Egypt. J. Chem. **Vol. 65,** No. SI13B pp. 1589 - 1594 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Improving micropropagation efficiency and biochemical content of pineapple (*Ananas Comosus*) using stevia extract

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Abstract

Shoot tips of pineapple (*Ananas comosus*) cultivar "Queen" were cultured individually on MS medium supplemented with 2.0 mg/L BA+0.1 mg/L NAA+0.4 mg/Lthiamine HCL for establishment stage. Clusters were re-cultured to study the effect of stevia extract on *in vitro* proliferation, rooting and biochemical content of pineapple plantlets. Sucrose at different concentration (10, 20 and 30g/L) and stevia extract at 7, 13 and 20 ml/L were tested during multiplication and rooting stage of *in vitro* growing pineapple plants. Moreover, different biochemical analysis such as total carbohydrates, total sugars, starch, phenols, flavonoids as well as chlorophyll A, B and AB were estimated. The obtained results assured that sucrose at 30 g/L is the optimum dose of carbon for shoot multiplication of pineapple. Meanwhile, during rooting stage, the highest roots number and roots length were obtained when MS medium was supplemented with 20 g/L sucrose combined with 7 ml/L stevia. This combination also increased total sugars, starch and chlorophyll B content of pineapple leaves. However, sucrose alone showed superiority in some chemical content under study (total carbohydrates, flavonoids and chlorophyll A).From this investigation, we can conclude that sucrose is an important source of carbon for *in vitro* multiplication of pineapple plants while, stevia extract is more efficient for plantlets growth in rooting stage.

Keywords: Biochemical, Sucrose, Stevia extract, Pineapple, Proliferation, Rooting.

1. Introduction

Pineapple (*Ananas comosus* L. Merr.) is a tropical fruit crop which originated in South America, then transferred to many countries. Egypt is a new culture land for this plant [1]. For spreading this important plant, tissue culture technique has been employed as a toolfor clonal propagation of numerous plants[2] and [3].

Growth and proliferation of shoots under *in vitro*conditions depend upon a number of factors. Carbon sources such as sucrose supplied in the culture medium play an important role in the *in vitro* growth of plant tissues during tissue culture process [4]. In fact, sucrose gave comparatively better results for *in vitro* culture [5]. Addition of sucrose (3%) under two sets of growth conditions of tomato plant (favor or disfavor for photosynthesis) was studied. The supply of sucrose under disfavor conditions increased plant growth and recorded higher photosynthetic rate, chlorophyll content and enzymes activities. In the contrary, supplying sucrose to plantlets developed under high favor for photosynthesis decreased growth and led to a lower photosynthetic rate [6]. Moreover, the absence of sucrose in the growth medium induced generation of leaves of *Alocasiaamazonica* plantlets. It is worth mentioned that, with increasing sucrose concentration *in vitro*, numbers of stomata, contents of various carbohydrates in the leaves were increased but size of stomata decreased in ex vitro conditions. In addition, water potential of plantlets leaves, which have been grown *in vitro*with a sucrose concentration more than 3%, was decreased. Otherwise, during acclimatization, maximum net CO_2 assimilation rate, transpiration, and stomatal conductance were observed in the findings of Jo *et al* [7].Hassan and Taha[4]reported that the type of carbon source may have different effect on plant regeneration. So, if we can provide the optimum source of carbon it will be benefit for growth specially in rooting stage.

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Receive Date: 01 March 2022, Revise Date: 24 March 2022, Accept Date: 27 March 2022
DOI: 10.21608/EJCHEM.2022.122900.5500

Stevia is known as natural non calories sweetener rich withstevioside in the leaves. It has been used in a wide range of processed food as a substitute for conventional sugars or artificial dietetics especially in Japan [8]. Using its extract in plant tissue culture process has not enough investigated.

Thus, the ultimate goal of this investigation was to study the effect of stevia extract on *in vitro*proliferation, rooting and biochemical content of pineapple plantlets.

2. Materials and Methods

This study was carried out at the Biotechnology and micropropagation Lab, Pomology Dept., and Tissue Culture Technique Lab, Central Laboratories Network, National Research Centre, during the period from 2019 to 2021.

Preparation of plant material

Shoot-tips of pineapple (*Ananas comosus*) cultivar "Queen" were collected from crown of mature fruits of about 20-25 cm in length. The older leaves were carefully removed. Shoot tip containing the apical meristem and 2-4 leaf primordial were washed under tap water for half an hour. The isolated shoot tip explants were soaked under aseptic condition in 15 % Clorox (5.25 % sodium hypochlorite) for 20 minutes with one drop of Tween 20. The explants were then rinsed several times using sterilized distilled water.

Establishment of explants

Sterilized shoot-tips were cultured on MS medium [9] supplemented with 2.0 mg/LBA, 0.1mg/LNAA, 0.4 mg/l thiamine-HCl, 30 g/L sucrose and 7 g/L agar [1] . The culture medium was distributed in culture jars (325 ml), where each jar contained 50 ml of the prepared medium. The culture jars were immediately capped with polypropylene closures and autoclaved at 121°C and 15 Ibs /ins^2 for 20 min. After eight weeks all survived explants were transferred and recultured on the same medium.

Preparation of stevia solution

Stevia leaves were dried and subjected (as 10 g/L) to a water extraction process with heating at lower temperature 60°C.The solution was filtered to remove any remains and added to the medium before autoclaving.

Effect of combinations of stevia extract with sucrose on multiplication stage

Sucrose and stevia solution were added at different concentration alone or in combinations to study their effect on multiplication as the following:

- 30 g/L sucrose +0 m/L stevia
- 20 g/L sucrose+ 7 m/L stevia
- 10 g/L sucrose 13m/L stevia
- 0 g/L sucrose+20 m/L stevia

Number of shoots, number of leaves and shoot length (cm) were recorded after four subcultures with four weeks interval.

Effect of combinations of stevia extract with sucrose on rooting stage

The above mentioned combinations of sucrose and stevia solution were used in rooting medium supplemented with1.0 mg/LIBA.

Chemical analysis

1. Leaf pigments content

The following pigments, *i.e.* chlorophyll a, b and total chlorophyll as mg/100g were determined by colorimetric determination in fresh leaf samples (0.5g) at wave length of 663 and 645 nm for a, b respectively and total chlorophyll according toRichardson *et al*[10]**.**

2.Leaf totalcarbohydrates content

A weight of 0.1 g from dried sample of leaves was extracted with 80% ethanol and used to determine total carbohydrates according to Simth *et al* [11].

3. Leaf total phenols content:

A methanol extraction was prepared from dried samples of pineapple shoots (0.1g) and then 0.5 ml of the extraction was added to 0.5 ml Folin, shaked and allowed to stand for 3 min. Then one ml of saturated sodium carbonate was added to each tube followed by distilled water, shaken and allowed to stand for 60 min. The optical density was determined at wave length of 725 nm using spectrophotometer as described by Danil *et al*[12].

5. Total flavonoids content

Total flavonoids content (mg/g dry weight) was estimated in pineapple shoots according to De-Losse[13].

6. Starch and Total soluble sugars

Starch content was determined in pineapple shoots according toA.O.A.C. [14].

Statistical design

Treatments were arranged in complete randomized design, each treatment was replicated three times, each replicate included three jars, and each contained three clusters (3-4 shoots) developed *in vitro* while, in rooting stages it contained three plantlets. Means were compared according to the method described bySnedecor and Cochran[15].

3. Results

Multiplication stage

Table (1) and Figure (1) show the effect of different carbon sources treatments on shoot number, shoot length and leaf number parameters of pineapple

*in vitro*growing pineapple. It is clear that supplementing the cultured medium with 30 g/L sucrose significantly increased shoot number followed by 20 g /L sucrose +7 ml /L stevia extract then 10 g/L sucrose +13 ml/L stevia as compared with 20 ml/L stevia in a descending order. Meanwhile, both 30 g/L sucrose and 10 g/L sucrose +13 ml/L stevia treatments have the highest significant effect on shoot length. However, supplementing the culture medium with 10 g/L sucrose +13 ml/L stevia significantly increased leaf number followed by 30 g/L sucrose then 20 g/L sucrose + 7 ml/L stevia as compared with 20 ml/L stevia. The obtained results indicated that sucrose (30 g/L) is important for shoot number while, sucrose at 10 g/L+ stevia at 13 ml/L are promoting leaf number and shoot length in multiplication stage.

Rooting stage

It is clear from Table (2) and Figure (2) that 20 g/l sucrose +7 ml /l stevia surpassed other treatments in enhancing number of roots.However, there are insignificant differences between the treatments; sucrose at 30 g/l and 20 g/l sucrose +7 ml /l stevia in shoot and root length. Moreover, sucrose at 30 g/l surpassed other treatments in enhancing number of leaves followed by 20 g/l sucrose +7 ml /l stevia.

Chemical analysis

Regarding the effect of different concentrations of carbon sources on chemical parameters of pineapple cultivar as tabulated in Table (3), it is clear that total sugars and starch were concentrated when combination between 20 g/L sucrose +7 ml/l stevia was used in the culture medium in relation to other treatments. However, total carbohydrates, flavonoids,chlorophyll A were significantly amplified when 30 g/L sucrose was used in the culture medium. Chlorophyll B was significantly accumulated when using 20 g/L sucrose + 7 ml/L stevia or 30 g /L sucrose only. On the other hand, statistical differences were lacked among used treatments when phenols and total chlorophyll (A+B) were concerned.

Discussion

Many researchers emphasized the importance of sucrose to the *in vitro*growing plants. It is considered the source of carbon as in vitro plant could not take the sufficient amount of CO_2 from the vessels environment and do the photosynthesis. The recommended concentration of sucrose is 30 g/L as mentioned in [9] .Meanwhile, some plants need higher concentration like date palm [16] and some others need reduction of the concentration due to hyperhydricity, injury or lower growth. Otherwise, some plants need other types of sugars as in jojoba[17], in date palm[18] and in fig[19].

The obtained results indicated that sucrose (30 g/L) is important for multiplication stage of pineapple which represented in shoot number and shoot length. While, sucrose at 10 g/L + stevia at 13 ml/L are important for leaf number. These results are in harmony with the findings of Yaseen et al [5] who demonstrated that sucrose gives comparatively better results for *in vitro*culture of apple root stocks. Also, combination of stevia solution with sucrose surpassed stevia alone in increasing shoot number, leaf number and shoot length of jojoba shoots[20]. Moreover, multiplication was decreased with absence of sucrose in the culture medium but leaves number was induced for *Alocasia amazonica*[7].Bahmani et al [21] found that the best medium for the highest rooting percentage, root number, root length as well as survival rate was MS medium in the presence of 88 mM sucrose for apple root stock. In addition, higher rooting percentage for date palm was obtained when MS medium supplemented with 45 g/L sucrose[22]. However, our results showed that sucrose at 30 g/L and 20 g/L sucrose +7 ml /L stevia both gave the highest shoot and root length. Moreover, the treatment of 20 g/L sucrose +7 ml /L stevia found to be distinguished as it showed superior root number and shoot and root length in rooting stage of pineapple. It is seemed that stevia extract helped as growth strengthen for rooting stage and subsequently acclimatization stage.

Moreover, our results summarized that both sucrose (30 g/L) and sucrose combined with stevia (20 g/L and 7 ml/L, respectively) showed superiority in some chemical compounds like total carbohydrates and flavonoids with sucrose only and total sugars and starch with the combination of sucrose plus stevia extract. These results confirm the finding of Jain and Babbar[23] as they assured that sucrose presence induced some biochemical content under study. Increasing sucrose concentration increased starch and sugars content during *in vitro*growth of*Alocasia amazonica* [7]. Our results indicated that increasing sucrose concentration from 0 to 30 g/L increased total carbohydrates in pineapple leaves. Similarly, Ferreira *et al* [24] assured the same results with in vitro Dendrobium shoots.

Table (1): Effect of different carbon sources treatments on multiplication stage of pineapple plantlets

Treatments	Shoot number (No)	Shoot length (cm)	Leaf number(No)
30 g sucrose+0 ml stevia	130.00	1.27	4.15
20 g sucrose+7 ml stevia	111.67	1.05	3.70
10 g sucrose+13ml stevia	35.00	1.22	6.11
0 g sucrose+20 ml stevia	9.00	0.67	2.04
LSD	2.667	0.075	1.646

Table (2) Effect of different carbon sources treatments on rooting stage of pineapple plantlets

Treatments	Shoot length(cm)	Leaf number(No)	Root number(No)	Root length(cm)
30 g sucrose+0 ml stevia	14.50	5.11	6.86	3.91
20 g sucrose+7 ml stevia	15.83	3.42	9.58	3.97
10 g sucrose+13ml stevia	16.75	1.58	6.00	2.49
0 g sucrose+20 ml stevia	13.75	1.65	5.49	1.99
LSD	NS	1.41	2.45	1.25

Table (3) Effect of different carbon sources treatments on biochemical content of pineapple leaves

Treatment	Total carbohydrates (%)	Total sugars (%)	Starch (%)	Phenols (%)	Flavonoids (mg/g DW)	Ch. A	Ch. B	Ch. A+B
30 g sucrose+0 ml stevia	48.25	1.87	1.02	1.52	4.44	0.64	0.61	1.25
20 g sucrose+7 ml stevia	37.92	2.82	1.33	1.48	2.70	0.51	0.64	1.15
10 g sucrose+13ml stevia	34.30	2.45	0.09	1.39	2.65	0.41	0.57	0.98
0 g sucrose+20 ml stevia	24.94	1.78	0.55	1.50	2.62	0.58	0.49	1.06
LSD	0.570	0.248	0.179	NS	1.144	0.096	0.05	NS

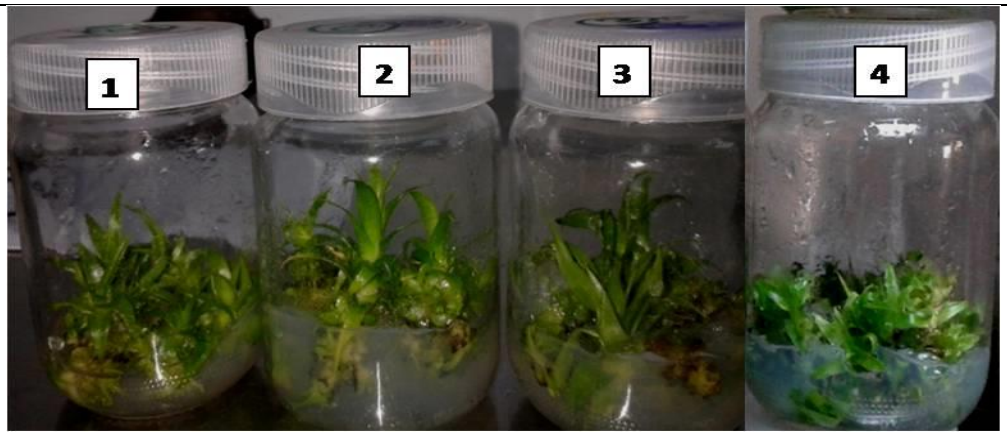

Fig. 1. Shoot multiplication of pineapple affected by sucrose and stevia extract. (1): 30 g/l sucrose, (2): 20 g/l sucrose+ 7ml/l stevia extract,(3): 10 g/l sucrose+13 ml/l stevia extract and(4): 20 ml/l stevia extract.

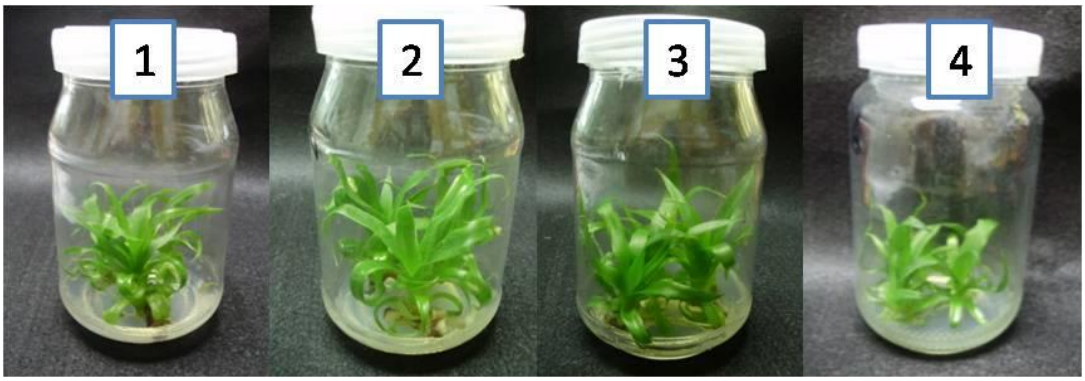

Fig. 2. Rooting stage of pineapple affected by sucrose and stevia extract. (1): 30 g/l sucrose, (2): 20 g/l sucrose+ 7ml/l stevia extract, (3): 10 g/l sucrose+13 ml/l stevia extract and (4): 20 ml/l stevia extract.

4. Conclusions

The obtained results indicated that sucrose (30 g/L) is important for multiplication stage of pineapple represented in shoot number and shoot length. Meanwhile, sucrose at 30 g/L or at 20 g/L+7 ml /L stevia both gave the highest shoot and root length. Moreover, the treatment of 20 g/L sucrose +7 ml /L stevia found to be distinguished for superior root number, shoot and root length in rooting stage of pineapple. It seems that stevia extract helped as enhancing plant growth for rooting stage and subsequently acclimatization stage.

5. Conflicts of interest

"There are no conflicts of interest to declare".

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