture of callus during the 30-day growth period.

Using different concentrations of sucrose in planting callus:

To study the effect of different concentrations of sucrose on the growth and maintenance of callus farms and based on the results of the (previous) paragraph, a solid MS medium supported with concentrations of 2.0 $mg.L^{-1}$ NAA interfered with 0.5 $mg.L^{-1}$ TDZ (considered the best medium to create callus) and contained three concentrations Of sucrose 30, 60 and 90 $g.L^{-1}$. The callus was cut into 1 g weight pieces and planted in a flask of 100 ml, and content of 20 ml from the medium, at the rate of ten replicates for each sample. The changes in the growth and shape of the callus were followed after 30 days, then the samples were dried and kept in dark plastic containers and in dry places until Extraction for determination of stevoiside.

Results and Discussion

Effect of different concentrations of NAA interfering with 0.5 $mg.L^{-1}$ BA on callus induction. From leaves of sugar leaf plant.

The results on table 1 showed the superiority of the MS medium supported with a concentration of 2.0 $mg.L^{-1}$ NAA with 0.5 $mg.L^{-1}$ of BA in giving the highest average wet weight of the induced callus of leaves which was 0.624g and 100% induction ratio, that did not differ

Table 1: Effect of different concentrations of NAA interfering with 0.5 mg.L^{-1} BA on induction of callus from leaves of sugar leaf plant.

Concentration of growth regulators (mg.L^{-1})		Induced pieces	Induction percentage	Average fresh weight (mg.Piece^{-1})
NAA	BA			
0.0	0.5	0	0 %	0 C. 0
0.5		2	25%	0.252 B
1.0		3	50%	0.372 B
1.5		5	100%	0.582 A
2.0		5	100%	0.624 A

The number of replication is 5 for each treatment.
Averages with similar letters are no statistically significant difference

Table 2: Effect of different concentrations of NAA interfering with 0.5 mg.L^{-1} TDZ on induction of callus from leaves of sugar leaf plant.

Concentration of growth regulators (mg.L^{-1})		Induced pieces	Induction percentage	Average fresh weight (mg.Piece^{-1})
NAA	TDZ			
0.0	0.5	0	%0	0.0 D
0.5		2	25%	0.262 C
1.0		3	50%	0.472 C
1.5		5	100%	0.722 B
2.0		5	100%	0.963 A

The number of replication is 5 for each treatment.
Averages with similar letters are no statistically significant difference

significantly from the treatment of 1.5 mg.L^{-1} NAA, which recorded a value of 0.582 g, while the rest of the treatments 0.5 and 1.0 mg.L^{-1} wet weights reached 0.252 and 0.372, with an induction ratio of 25% and 50%, respectively, compared to the control treatment that no response appeared, the induced callus was distinguished by its solid texture and light green color.

Effect of different concentrations of NAA interfering with 0.5 mg.L^{-1} TDZ on induction of callus from leaves of sugar leaf plant.

The results on table 2 showed the superiority of the MS medium supported with a concentration of 2.0 mg.L^{-1} NAA interfering with 0.5 mg.L^{-1} TDZ in giving the highest average wet weight of the induced callus from the real leaves which was 0.963 g and 100% induction ratio compared to the control treatment that No response appeared, while the treatment 1.5 mg.L^{-1} NAA recorded 0.722 g with an induction rate of 100%, and the treatment at a concentration of 0.5 mg.L^{-1} did not differ from the treatment with a concentration of 1.0 mg.L^{-1}. With an induction rate of 25% and 50%, respectively, the induced callus was distinguished by its fragile texture and light green color.

Effect of different concentrations of sucrose in the growth and maintenance of callus.

The results in table 3 showed that there is an inverse relationship between the concentration of sucrose and the mean growth in the medium, the MS medium exceeded which supported with concentration of 2.0 mg.L^{-1} NAA interfering with 0.5 mg.L^{-1} TDZ and containing sucrose 30 g.L^{-1} and giving the highest average wet weight of callus, which amounted to 0.770 g.Piece $^{-1}$, which did not differ significantly from the MS medium supported with the same growth regulators containing sucrose at a concentration of 60 g.L^{-1} which scored 0.554 g.Piece^{-1}, while the mean fresh weight in the MS medium equipped Concentrations of growth regulators containing sucrose at a concentration of 90 g.L^{-1} as it reached 0.363 g.Piece^{-1}.

Diagnosis and quantitative estimation of Stevoiside content by HPLC in callus culture.

The curves recorded from injection of heterogeneous

Table 3: Effect of different concentrations of sucrose in the growth of callus induced from the leaves of the sugar leaf plant which growing on MS medium at a concentration of 2.0 mg. L-1-NAA interfering with 0.5 mg. -Lt1-TDZ.

Concentration of sucrose (*g.L^{-1}*)	Average fresh weight (*g.Piece^{-1}*)
30	0.770 a
60	0.554 a b
90	0.363 b

The number of replication 10 for each treatment.
Averages with similar letters are no statistically significant difference.

Table 4: Retention time of Isolated Stevoiside and its Presence Ratio in Callus Culture of Sugar Leaf Plant cultivated in MS medium equipped with 2.0 mg.L^{-1} NAA + 0.5 mg.L^{-1} TDZ and equipped with different concentrations of sucrose.

Stevoiside source Callus Culture	Concentration of sucrose	Time of retention	Space package model	Concentration of the compound (µg. ml^{-1})
Standard Stevoiside	comparison	2.313	90196	100
Stevoiside	30	2.148	159159	661.72
	60	2.327	182705	759.61
	90	2.332	211003	877.26

samples into a high-performance liquid chromatography (HPLC) showed their Stevoiside content in terms of recorded retention time with standard sample retention time, The results of the diagnosis showed the presence of Stevoiside in the extract of the culture of callus from leaves in terms of retention time compared to the standard sample table 4. The induced callus on the MS medium supported at a concentration of 2.0 mg.L^{-1} NAA + 0.5 mg.L^{-1} TDZ and equipped with sucrose at a concentration of 90 g.L^{-1} for 30 days, recorded an increase above the percentage curve of the standard sample. and recorded a value of 877.26 µg. ml^{-1} of Stevoiside. Induced callus was recorded on the same medium which equipped with sucrose at a concentration of 60 g.L^{-1} a value of 759.61 µg. ml^{-1} of Stevoiside, while this value decreased in the induced callus on a medium MS supported with 2.0 mg.L^{-1} NAA + 0.5 mg.L^{-1} TDZ and equipped with concentration of 30 g.L^{-1} sucrose reached 661.72. µg. ml^{-1} of Stevoiside.

Callus consists of undifferentiated cells which is an irregular tissue that occurs at the wound site of plant parts (Raghavan, 2012). And one of the recent studies proved that callus has multiple colors, including white, light green or black, and the color and texture of this tissue depend on the group of growth regulators in the media (Mahmud and others, 2014). The successful induction of callus depends on several factors, including the genetic composition and developmental stage of the plant, as well as the type of plant part chosen and the growth regulators present in the food media (Namdari *et al.,* 2015). The results of the detection and evaluation of the stevoiside compound indicated the presence of the stevoiside compound in callus culture, as its concentration reached 877.26 in the medium supported with 2.0 mg.L^{-1}, NAA + 0.5 mg.L^{-1} -TDZ and containing sucrose at a concentration of 90 g.L^{-1} and this may be due to the fact that Callus culture are a slow-growing system that allows secondary metabolism compounds to accumulate in tissues (Ramawat, 2008). The results also indicated that the concentration of stevoiside increased gradually with the increase of the concentration of sucrose in the medium until concentration reached to the highest concentration at the concentration reached 90 g.Liters1- of sucrose, that the highest productivity given by plant cells when they are in a stable condition and under stress (El-Sumaidaey, 2017). In addition to that there are many factors that help callus to accumulate secondary metabolism compounds, including the concentration of the appearance, the period of exposure to it, the composition of nutrients, the age and conditions of the culture (Ganapathi and Kargi, 1990). In addition, the media in tissue culture work to provide the requirements for division and growth from nutrients as well as other regulators and other additions on the metabolic activities in cells, and in order to achieve the best productivity of secondary metabolism compounds, therefore an optimal medium must be provided which increases the biomass of cells (El-Sumaidaey, 2017).

Conclusions

1. The study was able to induce callus from the leaves for sugar leaf germination and sustain growth in the center of MS supported by a concentration of 2.0 Mg. L-1 NAA overlapping with TDZ with a concentration of 0.5 Mg. L^{-1}.

The study demonstrated the susceptibility of paper-derived callus cells to the production of Stevoiside.

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