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ENERGY SOURCES AFFECT *IN VITRO* PROPAGATION AND SUBSEQUENT ACCLIMATIZATION OF *ANANAS COMOSUS*, VAR. SMOOTH CAYENNE PLANTS

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ABSTRACT

Plant tissue culture is an inevitable technique to overcome healthy and limited planting materials problems using suitable energy sources. Different carbohydrates have diverse effect on *in vitro* growing plantlets in terms of growth performance, acclimatization and cost used for micro-propagation. Hence, this paper reports the effects of sucrose, fructose, glucose, table sugar and starch on pineapple *in vitro* mass propagation and acclimatization as well as the analysis of energy source required cost per a medium. A complete randomized design was used to compare analytic grade sucrose with other four energy sources at 2 and 3 % (w/v). The results revealed that the energy sources with varied concentration strongly influenced the *in vitro* growth and subsequent acclimatization of pineapple plantlets. Analytic grade sucrose and table sugar at 3 % performed well for *in vitro* survival rate (100%), shoot amplification (15.3-16.5 shoots), rooting ability (2.5cm long and 12 roots) and acclimatization (95.4-97%). However, fructose and glucose required high importation cost (229.1% and 121.9% over analytic grade sucrose, respectively), and have low growth and acclimatization performance next to starch and energy free medium. Thus, table sugar has found to be a suitable alternative energy source for pineapple mass propagation, which saved about 95-97% cost from that of laboratory grade sucrose.

Keywords: Acclimatization, *Ananas comosus*, cheap alternative, energy sources, *in vitro* mass propagation

INTRODUCTION

Ananas comosus L. is propagated asexually through different parts of the plant such as suckers, slips or crowns (**d'Eekenbrugge** and **Leel, 2003**). Using vegetative propaguels result in disease transmission, less uniformity and inadequacy for commercial production, which all are a bottleneck to satisfy pineapple fruit demands all over the world. However, *in vitro* propagation is become a crucial solution to obtain disease free, rapid, uniform and mass production of pineapple plantlets (**Teixeira *et al.*, 2001; Firoozabady** and **Gutterson, 2003; Abebe *et al.*, 2009**).

In vitro multiplication and subsequent growth of plant shoots are affected by several growth medium supplements. The type and levels of exogenous carbohydrate sources are among those major supplements that affect the *in vitro* plant growth and multiplication (**Hossain *et al.*, 2005**). The carbon sources serve as energy and osmotic agents to support the growth of plant tissues (**Lipavska** and **Konradova, 2004**). Several findings have been reported by many scientists with regard to the beneficial effects of various energy sources such as sucrose, fructose, glucose, table sugar, sugarcane juice to *in vitro* growth of plants (**Mauney, 1961; Bouza *et al.*, 1992; Bridgen, 1994; Cunha** and **Ferreira, 1999**). Since sucrose is efficiently up-taken across the plasma membrane, it has been used as the only energy source in most of the tissue culture studies with the concentration of 2-5% (**Bridgen, 1994**). Glucose also has various effects on *in vitro* growth of plants. Medium supplemented with 4% glucose or fructose results in highly embryonic culture along with higher somatic embryo frequencies and higher growth rate on *Linum usitatissium* (**Cunha** and **Ferreira, 1999**). Particularly, fructose is a crucial energy source for embryo (**Mauney, 1961**), stem segments and pollen culture (**Kaufman *et al.*, 1962; Dickinson, 1996**). However, use of fructose in the medium results in hyperhydricity which leads to low chlorophyll contents and abnormal nitrogen and sugar metabolism (**Bouza *et al.*, 1992**).

The growth of *in vitro* cultured plants and cost of medium are strongly influenced differentially by various energy sources. The highest costs of media come from the use of analytical tissue culture grade sucrose (**Demo *et al.*, 2008**). Recently, the use of high cost energy sources have been replaced by cheap and locally accessible carbohydrate sources such as table sugar, juices and plant extracts and showed promising responses. It has been reported that addition of plant extracts or juices of coconut, tomato, banana, orange, apple and yeast to the culture medium boosted the growth of tissues in many plant species (**He *et al.*, 2003**). Table sugar has also found to be a suitable alternative low cost medium component for *in vitro* micro-propagation of potato (**Demo *et al.*, 2008**).

Thus, the present work was conducted to study the influence of carbohydrate sources (sucrose, fructose, glucose, table sugar and starch) on *in vitro* multiplication and acclimatization of pineapple plantlets. An attempt was made to calculate the cost of medium that was reduced by supplementing cheap and locally available energy source.

MATERIAL AND METHODS

The study was conducted in the plant biotechnology research laboratory at the Jimma Agricultural Research Centre, Ethiopian Institute of Agricultural Research (EIAR), Ethiopia between February and October 2012.

Plant materials

Pineapple (*Ananas comosus* var. Smooth cayenne) slips were collected from pineapple plantation at horticulture field, Jimma Agricultural Research Center, Jimma, Ethiopia and sterilized followed by subsequent multiplication under *in vitro* condition as per the protocol established by **De Almeida *et al.* (2002)** and **Abebe *et al.* (2009)** in plant biotechnology laboratory, Jimma Agricultural Research Center. Healthy and uniform plantlets were sorted and used as source of culture for both multiplication and rooting experiments.

MS nutrient media supplemented with energy sources

The media were prepared using full strength **Murashige** and **Skoog (1962)** (MS) basal salts amended with 0.8% (w/v) agar (Sigma Chemical Co. Germany) and 2 mg/l benzyl aminopurine (BA) and 1mg/l Kinetin for multiplication phase. Similarly, MS salts with half strength supplemented with 3 mg/l indole-3-butyric acid (IBA) was prepared for rooting stage according to **Abebe *et al.* (2009)**. Then, five energy sources such as sucrose, glucose, fructose, starch (Sigma chemical company, Germany) and table sugar (local shop, Jimma, Ethiopia) with two different levels (2 and 3%) were supplemented to MS media. Energy source

free media were set as a control in parallel. Finally, the pH of the medium was adjusted to 5.8 using 1 N NaOH or 0.1 N HCl prior to agar supplementation and homogenization. Forty milliliter was dispensed in Jam jars followed by autoclaving at 1.06 kg /cm^2 and 121 degree Celsius for 20 min.

Shoot multiplication, rooting and acclimatization

Shoot multiplication experiment was conducted on media supplemented with five types of energy sources with two concentration levels. Shoot explants preparation for both multiplication and rooting were made according to **De Almeida *et al*. (2002)** and **Abebe *et al*. (2009)** protocols. Short and individual explants were excised and cultured into multiplication media, whereas long and strong explants were transferred into rooting media. Shoot multiplication and rooting experiments were kept in controlled growth rooms for 90 and 30 days after culturing, respectively. Five explants were cultured per a Jam jar for both multiplication and rooting experiments. Later, well rooted and vigor grown plantlets were acclimatized following procedures of **Mengesha *et al*., (2013)**.

Energy sources cost analysis

The cost analysis of media supplemented with table sugar and analytical grade sucrose, glucose, fructose and starch used to *in vitro* pineapple multiplication was conducted. The cost of energy sources was calculated per a litter of medium and per a kilogram of energy sources. Then, the total cost saved replacing one energy source to another per a litter of media was calculated according to **Mengesha *et al.* (2012)**.

$$\textit{Cost saved (\%)} = [100 - (\textit{ACS cost} / \textit{RCS cost}) \times 100]$$

ACS = Alternative Energy Source; RCS = Recommended Energy Source

Data collection and statistical analysis

Quantitative and qualitative data were collected from experiments. Numbers of multiplied shoots and rooting parameters (root number and length) were collected from multiplication and rooting experiments after 90 and 30 days, respectively. Apart from quantitative parameters, the growth status and colour of cultured shoots were evaluated. A complete randomized design was conducted with five replicate per treatment and three jars with five plantlets per experimental unit. The data were subjected to ANOVA using SAS, statistical software package (Version 8.01) (**SAS, 2001**) according to **Montgomery (2005)**. Significant mean values were compared using the procedure of REGWQ test (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*).

RESULTS AND DISCUSSION

Results

In vitro plantlet survival

The survival rate at 90 days after culturing was varied depending on the energy sources supplemented to media (Table 1). The media free from energy source and supplemented with analytic grade starch showed minimum survival rate (0%), whereas table sugar and analytic grade sucrose added media resulted in 100% survival rate regardless of concentrations used. Media supplemented with glucose and fructose also revealed high survival rates, ranging 94-98.5%.

Shoot proliferations

The energy sources were significantly different ($P < 0.01$) with respect to multiplied shoots per explants (Table 1). Analytical grade sucrose and table sugar with 3% concentration gave significantly higher mean number of shoots followed by 2% sucrose and table sugar in multiplication phase. In contrast, energy free and starch with both 2 and 3% concentration levels produced very low (almost null) shoots as compared to all other energy sources. Similar to multiplied shoots, the growth status and plantlets color were influenced by the type and levels of energy sources (Table 1, Figure 1). High concentrations (3%) of sucrose and table sugar in the media resulted in vigorously grown green plantlets, whereas low concentration (2%) showed stunted grown light green plantlets. Other energy sources, glucose and fructose, at two concentrations (3 and 2%) revealed stunted grown light green plantlets.

Table 1 Number of multiplied pineapple (var. Smooth cayenne) shoots on MS medium supplemented different energy sources after 90 days culture

Energy sources	Survival rate (%)	No. of shoots	Growth status	Color of plantlets
Control (0%)	0	0.0±0.00^e	No growth & died	No growth & died
Table sugar				
2%	100	14.0±1.69^b	Stunted growth	Light green
3%	100	16.5±1.85^a	Vigorous growth	Green
Sucrose				
2%	100	14.38±2.00^b	Stunted growth	Light green
3%	100	15.38±1.30^{ab}	Vigorous growth	Green
Glucose				
2%	96	10.75±1.91^c	Stunted growth	Light green
3%	98.5	11.63±1.60^c	Stunted growth	Light green
Fructose				
2%	94	8.0±1.69^d	Stunted growth	Light green
3%	98	8.5±1.41^d	Stunted growth	Light green
Starch				
2%	0	0.0±0.00^e	No growth & died	White
3%	0	0.0±0.00^e	No growth & died	White
CV		16.05**		

Legend: means followed by the same letter within the same column are not significantly different. **significant different at 1% probability level.

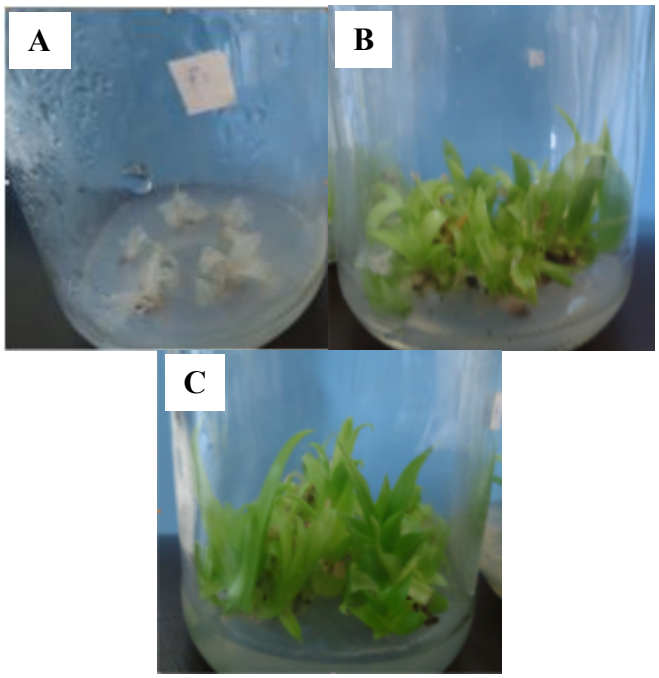

Figure 1 Growth status of *in vitro* multiplied pineapple shoots on MS media supplemented with different carbohydrate sources. **A**) Died and white shoots sample cultured; **B**) Light green shoots sample cultured; and **C**) Green shoots sample culture

Plantlet rooting

The rooting ability of *in vitro* raised pineapple plantlets were affected by different energy sources that supplemented into the rooting media. Significant differences ($p<0.05$) for root number and length were obtained among energy sources, but there was no significant difference between 2 and 3 % doses within every energy source (Table 2). Sucrose and table sugar produced higher root number and longer root length than other sources. Even though the concentration did not have significant effect on rooting ability, an increased concentration resulted in deep green and vigorously growing plantlets (Figure 2B). Meanwhile, glucose (3%) generated higher root number similar to sucrose and table sugar

than its lower dose (2 %), fructose and starch on root number. Unlikely, energy source free (Figure 2A) and starch (Figure 2D) added media had very low effect on root length and number (Table 2). This indicated that starch seems to be unsuitable carbon source for *in vitro* plantlet rooting ability.

Table 2 Different carbohydrate sources with 2 and 3 % concentration influence the rooting ability of *in vitro* multiplied pineapple plantlets regarding root number and length

Energy Source	Conc./L % (w/v)	Root Numbers	Root Length (cm)
Control	0	4.3±2.0^{cd}	0.38±.029de
Table sugar	2	13.1±2.97^{ab}	2.46±0.59^{abc}
	3	11.0±3.81^{ab}	2.73±0.83^{a}
Sucrose	2	15.4±5.42a	2.44±0.55^{abc}
	3	12.0±2.95^{ab}	2.52±0.58^{abc}
Glucose	2	8.0±2.82b^{cd}	1.69±0.99^{abc}
	3	10.4±2.20^{ab}	1.54±0.51^{bc}
Fructose	2	8.6±2.27^{bc}	1.6±0.51^{bc}
	3	7.8±2.83b^{cd}	2.62±0.82^{ab}
Starch	2	2.6±1.41^{d}	0.36±0.59^{de}
	3	4.1±1.07^{cd}	0.27±0.14^{e}
CV		0.70**	0.67**

Legend: means followed by the same letter within the same column are not significantly different. **Significant different at 1 % probability level. Conc./L= concentration per littre volume.

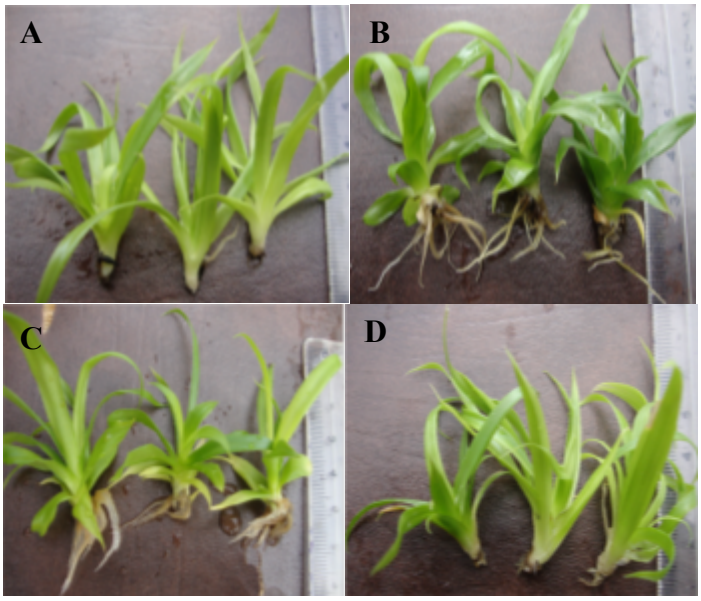

Figure 2 Rooting stage of pineapple plantlets growing with different type and concentration of carbohydrate sources. Sample plantlets rooting on (**A**) energy free; (**B**) on 3 % sucrose or table sugar; (**C**) on 2 % sucrose or table sugar; and on (**D**) starch (similar response was obtained from 2 and 3%).

Plantlet acclimatization and survival rate

In vitro raised pineapple plantlets with supplementation of diverse range of energy sources cause different survival performance during acclimatizing to external environment condition (Table 3). Plantlets growing on table sugar, sucrose, glucose and fructose with 2 and 3% concentration were survived about a range of 92-97 survival percentages. Plantlets grown on each of table sugar and sucrose supplemented media showed higher survival rate (95-97%) than other sources (Figure 3). However, those plantlets grown on energy source free and starch added media provided below 50% survival rate after 90 days acclimatization.

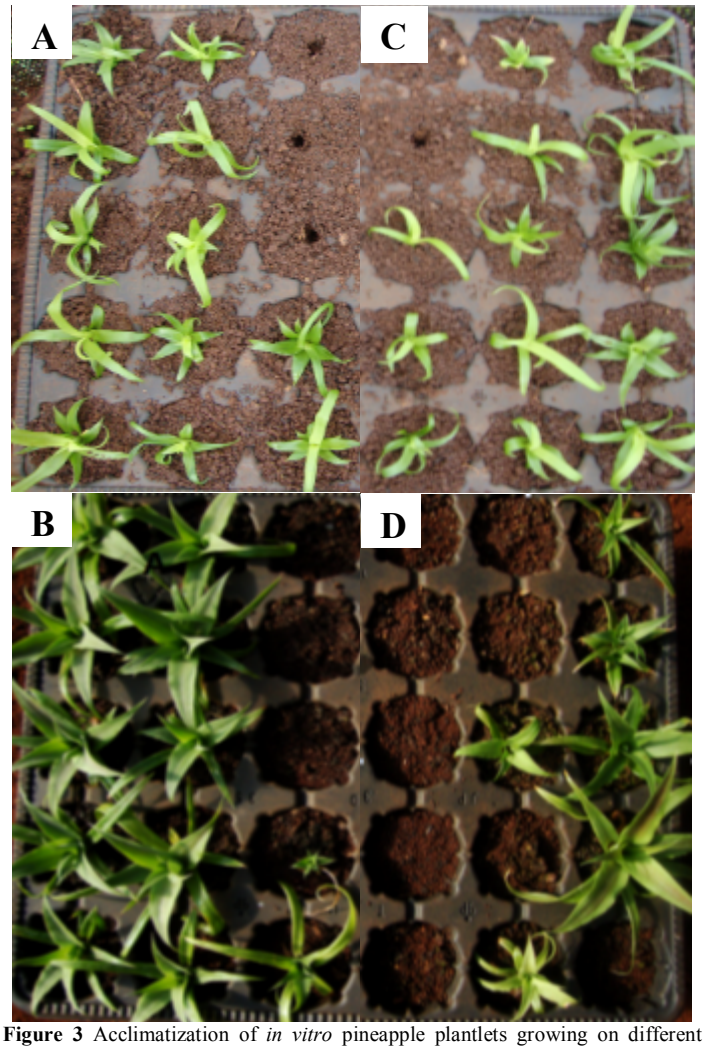

Figure 3 Acclimatization of *in vitro* pineapple plantlets growing on different energy sources supplemented media. Sample plantlets grown on table sugar transplanted into soil (**A**) before acclimatization and (**B**) after 90 days acclimatization in greenhouse. Other related sample grown on starch transplanted into soil (**C**) before and (**D**) after 90 days acclimatization. The survival rate of plantlets growing on table sugar was about 50% higher than that of starch and energy source free grown plantlets.

Table 3 *In vitro* grown pineapple plantlets survival in greenhouse after three months

Energy sources	Control	Table sugar		Sucrose		Glucose		Fructose		Starch	
Conc./L % (w/v)	0	2	3	2	3	2	3	2	3	2	3
Plant survival (%)	49.5	96	97	95.4	96	92	94.2	93.3	95.4	51.3	46.7

Legend: Conc. /L= concentration per litter of volume

Cost analysis

The total costs of media supplemented with different energy sources were analyzed. The costs used in the analysis were the current price in Ethiopian local market for table sugar and international market for analytical grade sucrose, glucose, fructose and starch. The cost of a liter MS medium energy sources using analytical grade sucrose, glucose, fructose and starch analyzed to be ETB 325.08, 721.44, 1069.56 and 378, respectively (Table 4). When replacing recommended analytic grade energy source (sucrose) by table sugar, 95-97% cost saves was achieved in medium. However, using other alternative analytic grade energy sources such as glucose, fructose and starch, the cost of a medium was increased by 16-229%. Fructose was found that the most expensive energy source (229%) followed by glucose (121%) and starch (16%) over analytic grade sucrose (Table 4).

Table 4 Cost analysis for carbohydrate sources using in pineapple micro-propagation

Energy source	Conc./L % (w/v)	Cost (ETB#)/kg	Cost (ETB)/L	Cost saved (%)/L
Table sugar	2	14.00	0.28	97.1
	3		0.42	95.7
Sucrose§	2	325.08	6.56	32.7
	3*		9.75	0
Glucose§	2	721.44	14.43	(-)48
	3		21.64	(-)121.9
Fructose§	2	1069.56	21.39	(-)119.4
	3		32.09	(-)229.1
Starch§	2	378.00	7.56	22.46
	3		11.34	(-)16.3

Legend: §analytical grade; #18.0 ETB ~ 1 USD; *recommended energy source and concentration which was used for cost analysis; the negative (-) indicated that the percentage of extra cost needed over the recommended energy source and its dose.

Discussion

Energy sources influence *in vitro* pineapple shoots proliferation and rooting ability

In vitro multiplication and growth of plants are affected by the dose and type of exogenous carbon sources that are supplemented to the medium (**Hossain *et al*., 2005**). Altered *in vitro* survival, shoot multiplication and rooting responses were observed on media supplemented with different energy sources as well as doses. The survival rate attributed to the availability of energy sources for maintaining the plant's normal growth. Carbohydrates are one of the major energy sources that play significant role on cell growth, maintenance and differentiation *in vitro* (**Romano *et al*., 1995; Vu *et al*., 1995**). The plant growth and development (e.g. root initiation) are highly energy demanding processes which can grow and develop using the existing energy source in the plant (**Calamar** and **de Klerk, 2002**). The effects of energy source types and levels on pineapple *in vitro* multiplication and rooting were in agreement with previous report on growth of *in vitro* Christmas tree (**Sull** and **Korban, 1998**) and patchouli (**Swamy *et al*., 2010**). MS media added with 3% analytic grade sucrose and table sugar multiplied more shoots followed by 2% of the same energy sources, whereas other energy sources resulted in reduced shoot proliferation at both 3 and 2% levels. This suggested that the translocation and assimilation of analytic grade sucrose and table sugar may be easier and quicker than others.

Addition of energy source types and levels into *in vitro* rooting MS media influenced pineapple root growth. Roots have an essential role and function in plant life and development through water and nutrients supply from the environment to the whole plant (**Schiefelbein *et al.*, 1997**). Well pineapple root growth was measured on MS media supplemented with analytic grade sucrose and table sugar. This root growth is in line with consistent root initiation of potato on MS media prepared with analytic grade sucrose and table sugar (**Demo *et al*., 2008**). The high number of roots per explants facilitated easy nutrients absorption from the medium, resulted in better plantlet growth and development.

In vitro and *ex vitro* survival rates of pineapple plantlets relayed on the type and level of energy sources

The energy sources, namely sugar, supplementation to the culture medium enhances *in vitro* plant growth and compensates low net photosynthetic rate due to poor photosynthetic ability **(Kubota *et al.*, 2001)**. Thus, overcoming photosynthetic problem through externally supplemented energy source, the survival rates of the explants on the medium can be increased. However, the survival rate of the cultures was varied depending on the energy sources supplemented to the media. The media free from energy source and supplemented with analytic grade starch showed minimum survival rate (0%), whereas table sugar and analytic grade sucrose added media resulted in 100% survival rate regardless of concentrations used followed by glucose and fructose, ranging 94-98.5%. This variation might be linked to less assimilation of the carbohydrate type and then led to scarcity of energy.

On the other hand, acclimatization and high percentage survival of pineapple plantlets is influenced by ability of plantlets to withstand transplanting stress and tendency to rapidly convert from heterotrophic or photomixotrophic to autotrophic growth (**Ziv, 1986**). The high percentage of acclimatization of plantlets (95-97%) that were grown on table sugar and analytic grade sucrose could be attributed to plantlets with functional root system, which continues to grow during *ex vitro* acclimatization **(Mengesha *et al*., 2013)**. The plantlets, therefore, grown on media supplemented with those energy sources were of high quality and vigorous with well developed leaves. In contrast, plantlets grown on starch supplemented media were light yellow and little rooted that seems to be weak to withstand external environment conditions.

Table sugar and analytic grade sucrose are preferable energy sources for *in vitro* pineapple growth

One of the disaccharide sugar called sucrose has been reported many times as the best energy source for *in vitro* plant proliferation and growth (**George, 1993, Hossain *et al*., 2005**). Interestingly, the evaluation of locally available table sugar and sucrose in the media for pineapple *in vitro* propagation showed almost similar results, suggesting sucrose can be replaced by table sugar for pineapple tissue culture. Similar results have been reported that table sugar is found to be a potential alternative energy source for *in vitro* propagation of plants (**Ganapati *et al*., 1995; Kaur *et al*., 2005; Demo *et al*., 2008**). Locally available table sugar at concentration of 3% (w/v) enhanced shoot proliferations and vigorous growth of plantlets similar to analytic grade sucrose (3%). This may be mainly due to easy translocation and assimilation of these energy sources available in medium by the explants resulting in cell division and then leading vigorous growth. In similar way, good performances of *in vitro* plantlets of banana, chrysanthemum, peanut, and chickpea in table sugar supplemented medium are reported (**Zapata, 2001; Gamborg, 2002**).

Table sugar as cheap and locally accessible energy source

Table sugar can be processed locally from commonly sugarcane. It can therefore find wide acceptability in developing countries needing to import analytical grade sucrose. Although it uses widespread, the cost of analytic grade sucrose is too high to justify the use at commercial level. Using locally accessible table sugar as an alternative energy source, the maintenance of *in vitro* propagation of pineapple and decline of cost required per a medium by 95.7 % were observed. This is in agreement with the successful reduction of analytic grade sucrose costs by 90% in banana tissue culture using table sugar (**Zapata, 2001**). Beside, utilization of locally available table sugar can reduce the cost of potato tissue culture by 34 to 51% without any quality problems of tissue cultured plants (**Demo *et al*., 2008**). Comparatively, table sugar has shown no side effect on *in vitro* plantlets and subsequent acclimatization whereas fructose causes hyperhydricity which leads to low cellulose and chlorophyll contents, less ethylene production and abnormal nitrogen and sugar metabolism (**Bouza *et al*., 1992**).

CONCLUSION

It can be concluded that various energy sources used in this study affected *in vitro* growth and subsequent acclimatization of pineapple plants. Among these different carbon sources used, table sugar performed well in terms of *in vitro* survival rate, shoot multiple, rooting ability, high acclimatization rate and cost reduction. Besides, 3 % table sugar can be used entirely as a replacement of 3 % analytical grade sucrose that was dominantly used energy source in most of the plant tissue culture. Since analytical grade sucrose is expensive next to glucose and fructose, this study suggests a table sugar as a cheap and locally accessible energy source for pineapple micro-propagation. However, further research is still needed to verify different quality table sugar effects on *in vitro* pineapple propagation, subsequent acclimatization and yield and its quality.

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