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THE EFFICIENCY OF PLANT FORM BIOREACTOR AS TEMPORARY IMMERSION SYSTEM IN BUD AND MULTIPLICATION SHOOT VIA DIRECT ORGANOGENESIS OF DATE PALM UNDER LED LIGHT

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Abstract

The study was conducted in the Date palm micropropagation Unit belonging to College of Agriculture, University of Kufa during the period from October 2015 to June 2018 in order to determine the effect of different concentrations of 2iP added to the nutrient media and testing the efficiency of the temporary immersion system using the plantform bioreactor in increasing the number of produced shoots and quality in the multiplication stage of buds and shoots under LED light. The results showed Temporary Immersion System (TIS) using the plantform bioreactor was excelled in the number of shoots, fresh weight of Biomass, and its content of sugars compared with solid media, while the solid media is excelled in shoot length. Addition of 0.4 mg.L^{-1} 2ip to the nutrient media led to effect on most indicators of the buds and multiplication shoots which led to increase of the number of shoots and their length, as well as the fresh weight of the biomass and dissolved sugars. Plantform bioreactor has shown remarkable efficiency in increasing the multiplication of shoots and other growth indicators and we recommend to incorporate it into the Date palm micropropagation protocol via direct organogenesis.

Introduction

Date palm (*Phoenix dactylifera* L.) cultivation is widespread in the Middle East and North Africa region which includes 3,000 cultivars (Johnson, 2011). There are 62 million palms in it from more than 100 million palms scattered around the world and over 1.3 million hectares (Al-Alawi *et al.,* 2017). The number of palms in Iraq has declined significantly where the number of palms reached 17 million palms during the year of 2014 compared to 1952 year, where the number of palms were 32 million palms (Central Bureau of Statistics, 2017). The dramatic decline in the number of palms due to war, neglect, high salinity and pests. The date palm propagation is commercialized through the cultivation of offset, but the number of offset produced by the single palm is few, especially the desired and rare cultivars, as well as that the offset in the palm is limited in the early years of the Palm age, so the technology of plant tissue culture is used to solve some of the problems, Through which one method is used to produce hundreds of intracellular filaments, as well as the possibility of using rare palms in the production of palm *in Vitro*. The propagation of the dates by tissue culture is conducted by two pathways Somatic Embryogenesis (direct and indirect) and Organogenesis (direct and indirect) due to the emergence of some problems in the indirect pathways due to the passage of Callus stage which may cause the production of plants that are not fully identical to the mother, so the current focus has been on using the direct organogenesis pathway (Al-Khayri and Naik, 2017). Growth regulators, including Auxins and cytokinines, that added to nutrient media is considered as major factors that influence the multiplication of buds and shoots in date palms. Khierallah and Bader (2007), Bader and Khierallah (2009) succeeded in multiplication the shoots of date plams (Maktoom and Barhee cultivars) when using 2 mg BAP, 4 mg 2ip, 1 mg NAA and 1 mg NOA. Bekheet (2013) was able to obtain the best multiplication of the shoots by adding 5 mg 2ip

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from the regulators and 2 mg Kin to the multiplication media of Zaghlool cultivar. Solid nutrient medias are commonly used for the propagation of the date palm, either through organogenesis or Embryogenesis. In this way, it is difficult to introduce automatic devices into the large commercial propagation process (Vinocur *et al.,* 2000). Therefore, recent serious attempts have been made to use temporary immersion system (TIS) in commercial propagation because they have advantages compared to the solid medias (Zobayed and Saxena, 2003). Therefore, modern studies have attempt to the development of micropropagation programs using modern Culture system in their production, including the Culture system in the liquid media, which is conducted in larger containers because it is more easily in terms of application and need less time where it is not required to be placed the explant in a specific direction, In order to avoid the continuous immersion of the plant separator in the liquid media and to increase the amount of ventilation around the explant. This Culture system (TIS) has been successfully employed in commercial propagation of many economically important plants. The plantform bioreactor is one of the bioreactors used as a temporary immersion system (TIS). This bioreactors has been used in the micropropagation for many plants successfully including bananas, chestnuts, raspberries, apples, olives and other zina plants (Welandera *et al.,* 2014). Othmani *et al.,* (2009) and AL-Mayahi (2015) and Al-Mayahi and Moussawi (2017) studied the effect of temporary immersion of embryonic Callus in the production of somatic embryos for date palms and found that the temporary immersion gave the highest number of embryos compared to the solid media. A review of the available references indicates that a rare studies about the effect of temporary immersion systems using plantform bioreactor in direct organogenesis for date palm micropropagation. The aim of study to test the efficiency of plantform bioreactor as a temporary immersion system (TIS) and different concentrations of added cytokinins (2 iP) to the nutrient media in increasing the number of produced shoots and their quality in multiplication of buds and shoots under LED lamps.

Materials and Methods

The study was conducted in the laboratories of Date palm micropropagation Unit at the College of Agriculture, University of Kufa during the period from October 2015 to June 2018 to test the efficiency of a temporary immersion system (TIS) consisting of a plantform bioreactor and 2ip concentrations in multiplication of date palm shoots via direct organogenesis by culture the quarters of the apical meristem. In the study, the offset for the date palm (Barhee cultivar) with age of (3-4 years) was used, which weighs 8-12 kg and growing in private orchard in Al-Hindeia District in Karbala province, a source for the establishment of date palm cultures. Barhee is considered one of the best cultivars. Offset were brought to the laboratory, and dissected in the anatomy room by a knife by removing their leaves and fibers upward until reaching the Shoot tip to avoid damage to the apical meristem, where the final size of the bud is 3-4 cm diameter and its length (6-8 cm) after it was placed in an anti-oxidation solution (which is composed of 100 $mg.L^{-1}$ Ascorbic Acid and 150 $mg.L^{-1}$ Citric Acid, after it was kept in the refrigerator at 5°C until surface sterilization process (Tisserat, 1988).

Surface sterilization for the apical meristem

Shoot tip was sterilized according to method was used by Badr and Ali (2014). In Laminar air flow cabinet. Shoot tip was placed in sodium hypochlorite solution with a concentration of 20% for 20 min. shoot tip was then transferred to the same solution but at 10% concentration for 15 minutes. The damage tissues were then removed and then sterilized again with the same concentration of the previous solution but for 10 minutes. The tissue was then washed with sterilized distilled water 3-4 times. The longitudinal bud was then divided into four quadrants for the purpose of culture. After conducting the sterilization process for the apical buds and cutting it longitudinally into four parts, each part cultured in a culture container containing 50 ml of the nutrient media for the growth of buds containing the media (MS) (Murashige and Skoog, 1962) with 0.5 $mg.L^{-1}$ NAA, 0.5 $mg.L^{-1}$ NOA, 1 $mg.L^{-1}$ BA and 1 $mg.L^{-1}$ 2 ip (Rad *et al.,* 2015). Then the cultures were incubated in the dark at a temperature of 27 °C for 12 months, being transferred every two months.

The multiplication stage of buds and shoots

At this stage the buds were moved to the MS with 0.5 $mg.L^{-1}$ Kinetin, 0.5 $mg.L^{-1}$ BAP and 1 mg. L^{-1} NAA. The cultures were incubated at 27°C, a light intensity of 1000 lux and a period of 16 hours daily. This stage was repeated several times for the purpose of multiplying buds from the previous stages and then obtained a groups of buds and shoots.

Efficiency Test of Plantform bioreactor as TIS

Plantform bioreactor system is used in the research as shown in Figs. 1 and 2. The system consists of 12 cultures containers with the size of 180×160×150mm, At the base of each container is basket with holes of 1 mm in size is placed above container that controls the medium flow. This basket holds the plant material. A frame with four leg sisplaced above the basket to avoid the basket to

Fig. 1: The main parts of the Plantform bioreactor system

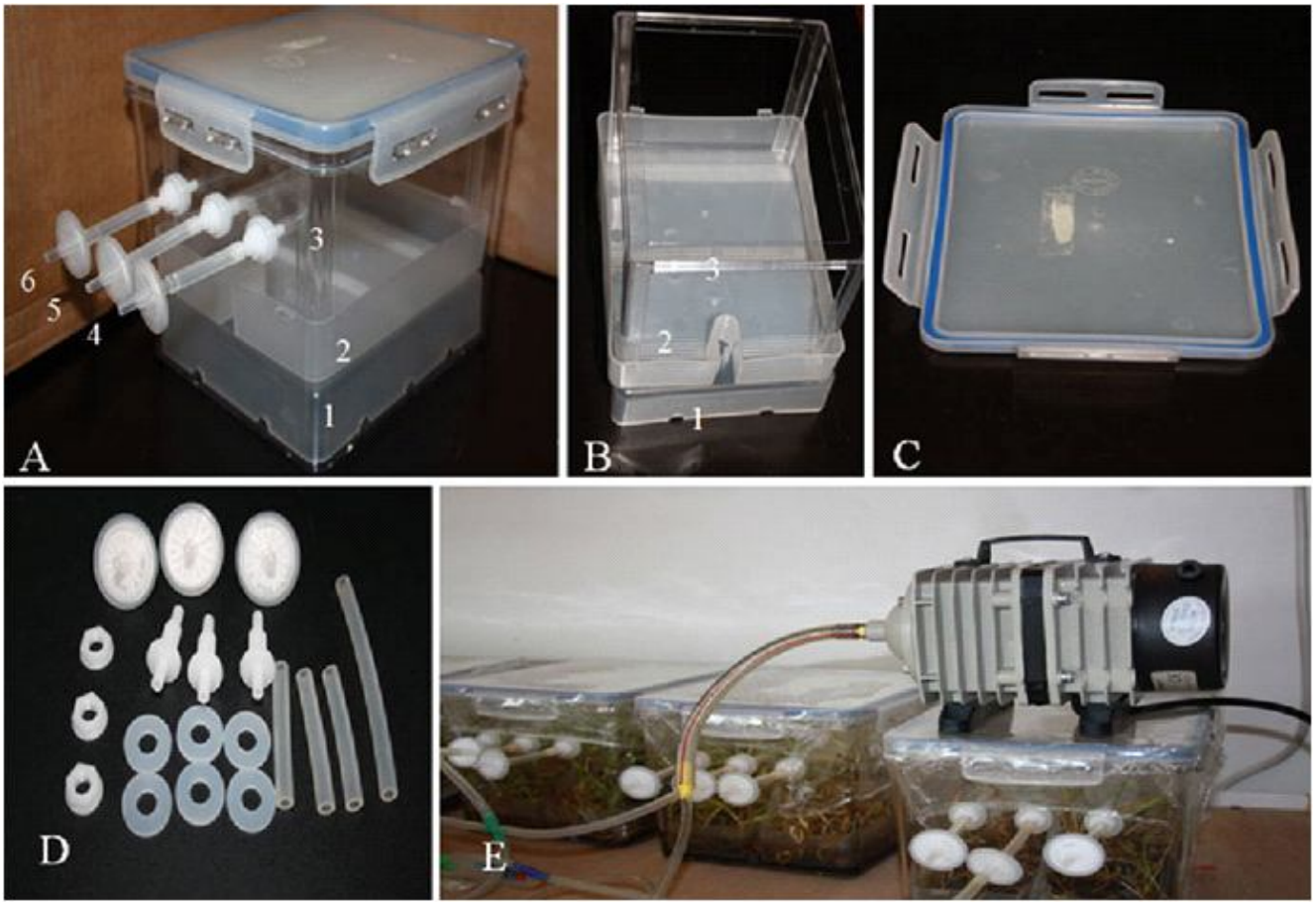

Fig. 2: Agriculture container and side port, The container base (A-B-C), filtration filters (Millipore), air transfer tubes (D) and air pump (E).

screws Provided with Silicone seals Are fitted Tightly within The holes. Connected To these screws are flexible plastic Tygon tubes, With an Inner diameter of 3.2 mm, and 0.22 ìm poly tetra fluoro ethylene (PTFE) Filters. The Filters make sure that the airflow in and out of the bioreactors is sterile. The principle of its work is being by placing nutrient liquid media in the container by 500 ml per container and place the non-perforated basket above it and rise when air pressure is applied to the bioreactor. The construction and placement of the basket is made so that the Plants are Only immersed into the Liquid medium when air Pressure is Applied to the bioreactor. Further more, the Bioreactor has Three opening Holes for Medium supply, Aeration and ventilation. Specially designed Hollow then the perforated basket, which will place the plant parts in it and then govern the lid of the container well. The air is then pushed into the nutrient media under the basket and above the basket from the air pump attached to the timer. It is usually operated for several minutes every several hours, so that the media rises, the cultivated

parts are submerged then exposure to air.

A 500 mL of liquid MS media supplemented with (0.5 mg.L^{-1} Kinetin and 0.5 mg.L^{-1} BAP and 1 mg.L^{-1} NAA) was place in each container of this system after adding 2ip at concentrations 0.1, 0.2, 0.3, 0.4 mg.L^{-1}. In addition to the control treatment (solid medium without 2ip). Five plant parts (biomass) were cultivated in each container, Each biomass weighs 1 g so that the container contains five grams of cultivated plant tissue. The containers were placed and operated in the growth rooms under the light of the LED lamps (mixture of two color red and blue with ratio of 18 for red color and 2 blue color) as shown in Fig. 3 for 4 months with 16 hours illumination followed by 8 hours of darkness and 4-minute operation every one hours. At the end of this experiment, three container were chose and collected according to the average number of shoots, the average length of formed shoots, the average of fresh weight for the biomass (g) and the content of shoots of total soluble sugars (mg.g^{-1} dry weight) according to the proposed method by (Herbert *et al.* 1971) using spectrophotometer spectros copy at a wavelength of 488 nm.

Experimental design and statistical analysis

The experiment was performed as a factorial experiment using Complete Randomized Design (CRD), with two factor (2ip concentration and culture system). The averages of the treatments were compared using a Least Significant Differences (L.S.D) at a probability level of 0.05 to test the differences between the averages.

Results

Number of shoots

Table 1 indicates that there are significant differences between the two Culture system in trait of the average number of shoots. The TIS system by using Plantform Bioreactor was significantly excelled on cultivation in the solid media by giving it 33.82 shoots compared to 24.51 shoots for the solid media. There were also significant differences between the 2ip concentrations in the number of shoots where the number of shoots increased with increase the 2ip concentration. The concentration of 0.4 mg.L^{-1} 2ip was significantly excelled on the other concentrations by giving it 44.65 branch, while the concentration of 0.0 mg.L^{-1} (control) gave the lowest number of shoots reached 9.97 shoots. The results of the interaction in the same table indicate significant differences between the Culture system and the 2ip concentrations. The cultures of the interaction treatment between temporary immersion system and 0.4 mg.L^{-1} 2ip was significantly excelled on the other interaction treatments by giving it 52.25 shoots as shown in Fig. 4. While the

Fig. 3: Used LED system (A) and distribution of immersion system under LED lamps.

interaction treatment of (culture in the solid media and 0.0 mg.L^{-1} 2ip) gave the lowest average of the number of shoots amounted to 9.5 shoots.

Length of shoots

Table 2 indicates that there are significant differences between the culture systems used in the study for the length of the shoots. The culture system in the solid media is excelled to the temporary immersion system by using a plantform bioreactor with an average of 2.033 cm while cultivation with the temporary immersion system gave 1.368 cm. This may be due to the fact that the culture system with temporary immersion has encouraged the production of shoots in large numbers, which led to the lack of lengths to its compete for nutrients, and this is evident in the results of the study in table 1 related to the number of formed shoots. It is clear from table 2 that there are significant differences between the 2ip concentrations for the length of shoots, where the average number of shoots increased with increase a concentration, where the concentration of (0.4 mg.L^{-1} 2ip) was significantly excelled on the rest of the other concentrations by giving it 3.277 cm while the concentration of (0.0 mg.L^{-1} 2ip) (control) gave the lowest average for the length of branch (0.570 cm). As for the results of the interaction in the same table, there were significant differences between the interactions of the type of the culture system and the 2ip concentrations. The interaction treatment (cultivation in solid was and 0.4 mg.L^{-1} 2ip) was significantly excelled on the other interaction treatments which gave 3.990 cm, While the

Table 1: The effect of the culture system and the concentration of 2ip and their interactions in the number of multiplicated shoots for date palm (Barhee cultivar) after 8 weeks under the LED lamps (18 red: 2 blue).

Culture system	Concentration of 2ip ($mg.L^{-1}$)					The average of Culture system
	0	0.1	0.2	0.3	0.4	
Cultivation in a solid media	9.5	16.15	27.55	32.3	37.05	24.51
Cultivation in a liquid media using temporary immersion system using Plantform Bioreactor	10.45	23.75	38	44.65	52.25	33.82
The average concentration of 2ip	9.97	19.95	32.77	38.47	44.65	
LSD (0.05)	Concentration of 2ip = 2.04 Culture system = 1.29 Interaction = 2.88					

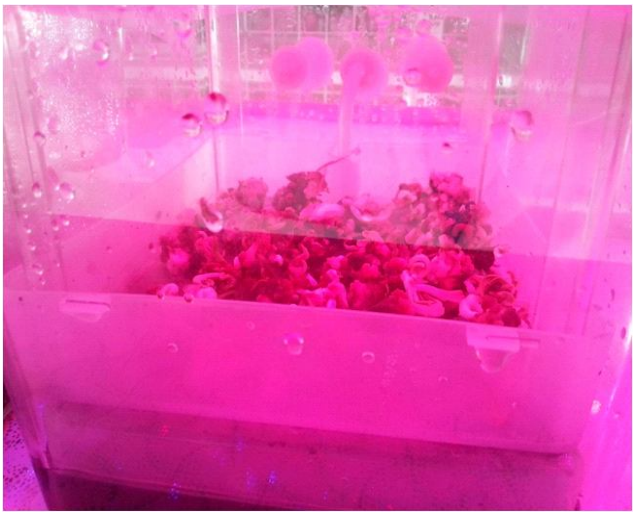

Fig. 4: Number of bud and multiplicated shoots under the temporary immersion in the culture container of the plantform bioreactor under LED lamps.

interaction treatment (temporary immersion system and 0.0 $mg.L^{-1}$ 2ip) gave the lowest length of branch (0.475 cm).

Average weight of biomass (g)

Table 2 indicates that there are significant differences in the weight of the biomass. The cultivation with the temporary immersion system by using Plantform Bioreactor excelled on cultivation with the solid media by giving it 34.46 g. The same table shows that there are significant differences between the 2ip concentrations of this trait, where the treatment of (0.4 $mg.L^{-1}$) excelled on the rest of the treatments by giving it 46.22 g. While the control treatment gave the lowest weight of biomass (13.25 g). The results of the interaction in the same table indicate that there are significant differences between the interaction treatments. The interaction treatment (the temporary immersion system and 0.4 $mg.L^{-1}$ 2ip) was significantly excelled on the other interaction treatments which gave 60.42 g. While the interaction treatment (cultivation in the solid media and 0.0 $mg.L^{-1}$ 2ip) (control) has gave the lowest average weight of biomass, which amounted of 11.88 g.

The shoots content of the total soluble sugars

Table 4 showed significant differences between the culture systems under study. The results showed that the superiority of culture in the liquid media with the temporary immersion system significantly in the shoots content of the total soluble sugars which reached (2.112 $mg.g^{-1}$ dry weight) in culture on the solid media, which gave (1.115 $mg.g^{-1}$ dry weight). The same table indicates significant differences between 2ip concentrations added to the average in the shoots content of the total soluble sugars. The average of total accumulation of sugars increased with increase a concentration of 2ip in the media, the concentration of (0.4 $mg.L^{-1}$) gave the highest level of sugar accumulation amounted of (2.527 $mg.g^{-1}$ dry weight) and thus recorded a significant superiority over the remaining 2ip concentrations added to the media while the concentration of (0.0 $mg.L^{-1}$) (control) gave the lowest shoots content of the total soluble sugars (0.432 $mg.g^{-1}$ dry weight). The results of the interaction in the same table showed significant differences between the interactions of the culture system and the 2ip concentrations. The interaction treatment (cultivation with the temporary immersion system and concentration of 0.3 $mg.L^{-1}$ 2ip) excelled on the other interaction treatments by giving it (3.210 $mg.g^{-1}$ dry weight). The lowest content of total soluble sugars at interaction (cultivation in the solid media and concentration of 0.0 $mg.L^{-1}$.2ip), which amounted to (0.396 $mg.g^{-1}$ dry weight).

Discussion

Tables 1, 2, 3, 4 related to testing of mounting concentrations of the 2ip growth regulator in the multiplication stage, The increase in concentration of the growth regulator 2ip was accompanied by an increase in the number of multiplicated buds, its length and weight. The effect of different cytokinines, including 2ip, in the events of vegetative multiplication comes through their role in cell division and forming organs, The presence of

Table 2: The effect of the culture system and the concentration of 2ip and their interactions in the average length of shoots for date palm (Barhee cultivar) after 8 weeks under the LED lamps (18 red: 2 blue).

Culture system	Concentration of 2ip (mg.L^{-1})					The average of Culture system
	0	0.1	0.2	0.3	0.4	
Cultivation in a solid media	0.665	1.140	1.615	2.755	3.990	2.033
Cultivation in a liquid media using temporary immersion system using Plantform Bioreactor	0.475	0.855	1.235	1.710	2.565	1.368
The average concentration of 2ip	0.570	0.998	1.425	2.232	3.277	
LSD (0.05)	Concentration of 2ip = 0.126 Culture system = 0.080 Interaction = 0.178					

Table 3: The effect of the culture system and the concentration of 2ip and their interactions in the average fresh weight of biomass (g) for date palm (Barhee cultivar) after 8 weeks under the LED lamps (18 red: 2 blue).

Culture system	Concentration of 2ip (mg.L^{-1})					The average of Culture system
	0	0.1	0.2	0.3	0.4	
Cultivation in a solid media	11.88	14.15	18.24	22.73	32.02	19.80
Cultivation in a liquid media using temporary immersion system using Plantform Bioreactor	14.63	20.30	31.08	46.76	60.42	34.46
The average concentration of 2ip	13.25	17.22	24.66	34.75	46.22	
LSD (0.05)	Concentration of 2ip = 1.96 Culture system = 1.24 Interaction = 2.78					

cytokinin as part of the t-RNA transporter has an important role in linking RNA-t with m-RNA during protein synthesis. T-RNA free of the Adeninisopentenyl side chain is ineffective and the addition of 2ip activates RNA, cytokinines regulate the genetic expression at the level of RNA replication, it was observed that a slight increase in the level of internal cytokinin was accompanied by a significant increase in the level of mRNA (Mohammed and Yunis, 1991). These results agree with (Al-Marri and AL-Ghamdi, 1995; Bekheet and Saker, 1998; Hamid, 2001; Al-Khateeb *et al.,* 2002; Khierallah and Bader, 2007). The presence of BA and 2iP is necessary for multiplication of date palm tissue. As for the increase in sugars with 2ip, cytokinines work as a sink for nutrients and sugars found in the nutrient media, which is important building processes in tissue, thus accumulating sugars (Davies, 2004). The results of the same tables indicate that the superiority of cultivation in the liquid media with temporary immersion on the culture system in the solid media may be due to increasing nutrient availability and absorption in the liquid media compared to the solid media, as well as the movement of the implanted part leads to increased gas exchange and avoid the lack of elements that occur in the solid media (Pierik, 1999). The temporary immersion system is characterized by the use of the plantform bioreactor to allow the gas exchange and ventilation, which are very important in tissue culture. The temporary immersion system contributes to the increase of oxygen, which led to the growth and development of buds and shoots due to the movement generated by the processing of external air by the temporary immersion system. Increasing the surface area of cells exposed directly to the nutrient media has increased the number of formed shoots (Mehrotra *et al.,* 2007). Roels *et al.,* (2005 and 2006) noted that TIS in the liquid media prevents the accumulation of carbon dioxide and ethylene oxide, which have detrimental effects on obtained cultured tissue as a result of cultivation over solid media. This system allows by rich the cultures with oxygen and the elimination of plant-damaging gases that hinder their growth, such as ethylene (Maurizio *et al.,* 2015), which is reflected by getting good buds and seeds. It is also possible to explain the superiority of the growing cultures on the liquid media in the content of their shoots of the total soluble sugars to the fact that the liquid medias in the temporary immersion system allow gas exchange and ventilation as well as increase the availability of nutrients and sucrose, Which the tissue benefit from it in the construction of sugars. The trend towards agriculture with temporary immersion systems is to prevent nutrient deposition and to allow the optimal growth of plant tissues (Scragg, 1992), the content of the macro-elements are considered responsible for carbohydrate accumulation and formation of buds. The availability of carbohydrates in the agriculture media can also increase the internal levels of the plant tissue of sugars

Table 4: The effect of the culture system and the concentration of 2ip and their interactions in the shoots content of the total soluble sugars for date palm (Barhee cultivar) after 8 weeks under the LED lamps (18 red: 2 blue).

Culture system	Concentration of 2ip (mg.L^{-1})					The average of Culture system
	0	0.1	0.2	0.3	0.4	
Cultivation in a solid media	0.396	0.410	1.276	1.312	2.182	1.115
Cultivation in a liquid media using temporary immersion system using Plantform Bioreactor	0.468	1.808	2.203	3.210	2.782	2.112
The average concentration of 2ip	0.432	1.109	1.740	2.261	2.527	
LSD (0.05)	Concentration of 2ip = 0.119 Culture system = 0.075 Interaction = 0.168					

(Borisjuk *et al.,* 2004). The results of the study agreed in terms of the superiority of temporary immersion using plantform bioreactor in the average number of shoots and their lengths compared to the traditional methods of propagation (culture on solid media) in other plants with (Welander *et al.,* 2014; Sacco *et al.,* 2015; Carla *et al.,* 2015). It also agreed with (Yan *et al.,* 2010; AlKhateeb and Alturki, 2014) in terms of increasing the shoots fresh and dry weight in the liquid medias under temporary immersion compared to the solid media.

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