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In vitro somatic embryogenesis response of date palm *cv.* Sukkary to sucrose and activated charcoal concentrations

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Abstract

The rational of the study was to determine the effect of different concentrations of sucrose (20 and 40 g/L) and activated charcoal (0.5, 1 and 1.5 g/L) alone (control) and in combinations on the somatic embryogenesis of date palm *cv.* Sukkary**.** The results of two-factorial (sucrose and activated charcoal) randomized complete design indicated that no embryogenesis growth occurred when MS media was used without the addition of carbon source. The individual and combined effects of 40 g/L sucrose and 1 g/L activated charcoal produced sturdy embryogenesis and its related traits. The use of 40 g/L sucrose caused significant improvement in the number of somatic embryos (49.92), length of somatic embryos (1.08 cm), fresh weight of somatic embryos (1.54 g), dry weight of somatic embryos (0.148 g), germination of somatic embryos (4.92), and length of leaflets (2.14 cm). Similarly, use of 1 g/L of activated charcoal significantly increased the number (42.89), length (0.99 cm), fresh weight (1.29 g), dry weight (0.156 g), germination (3.22) of somatic embryos, and length of leaflets (1.47 cm) as compared to other treatments. Results also showed that the combined application of 40 g/L sucrose and 1 g/L activated charcoal significantly enhanced the number of somatic embryos (69.67), length of somatic embryos (1.37 cm), fresh weight of somatic embryos (2.18 g), dry weight of somatic embryos (0.262 g), germination of somatic embryos (6.33), and length of leaflets (2.57 cm) as compared to other treatment combinations. However, the same sucrose level with 0.5 g/L activated charcoal concentration also showed promising results.

Key words: Date palm, *Phoenix dactylifera*, Sukkary, tissue culture, *in vitro*, sucrose, activated charcoal, embryogenesis

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most resilient woody crops in the deserts, which remains productive for many years with limited inputs (Aleid *et al.*, 2015). The demand for offshoots in the date palm growing countries is rising up to 1-2 million per year (Jain, 2007), however, a female date palm produces only 10-20 offshoots in its entire life (Zaid and deWet, 1999), which is a major restraint for propagating desirable cultivars conventionally. Production of plants through *in vitro* culture is successfully introduced in many species (Munir *et al.*, 2015). Therefore, date palm plantlets produced through *in vitro* method has become an ideal alternative for largescale production to fulfill the market demand (Saker and Moursi, 1999). The technique is not only applied for commercial production of the planting materials but it is also aimed for solving hybridization problems, production of disease free plants and germplasm conservation (Al-Khalifah and Shanavaskhan, 2012).

Date palm *in vitro* propagation techniques from meristem shoot-tip, axillary buds, zygotic embryos, and immature leaves are already developed (Reuveni, 1979; Reynolds and Murashige, 1979; Tisserat, 1979; Tisserat and Demason, 1980; Daguin and Letouze, 1988; Huong *et al.*, 1999). However, the development of an embryo from vegetative cells of the plants (somatic embryogenesis) is very common technique, which allows many practical and commercial applications, particularly for *in vitro* clonal micropropagation, which is widely used for several date palm cultivars (Fki *et al.*, 2011).

The success of plant *in vitro* culture is mostly dependent on the use of suitable nutrient media constituents. Among these constituents, sugar is a significant ingredient in the nutrient medium for essential *in vitro* growth and development, as poor photosynthesis can not fulfill the energy need of the growing explants. Sucrose, glucose and fructose are used as carbon sources, depending on the age and type of plant materials (Pan and van Staden, 1999). Previous studies showed that application of 30 to 70 g/L of sucrose in the media increased *in vitro* plantlets production of five cultivars of date palm (Al-Maarri and Ghamdi, 1997). Al-Maarri and Alghamdi (1998) found similar results when 60 g/L of sucrose was added than the 30 g/L. However, Hamid (2001) reported that 45 g/L of sucrose enhanced the elongation of buds from shoot-tip explant of date palm *cv.* Maktoum. Taha *et al.* (2001) observed that 40 g/L sucrose increased the length of plantlets and number of leaves as compared to 30 g/L concentration. Abdulwahed (2013) obtained higher elongation and multiplication ratio of plantlets when 65 g/L sucrose was used.

Similarly, activated charcoal (AC) is also used in the tissue culture media, which can adsorb many substances because of its large surface area of fine pores. It improves cell growth and development (Dharishini *et al.*, 2015). Its stimulating effects on morphogenesis may be due to its irreversible adsorption of inhibitory compounds present in the medium and significantly reducing the toxic metabolites, phenolic exudation and brown exudate accumulation. It also releases the natural substances present in it which promote growth, alteration and darkening of culture media, and adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene. It also enhances the uptake of plant (Eymar *et al.*, 2000;

Thomas, 2008). Taking into account the significant role of sucrose and activated charcoal on somatic embryogenesis and further regeneration, present research is designed to investigate the effect of adding different levels of sucrose and activated charcoal on somatic embryogenesis and shoot proliferation of date palm *cv.* Sukkary under *in vitro* conditions.

Materials and methods

The study was conducted in the Tissue Culture Laboratory, Plant Biotechnology Department, College of Agriculture Sciences and Food, King Faisal University, Kingdom of Saudi Arabia. Three years old date palm offshoot of *cv.* Sukkary weighed 6 kg were separated from healthy mother tree. Young offshoot was thoroughly cleaned with double distilled water and outer leaves were carefully removed to expose the central meristem shoot-tip. The exposed region was excised and immediately placed in antioxidant solution containing 150 mg/L ascorbic acid and 100 mg/L citric acid. The central meristem shoot-tip was sterilized in 20% v/v Clorox solution for 15 minutes, followed by rinsing 3 times with double distilled water. The meristem explants were kept in the antioxidant solution until the culturing was completed. The meristem shoot-tip was cut into 1 cm pieces (explants) and were cultured using organogenesis media as described by Al Khateeb and Ali-Dinar (2002). Two somatic embryos resulted from direct organogenesis were transferred to 250 mL flask filled with 100 mL of modified Murashige and Skoog (1962) medium supplemented with 170 mg/L sodium hydrogen orthophosphates ($NaH_2PO_4.2H_2O$), 125 mg/L inositol, 200 mg/L glutamine, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, 1 mg/L biotin, 1 mg/L calcium pantothenate, 7 g/L agar and 30 g/L sucrose. Different sucrose (0, 20, and 40 g/L) and activated charcoal (0, 0.5, 1, and 1.5 g/L) concentrations were added to the MS media.

Each treatment combination was represented by five replicates having two meristem shoot-tip explants per replicate in a 2-factorial completely randomized design arrangement. Cultures containing 20 mL media in 125 mL conical flasks were incubated at 25±2 °C under the light intensity of 1000 lux at 16 hours a day photoperiod provided with three 40W fluorescent lamp.

Data regarding number of somatic embryos, length of somatic embryos, fresh and dry weight of somatic embryos, number of somatic embryos germinated and length of leaflets were determined after 8 weeks of culture. The data were statistically analyzed according to the technique of analysis of variance for 2-factorial completely randomized design using Statistical Analysis System software (SAS, 2001). The treatment means were compared using least significant difference (LSD) test at 5% level of probability.

Results and discussion

The effect of different concentrations of sucrose: Results in Table 1 revealed a significant ($P \leq 0.05$) effect of sucrose levels on number of somatic embryos, length of somatic embryos, fresh weight of somatic embryos, dry weight of somatic embryos, germination of somatic embryos, and length of leaflets of date palm *cv.* Sukkary. The addition of 40 g/L of sucrose in the *in vitro* media helped to raise the number of somatic embryos (49.92) followed by 20 g/L (32.58) and control (8). Similarly, maximum length of somatic embryos (1.08 cm) was observed when 40 g/L sucrose was used followed by 20 g/L of sucrose (0.78 cm) and control (0.56 cm). A similar trend was noticed in fresh and dry weights of somatic embryos, that is highest fresh weight when 40 g/L sucrose was applied in the media (1.54 g) followed by 20 g/L sucrose (1.17 g) and control (0.40 g). Highest dry weight was in 40 g/L sucrose treatment (0.148 g) followed by 20 g/L sucrose (0.086 g) and control (0.032 g). No shoot regeneration was observed in somatic embryos produced in control, however, somatic embryos produced in the media containing 40 g/L sucrose showed maximum number of germination (4.92) followed by 20 g/L sucrose treatment (2.25). Similarly, maximum length of leaflets (2.14 cm) was measured when 40 g/L sucrose was used in the media followed by 20 g/L sucrose treatment (1.40 cm).

Findings of present study revealed 75 and 84% increase in number of embryos when 20 and 40 g/L sucrose was added in the medium respectively as compared to control. Similar trend was observed in other parameters such as 28 and 48% bigger sized embryos, 66 and 74% more fresh weight and dry weight increased by 63 and 78% at 20 and 40 g/L addition of sucrose, respectively. These results clearly indicated the significance of the use of sucrose in the medium for date palm. It is well-known that *in vitro* plant cells and tissues need carbohydrates to keep up the osmotic potential and to serve as energy and carbon source for developmental processes like shoot multiplication, root formation, embryogenesis and organogenesis, which are very energy demanding biological processes (Yaseen *et al.*, 2013). The results reported by Taha *et al.* (2001) showed that 50 g/L sucrose had significant effect on *in vitro* plantlet height and leaves number of date palm. Similarly, addition of sucrose at 60 g/L in the *in vitro* medium produced the highest number of somatic embryos and longest shoot (Alkhateeb, 2008). Sucrose at 45-90 g/L also encouraged morphogenesis in date palm (Asemota *et al.*, 2007; Hassan *et al.*, 2008)

The effect of different concentrations of activated charcoal: Table 2 indicated significant ($P \leq 0.05$) differences of activated charcoal concentrations regarding number of somatic embryos, length of somatic embryos, fresh weight of somatic embryos, dry

Table 1. Individual effect of different concentrations of sucrose on number, length, fresh weight, dry weight and germination of somatic embryos and length of leaflets of date palm *cv.* Sukkary

Sucrose (g/L)	Number of somatic embryos	Length of somatic embryos (cm)	Fresh weight of somatic embryos (g)	Dry weight of somatic embryos (gm)	No. of somatic embryos germinated	Length of leaflets (cm)
0	8.00±1.13	0.56±0.05	0.40±0.42	0.032±0.016	0.00±0.00	0.00±0.00
20	32.58±5.23	0.78±0.07	1.17±0.14	0.086±0.016	2.25±0.48	1.40±0.32
40	49.92±6.62	1.08±0.12	1.54±0.25	0.148±0.041	4.92±0.55	2.14±0.21
LSD ($P \leq 0.05$)	5.39	0.17	0.23	0.026	0.67	0.25

± standard error within replicates.

Table 2. Individual effect of different concentrations of activated charcoal on number, length, fresh weight, dry weight and germination of somatic embryos and length of leaflets of date palm *cv.* Sukkary

Activated charcoal g/L)	Number of somatic embryos	Length of somatic embryos (cm)	Fresh weight of somatic embryos (g)	Dry weight of somatic embryos (g)	Number of somatic embryos germinated	Length of leaflets (cm)
0	24.56±10.30	0.69±0.11	0.81±0.25	0.063±0.025	2.00±1.07	1.06±0.53
0.5	28.11±11.11	0.80±0.17	1.05±0.36	0.074±0.040	2.00±1.53	0.90±0.68
1	42.89±17.01	0.99±0.21	1.29±0.50	0.156±0.055	3.22±1.83	1.47±0.77
1.5	25.11±10.30	0.74±0.16	1.00±0.35	0.060±0.023	2.33±1.35	1.29±0.66
LSD ($P \leq 0.05$)	6.23	0.20	0.26	0.030	0.77	0.29

± standard error within replicates.

weight of somatic embryos, germination of somatic embryos, and length of leaflets of date palm *cv.* Sukkary. Media containing 1 g/L of activated charcoal produced highest number of somatic embryos (42.89) followed by 0.5 g/L (28.11), 1.5 g/L (25.11) and control (24.56). However, there was non-significant difference between the 0.5 g/L and 1.5 g/L activated charcoal and control treatments. Similarly, maximum length of somatic embryos (0.99 cm) was observed in media containing 1 g/L activated charcoal followed by 0.5 g/L (0.80 cm), 1.5 g/L (0.74 cm) and control (0.69 cm). All treatments were at par excluding 1 g/L one. Highest fresh weight of somatic embryos (1.29 g) was recorded when 1 g/L activated charcoal was used followed by 0.5 g/L (1.05 g), 1.5 g/L (1 g) and control (0.81 g). Activated charcoal treatment 0.5 and 1.5 g/L statistically behaved alike. Similarly, maximum dry weight of somatic embryos (0.156 g) was recorded when 1 g/L activated charcoal was put into the media followed by 0.5 g/L (0.074 g), control (0.63 g) and 1.5 g/L (0.60 g). Minimum number of somatic embryos germination (2) was observed in control, 0.5 g/L and 1.5 g/L treatments. However, maximum (3.22) somatic embryos were germinated when 1 g/L activated charcoal was used in the media. Similarly, highest length of leaflets (1.47 cm) was estimated in media containing 1 g/L of activated charcoal followed by 1.5 g/L (1.29 cm), control (1.06 cm) and 1 g/L (0.90 cm) treatments.

The above-mentioned results indicated that the application of 1 g/L activated charcoal increased the number of somatic embryos, length of embryos, fresh and dry weight of embryos, embryo germination and leaflet length by 43, 30, 38, 59, 38 and 28%, respectively as compared to control. These outcomes showed the importance of the application of activated charcoal in the medium to improve cell growth and development of date palm. Abdulwahed (2013) reported that 0.75 g/L of activated charcoal in the medium caused a significant improvement in the shoots multiplication and elongation in date palm. Moreover, the addition of 2,4-D and activated charcoal at 300 mg/L in the liquid medium promoted the differentiation of somatic embryos in date palm *cv.* Boufeggous (Othmani *et al.*, 2009). Naik and Al-Khayri (2016) obtained date palm *in vitro* callus proliferation when 1.5 g/L activated charcoal was used in the media. It has been reported that activated charcoal improves *in vitro* medium pH to an optimum level for morphogenesis and removes inhibitory chemicals (Pan and van Staden, 1999). Eymar *et al.* (2000) observed that the pH-optimizing role of activated charcoal encouraged the uptake of NO_3^- or NH_4^+ from the media, which eventually enhanced morphogenesis. Similarly, Fki *et al.* (2011) stated that the media supplemented with activated charcoal can overcome the issue of substantial release of phenolic compounds in many date palm cultivars.

The effect of interaction between sucrose and activated charcoal: Findings of present study showed that the number of somatic embryos, length of somatic embryos, fresh weight of somatic embryos, dry weight of somatic embryos, germination of somatic embryos, length of leaflets were significantly increased when a combined application of 40 g/L sucrose and 1 g/L activated charcoal was used in the media. Media containing zero sucrose at all combinations of activated charcoal showed weakest performance regarding all parameters studied. Fig. 1 depicted that maximum number of somatic embryos (69.67) were counted when 40 g/L sucrose and 1 g/L activated charcoal was applied followed by 20 g/L sucrose and 1 g/L activated charcoal (47.67) which was statistically non-significant with 40 g/L sucrose and 0.5 g/L activated charcoal (45.33), 40 g/L sucrose and 1.5 g/L activated charcoal (42.67) and 40 g/L sucrose alone (42) treatments. Similarly, maximum length of somatic embryos (1.37 cm) were estimated in 40 g/L sucrose and 1 g/L activated charcoal combination followed by 40 g/L sucrose and 0.5 g/L activated charcoal (1.13 cm) and 40 g/L sucrose and 1.5 g/L activated charcoal (1.02 cm) which were statistically non-significant to each other (Fig. 2). Fig. 3 indicated that somatic embryos formed under 40 g/L sucrose and 1 g/L activated charcoal media had maximum fresh weight (2.18 g) followed by 40 g/L sucrose and 0.5 g/L activated charcoal (1.70 g) which was statistically non-significant with 20 g/L sucrose and 1.5 g/L activated charcoal (1.54 g) treatment. Maximum dry weight (0.262 g) of somatic

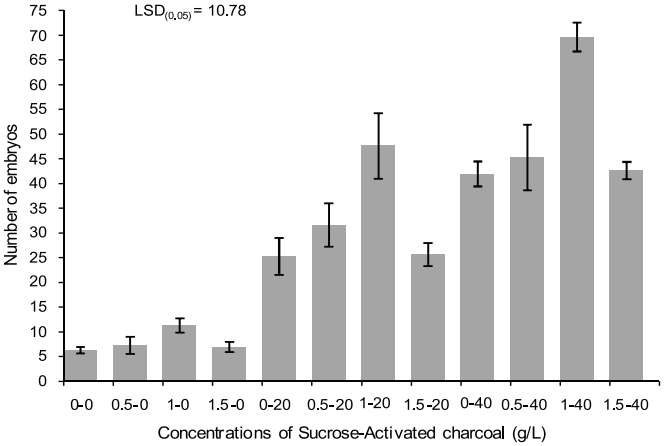

Fig. 1. Combined effect of sucrose (0, 20 and 40 g/L) and activated charcoal (0, 0.5, 1 and 1.5 g/L) concentrations on the number of somatic embryos of date palm *cv.* Sukkary. Y-bar indicated the sample variability whereas LSD value ($P \leq 0.05$) is the least significant difference among treatment means.

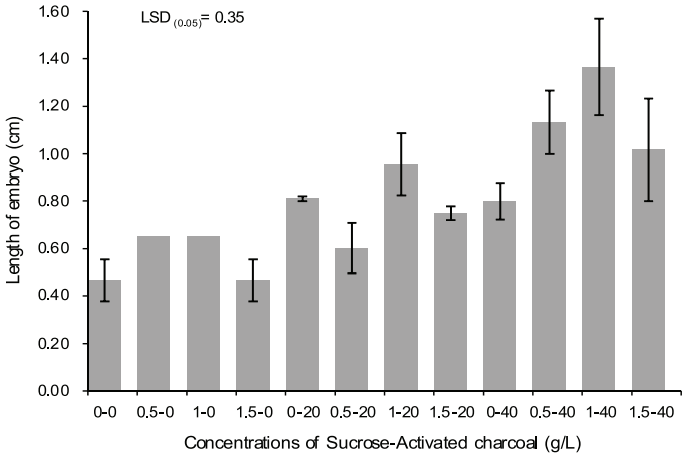

Fig. 2. Combined effect of sucrose (0, 20 and 40 g/L) and activated charcoal (0, 0.5, 1 and 1.5 g/L) concentrations on the length of somatic embryos (cm) of date palm *cv.* Sukkary. Y-bar indicated the sample variability whereas LSD value ($P \leq 0.05$) is the least significant difference among treatment means.

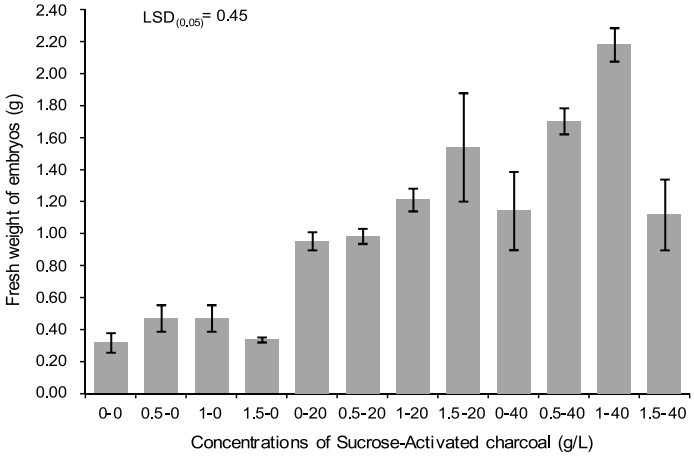

Fig. 3. Combined effect of sucrose (0, 20 and 40 g/L) and activated charcoal (0, 0.5, 1 and 1.5 g/L) concentrations on the fresh weight of somatic embryos (g) of date palm *cv.* Sukkary. Y-bar indicated the sample variability whereas LSD value ($P \leq 0.05$) is the least significant difference among treatment means.

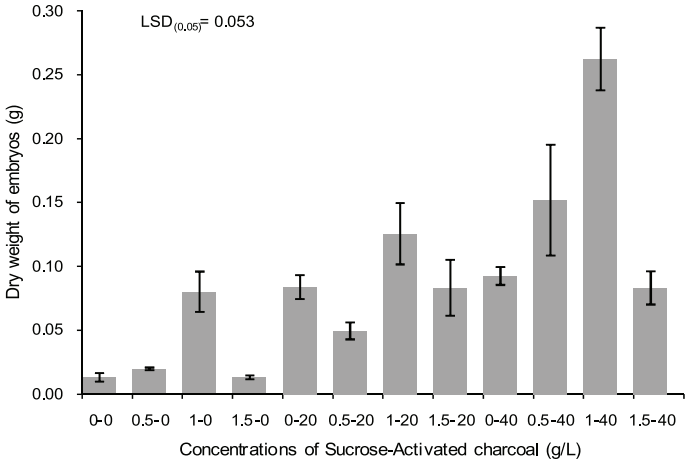

Fig. 4. Combined effect of sucrose (0, 20 and 40 g/L) and activated charcoal (0, 0.5, 1 and 1.5 g/L) concentrations on the dry weight of somatic embryos (g) of date palm *cv.* Sukkary. Y-bar indicated the sample variability whereas LSD value ($P \leq 0.05$) is the least significant difference among treatment means.

embryos was assessed when 40 g/L sucrose and 1 g/L activated charcoal were applied in the media followed by 40 g/L sucrose and 0.5 g/L activated charcoal (0.152 g) which was statistically non-significant with 20 g/L sucrose and 1 g/L activated charcoal (0.126 g) treatment (Fig. 4). The use of the same treatment (40 g/L sucrose and 1 g/L activated charcoal) also showed the highest (6.33) number of somatic embryos germination (shoot formation) closely followed by 40 g/L sucrose and 0.5 g/L activated charcoal (5) which were statistically non-significant to each other. Combination of 40 g/L sucrose and 1.5 g/L activated charcoal (4.67) also gave promising results (Fig. 5). Similarly, Fig. 6 illustrated that maximum length of leaflets (2.57 cm) was determined when 40 g/L sucrose and 1 g/L activated charcoal was applied in the media closely followed by the same sucrose concentration with 1 (2.22 cm) and 1.5 g/L (2.17 cm) activated charcoal. All these three treatments were at par statistically. Similar results were reported by Abdulwahed (2013) where combined application of sucrose (65 g/L) and activated charcoal (0.75 g/L) increased multiplication of shoots of date palm *cv.* Sufedy. Dahab *et al.* (2005) reported that adding activated

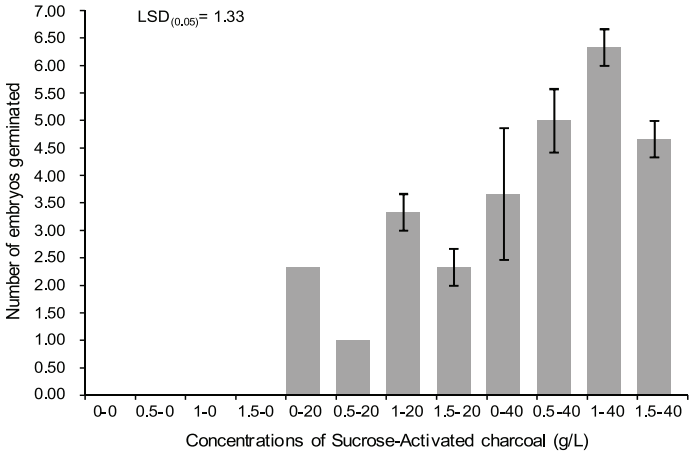

Fig. 5. Combined effect of sucrose (0, 20 and 40 g/L) and activated charcoal (0, 0.5, 1 and 1.5 g/L) concentrations on the number of somatic embryos germinated of date palm *cv.* Sukkary. Y-bar indicated the sample variability whereas LSD value ($P \leq 0.05$) is the least significant difference among treatment means.

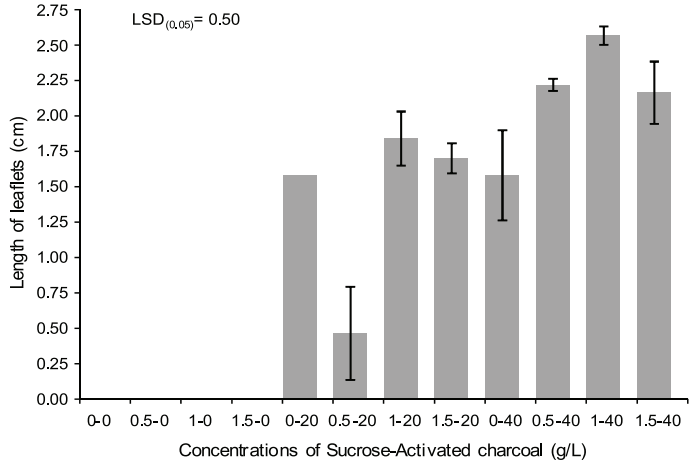

Fig. 6. Combined effect of sucrose (0, 20 and 40 g/L) and activated charcoal (0, 0.5, 1 and 1.5 g/L) concentrations on the length of leaflets of date palm *cv.* Sukkary. Y-bar indicated the sample variability whereas LSD value ($P \leq 0.05$) is the least significant difference among treatment means.

charcoal along with sucrose was the best treatment to obtain the longest root and shootlet in *Ruscus hypoglossum*. Similarly, Martínez *et al.* (2015) obtained the best embryo proliferation in MS medium containing 0.4% charcoal with 6% sucrose for *Quercus alba* and 3 % sucrose for *Q. rubra*.

In this study, it has been concluded that the individual impact of sucrose and activated charcoal for best somatic embryogenesis and development (number of somatic embryos, length of somatic embryos, fresh and dry weights of somatic embryos, germination of somatic embryos and length of leaflets) was observed when they were used at 40 and 1 g/L, respectively. However, the combined effect of sucrose (40 g/L) with activated charcoal (1 g/L) was highly significant than the individual effects of these carbon sources. The same concentration of sucrose with 0.5 g/L activated charcoal also gave promising results. The findings of the experiment also showed that without carbon source no somatic embryogenesis and developmental processes occurred.

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