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Research Article

# A comparative study between temporary immersion system and semi-solid cultures on shoot multiplication and plantlets production of two Moroccan date palm (*Phoenix dactylifera* L.) varieties *in vitro*

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**Abstract**

Date palm micropropagation is commonly performed on gelled media. However, it's typically a labour-intensive system and consequently plantlets production cost is very high. Therefore, it is necessary to develop cost effective alternatives without compromising the quality of produced plant material. New technologies based on liquid media in bioreactors have been developed to reduce the handling time, while increasing the multiplication rates and plant quality. The present research focuses on the comparison between Temporary Immersion System (TIS) and gelled media (GM) culture systems of two Moroccan date palm varieties 'Mejhool' and 'Boufeggous'. Obtained results indicated that shoot and root lengths as well as shoot fresh and dry weights were significantly ($P < 0.05$) higher in TIS compared to GM. Moreover, the vigour of obtained shoots was better in TIS compared to GM. Therefore, TIS-derived plantlets have shown an acclimatization rate of 95% while this rate for GM-derived plantlets was 82%. Hence, bioreactors, as a growing system based on TIS, can be a valid alternative to conventional systems for *in vitro* culture, resulting in a reduction of cost, shelving area requirements, labour and time for the mass propagation of date palm cultivars.

***Keywords:*** bioreactor; liquid medium; micropropagation; organogenesis; shoot proliferation

## Introduction

Date palm is an important subsistence crop of the desert regions and is a rich source of nutrition, contributing to food security. *In vitro* clonal propagation is an effective and efficient alternative for conventional vegetative propagation, to ensure rapid multiplication and establishment of true-to-type plants of elite cultivars (Georgieva *et al.*, 2016). However, the present cost of production needs to be reduced drastically for popularizing tissue culture propagation of date palm. Indeed, the cost of date palm plantlet production is notoriously high as compared to that of other horticultural crops. For example, it is more than 100 times that of banana (Rajmohan, 2011). Hence, there is a pressing need to improve the protocols, to bring down the plant price to affordable levels of the ordinary farmers. Agar is the most widely used gelling agent, and accounts for 10-20% of the cost of the culture medium (Vyas *et al.*, 2008). Besides, agar can contain impurities leading to inconsistent responses. In addition, cultivation on agar-gelled medium requires labour-

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intensive steps including repeated sub-culturing. Indeed, labour generally accounts for 40-60% of production costs (Nagori *et al.*, 2009). Besides, the low multiplication rate of shoots obtained in agar solidified-medium is a critical problem facing the success of commercial tissue culture in date palm propagation (Ibraheem *et al.*, 2013). Overall, the parameters most involved in reducing production costs include: (1) the drastic reduction in work; (2) reduction in shelving area; (3) reduction in the number of containers used; (4) better biological yields (Etienne and Berthouly, 2002).

The conventional method, using semi-solid medium, has been widely used due mainly to its simplicity. However, the huge amount of labour is a major disadvantage (Arigundam *et al.*, 2020). New technologies have been developed to reduce the handling time, while increasing the multiplication rate and plant quality. Besides, liquid cultures are more amenable to automation necessary in commercial scaling-up production systems (Othmani *et al.*, 2011). As a result, the Temporary Immersion System (TIS), also called Temporary Immersion Bioreactor (TIB), was created in the 1980's. The principle of TIS technology is that plant material is immersed in growth media for short periods and at regular intervals. These immersions are sufficient for the plants to take up the nutrients. TIS technology makes use of the advantages of liquid cultures, while growing the plant material under high gas-exchange environment. Indeed, the applied air pressure is also functioning as ventilation due to created bubbles during this process. Excessive air, as well as released gases, can deplete from the container through a ventilation tube connected with a filter (Welander *et al.*, 2014).

TIS provide a rapid and efficient plant propagation system for many agricultural and forestry species. In fact, many research works have been published in *Phoenix dactylifera* L. (Fki *et al.*, 2011; Othmani *et al.*, 2017; Almusawi *et al.*, 2017), *Elaeis guineensis* Jacq. (Marbuna *et al.*, 2015), *Olea europea* L. (Benelli and De Carlo, 2018), *Ananas comosus* L. (Ramli, 2018), *Prunus avium* L. (Godoy *et al.*, 2017). Generally, plantlets propagated in TIS have better performances than those propagated by conventional methods of micropropagation. It results in increased shoot vigour and in the frequency of morphologically normal or natural-like plantlets. This is a result of a better handling of the *in vitro* atmosphere and the nutritional status of cultured plantlets. Hence, liquid medium favours the easy uptake of nutrients and growth regulators resulting in enhanced vegetative growth of cultured explants while in semi-solid media, agar is an adsorbent agent that complicates the movement of nutrients (Sandal *et al.*, 2001). In addition to diminishing production costs regarding labour force, TIS can save energy, augment micropropagation productivity and efficiency (Lyam *et al.*, 2012). The use of bioreactors in date palm resulted in an improved multiplication rate and reduced micropropagation time. It also reduces the cost of saleable units and thus improves economic return for commercial micropropagation (Almusawi *et al.*, 2017; Carvalho *et al.*, 2019).

Plantform™ bioreactor (*www.plantform.se*) is a relatively recent developed TIS made of transparent polycarbonate. The size of the bioreactor (180 × 160 × 150 mm) allows a larger amount of plant material in each unit and hence reduces labour costs in large-scale plant production. Immersion and ventilation cycles are regulated by two separate air pumps, each connected to a timer. One more advantage of this bioreactor is that it has a relative greater interior bottom in a suitable size for easy handling of plant material. Besides, such bioreactors could be placed above each other for saving culturing space, which is more attractive in commercial production (Welander *et al.*, 2014). Furthermore, the amount of nutrient supply (up to 500 ml) can be adapted according to the different growth phases, as bigger plants require more nutrients than small ones. In addition, enrichment of oxygen and other gases as well as purging of deleterious gases can be easily monitored in this system. When exhausted, media could be easily replaced under laminar flow hood without changing the bioreactor. Subsequently, the objective of this study was to evaluate the feasibility of using the Plantform™ bioreactor to micropropagate plant material, in comparison to routinely used method performed on semi solid media, in two Moroccan varieties 'Mejhool' and 'Bougeggous' producing high fruit quality.

## Materials and Methods

*Plant material*

Plant material is produced by using organogenesis technique from offshoot shoot tip explants of date palm 'Mejhool' (MJHL) and 'Boufeggous' (BFG) varieties. Shoot tips removed from 3-4 years-old offshoots were disinfected before transferring on culture media according to the protocol used in our laboratory (Abahmane, 2017). Shoot tip explants were cultured on Murashige and Skoog (1962) medium (MS) supplemented with 0.5 mg $l^{-1}$ of 1-naphthaleneacetic acid (NAA), 0.5 mg $l^{-1}$ of naphthoxyacetic acid (NOA), 1 mg $l^{-1}$ of 6-(dimethylallylamino) purine (2-iP) and 1 mg $l^{-1}$ of benzylaminopurine (BA) (Rad *et al.*, 2015*). In vitro* cultures were incubated for 6 months under dark conditions at 26 ± 1 °C and monthly sub-cultured on fresh media. Obtained vegetative buds were proliferated on multiplication medium consisting in MS/2 salts supplemented with 0.25 mg $l^{-1}$ of 2-iP, 0.1 mg $l^{-1}$ of NOA and 0.2 mg $l^{-1}$ of indole acetic acid (IAA) under 16 hours light photoperiod, supplied by LED tubes (25 µmol $m^{-2}$ $s^{-1}$). After successive transfers on multiplication media, obtained clusters of buds were used as initial plant material for the experiments.

All culture media were supplemented with $NaH_2PO_4$ (170 mg $l^{-1}$), myo-inositol (100 mg $l^{-1}$), adenine (40 mg $l^{-1}$), glutamine (200 mg $l^{-1}$), Nicotinic acid (0.1 mg $l^{-1}$), Pyridoxine-HCl (0.1 mg $l^{-1}$), Biotin (0.01 mg $l^{-1}$), sucrose (30 g $l^{-1}$) and agar (Satiagel AMP45) at 8 g $l^{-1}$. The pH of media was adjusted to 5.8 ± 0.1 before autoclaving during 20 minutes at 1 bar pressure.

*Effect of growing system on growth and development of plant material*

Plantform™ bioreactor based on temporary immersion system was compared to conventionally culture on gelled medium (Figure 1A). The Plantform™ is a propagation bioreactor where shoots undergo periodic immersions in liquid medium alternated with dry periods; avoiding gas accumulation through forced ventilation. The immersion regime used in this experiment was set at 4 hours cycle with 5 min immersion periods daily. 500 ml of liquid medium were dispensed in each bioreactor while 100 ml of gelled medium were dispensed in 270 ml jars for comparison.

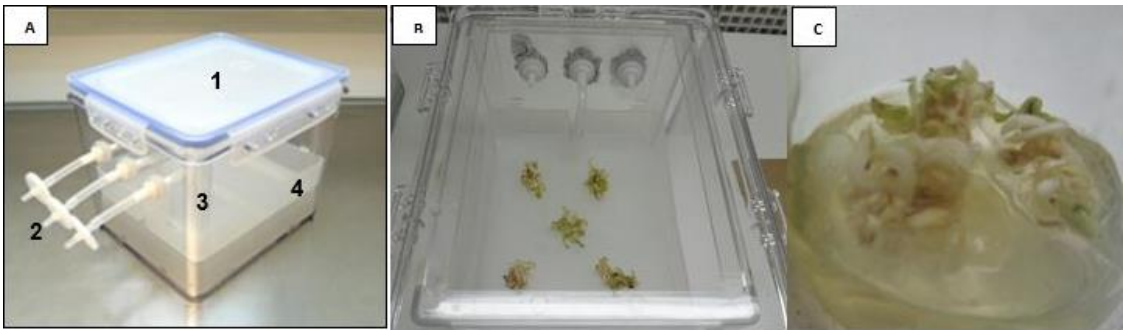


**Figure 1.** Plantform™ bioreactor: (A1) lid, (A2) Inlets/outlets for gas exchange and medium supply, (A3) Bioreactor container, (A4) Basket for plant material support. Plant material in bioreactor (B) and in gelled medium (C)

After *in vitro* culture of plant material for 12 weeks, several growth parameters were assessed including shoot and root numbers, shoot and root length, fresh and dry weight of both shoots and roots. Shoot and root length was measured respectively for the longest leaves and roots. Dry weight was determined after drying the plant material at 70 °C for 72 hours.

*Plant acclimatization*

Regenerated plantlets with 3 to 4 leaves and well-developed root system (4-5 roots) were transferred from culture media and the root system was washed with tap water then the whole plantlets were soaked for 3 min in a solution of systemic fungicide with broad spectrum (Pelt 44: methyl thiophanate) at 1 g $l^{-1}$. The plantlets were then transferred in plastic bags (8 × 13 cm) filled with substrate made of mixture of peat moss and fine gravel (2:1) for acclimatization. The potted plantlets were incubated under micro tunnel covered with transparent polyethylene film. After 4 weeks of acclimatization, the plastic film was gradually removed to allow plantlets hardening under glasshouse conditions (28 °C and Relative Humidity of 75%).

*Experimental design*

Experiments were set up in a completely randomized design. Three bioreactors for each variety were used with each bioreactor as one replicate. Each bioreactor contained five homogeneous shoots at the multiplication stage (Figure 1B). Meanwhile, the same number of explants grown in five jars on gelled medium was included for comparison (Figure 1C). Collected data were analysed by using ANOVA with two factors (2 varieties and 2 growing systems) performed with SPSS 16.0 for windows (IBM Software) and the means were compared using LSD at 5%.

## Results and Discussion

*Effects of growing system on shoot and root production*

The effectiveness of the Plantform™ bioreactor in micropropagation of date palm 'Mejhool' and 'Boufeggous' varieties was examined through measurement of biomass production during subcultures period in comparison to gelled medium culture. Collected data concerning shoot multiplication have shown that the number of proliferated shoots is slightly high on GM compared to TIS for both varieties (Figure 2). However, the observed differences between the two systems were not statistically different ($p < 0.05$). Besides, the two varieties have shown the same behaviour and no statistical differences have been detected. Otherwise, the growing system had significant effects ($p < 0.05$) on leaf length (Figure 3). Indeed, liquid media stimulated leaf length compared to gelled media. Besides, significant differences ($p < 0.05$) were observed between the two varieties. The longest leaves (11.67 cm and 7.74 cm) were recorded respectively in TIS and GM in 'Mejhool' variety.

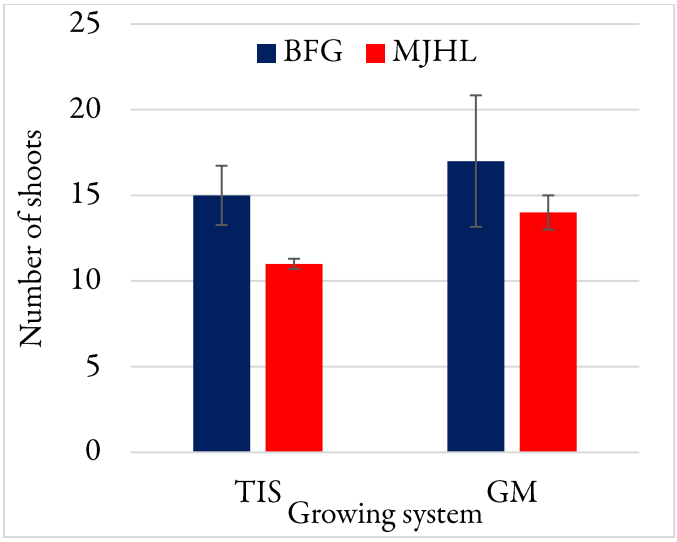


**Figure 2.** Effect of growing system on shoot number

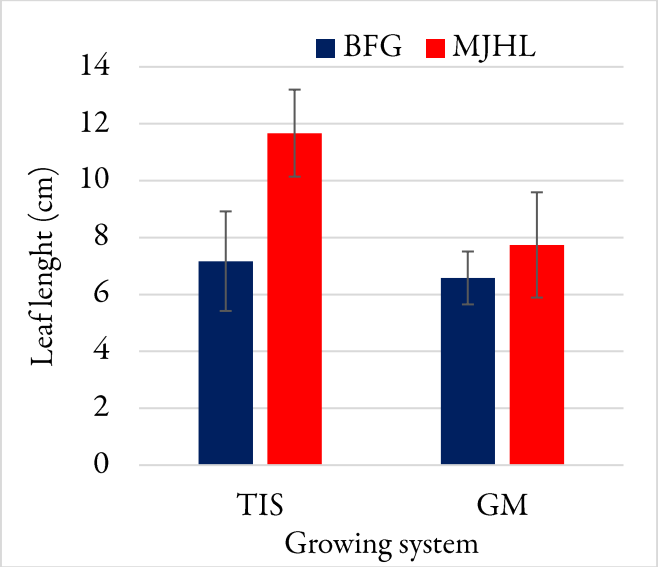


**Figure 3.** Effect of growing system on leaf length

Concerning root production, it was noticed that GM increased the number of produced roots (6.19 roots/shoot) in comparison to TIS (5.48 roots/shoot) but only in 'Mejhool' variety (Figure 4). In 'Boufeggous' variety, the situation was reversed as recorded data showed 3.13 and 2.76 roots per shoot respectively in TIS and GM. However, root length was globally stimulated in TIS compared to GM (Figure 5). The highest root length (17.17 cm) was recorded in 'Mejhool' variety on liquid medium compared to 13.09 cm registered on gelled medium. Statistical analysis has revealed significant differences ($p < 0.05$) between the two studied varieties as 'Mejhool' variety has shown the longest roots and the highest number of roots per shoot.

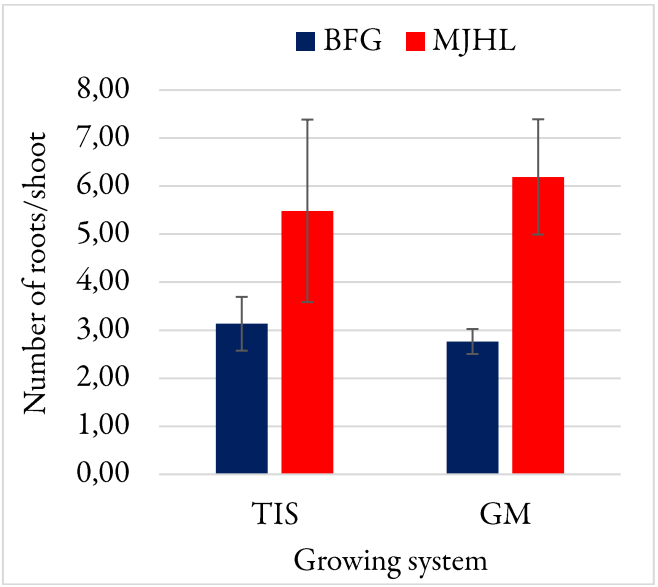


**Figure 4.** Effect of growing system on root number

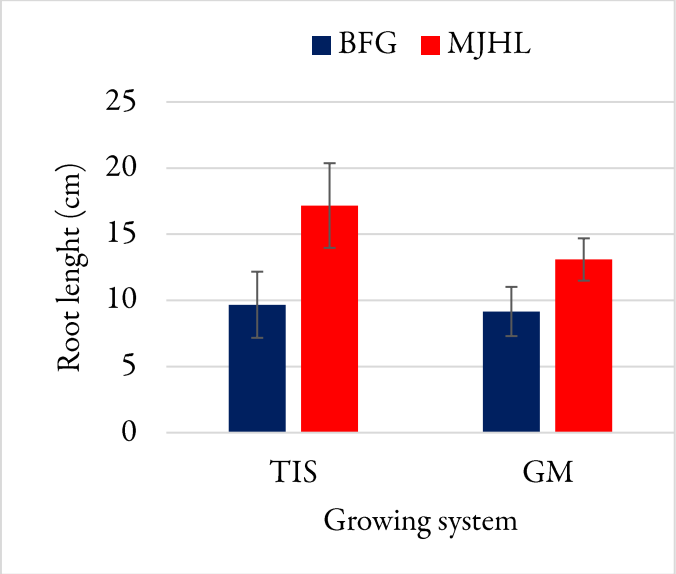


**Figure 5.** Effect of growing system on root length

Obtained results were in accordance with reported data of AlKhateeb and Alturky (2014) in 3 date palm cultivars ('Sukary', 'Mejhool' and 'Reziz'). In fact, they stated that production of buds on semi solid media was higher compared to liquid media in all studied varieties. In the same study, leaf length was notably stimulated on liquid media for all studied varieties. However, Fki *et al.* (2011) and Al-Khayri and Naik (2017) reported a significant promoting effect of TIS on date palm shoot proliferation when compared to cultivation on solid media. Similar results were also reported by Khierallah and Bader (2007) and Othmani *et al.* (2017) who stated that transfer of date palm shoot clusters in temporary immersion bioreactor clearly improved the yield of regenerated shoots 5.5-fold in comparison with those regenerated on agar-solidified medium. Besides, shoots elongated faster to become vigorous and gradually produced new shoots as long as they were kept in the multiplication medium in comparison with those cultured on agar-solidified medium (Othmani *et al.*, 2011). Persson (2012) and Welander *et al.* (2014) reported similar results in *Digitalis lutea*, *Echinacea purpurea* and *Rubus idaeus* cvs. micropagated either in TIS and agar media as in overall, the shoot multiplication ratio was generally better in TIS compared to solid or non-TIS liquid medium. The frequent air replenishment and direct access of the cultures to nutrient medium are supposed to be the explanations for the better growth and higher shoot multiplication ratio for TIS (Etienne and Berthouly, 2002). According to Chakrabarty *et al.* (2003), the higher rate of multiplication registered in liquid medium can be explained by the fact that, in this system, there is a larger surface area for the absorption of cytokinins which, as a consequence, inhibits the apical dominance of shoots and increases the formation of axillary shoots. Moreover, in the liquid medium the components are taken up by plants with a better translocation through leaves via stomata and aqueous pores (Schönherr, 2006), and are transferred to the growing regions over a shorter distance (De Klerk and Ter Brugge, 2011). Therefore, uptake of medium ingredients and plant growth regulators over the whole plant surface improves the growth of plant material in liquid medium with TIS (Akdemir *et al.*, 2014).

Otherwise, Al Khateeb and Alturki (2014) reported that the number of roots was significantly higher on semi solid media compared to liquid media in 'Mejhool' variety. In the same study, the 'Reziz' variety showed a significantly higher number of roots on liquid media while there were no significant differences in 'Sukary' cv. between the two systems. They concluded that these differences in number of roots could be genotype-dependent. They also reported a highly significant effect of liquid media on root length in 'Sukary' variety. In addition, it was noticed that date palm cv. 'Deglet Bey' plants derived from TIS grew faster and rooted earlier than those derived from agar-solidified medium (Othmani *et al.*, 2009).

*Effects of growing system on shoot and root fresh and dry weights*

Fresh weight increases of cultures differed between bioreactor and gelled medium (Figure 6). Both varieties gained significantly more weight during cultivation in TIS compared to agar media. However, 'Boufeggous' variety produced significantly more fresh weight in both culture systems (72.03 g and 50.89 g) compared to 'Mejhool' variety (51.81 g and 35.85 g) respectively on TIS and GM. Moreover, the growing culture system had significant ($p < 0.05$) effects on shoot dry weight for both varieties (Figure 7). Indeed, accumulation of shoot dry weight was higher in TIS compared to GM.

Root fresh weight seems to be not significantly affected by growing system. In fact, the two cultivars have shown different behaviour. While a high root fresh weight (7.91 g) was observed on GM in 'Mejhool' variety, TIS system has shown slightly more root fresh weight (4.44 g) compared to GM (3.27 g) in 'Boufeggous' variety. However, root dry weight was globally higher in TIS compared to gelled medium for both varieties.

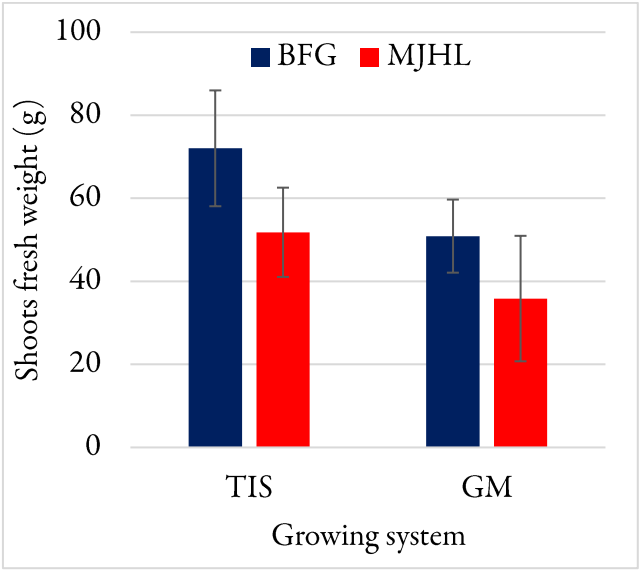


**Figure 6.** Effect of growing system on shoot fresh weight

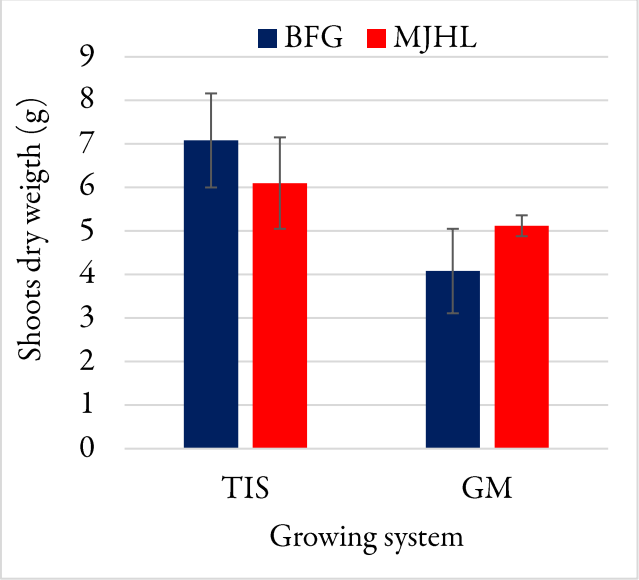


**Figure 7.** Effect of growing system on shoot dry weight

Similar results were reported by Al Khateeb and Alturki (2014) who stated that 'Reziz' variety produced more fresh and dry weights in liquid media compared to semi solid media. In addition, Farahani and Majd (2012) reported that the highest multiplication rate and weight gains of banana (*Musa*, cv. 'Dwarf Cavendish') were observed in the TIB system. Welander *et al.* (2014) reported that fresh weight gained in TIS culture was higher than in GM in both *Digitalis lutea* and *Echinacea purpurea*. The frequent air replenishment and direct access of cultures to nutrient medium are supposed to be the explanations for the better growth and fresh weight accumulation observed in TIS. Moreover, toxic metabolites, which may accumulate in the vicinity of the tissues, are more effectively dispersed by liquid immersions (McAlister, 2003). Concerning root development, obtained results were in accordance with reported data by Aragón *et al.* (2014) who stated that TIS improved rooting of plantain plantlets and gave rise to longer roots and higher dry mass. Moreover, Yan *et al.* (2010) reported that TIS promoted the growth and quality of *Siraitia grosvenorii* plantlets. Proliferation rate, shoot length, fresh weight and dry weight of shoots, and total biomass production were significantly ($P < 0.05$) higher respectively in TIS than in gelled and liquid medium.

*Effects of growing system on shoot quality*

The general appearance of obtained shoots was different in TIS and GM cultures. Shoots from TIS have a healthy appearance, with dark green and comparable leaves to seedlings-derived ones. Hence, TIS derived plantlets showed better performances mainly in terms of leaf expansion compared to GM (Figure 8).

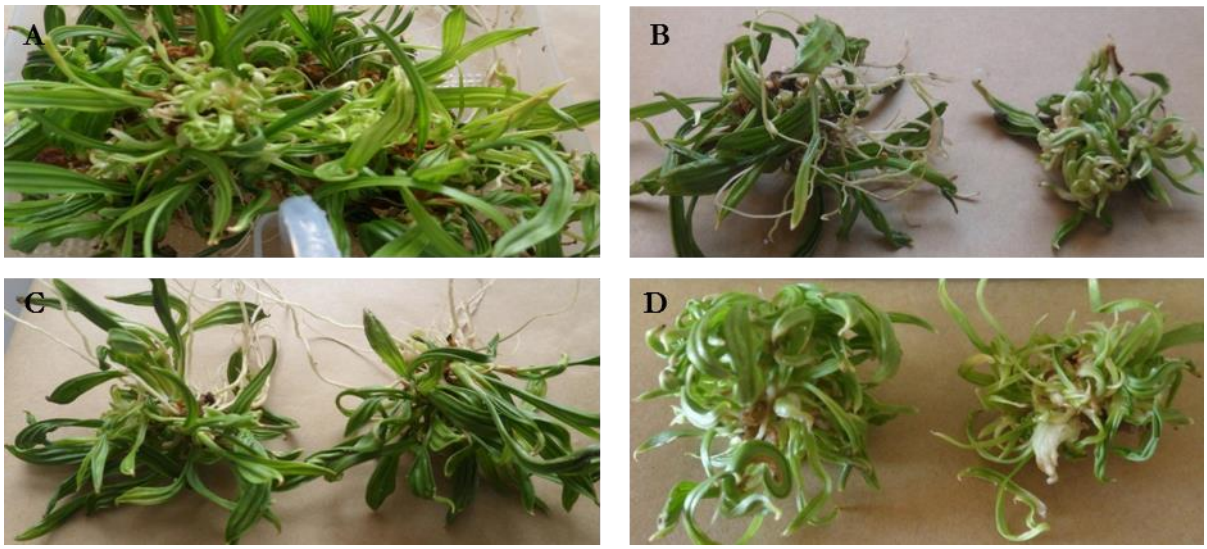


**Figure 8.** Appearance of date palm *in vitro* shoot buds cultured in TIS and GM; A) 'Mejhool', TIS; B) 'Mejhool', GM; C) 'Boufeggous', TIS; D) 'Boufeggous', GM

The positive effects of TIS on shoot growth have been demonstrated by many authors in earlier studies. Benelli and De Carlo (2018) reported that Plantform™ bioreactor improves *in vitro* culture of *Olea europea* cv. 'Canino', showing higher proliferation, shoot length and better vigour of shoots. Farahani and Majd (2012) reported that banana plantlets propagated in TIS showed better performance than those propagated by conventional methods. In addition, Yang and Yeh (2008) reported that during *ex vitro* acclimatization, *Calathea orbifolia* plants, produced in TIS, had much higher photosynthetic rates and subsequently higher leaf area, fresh and dry weights than those from semi-solid media. Gatti *et al.* (2017) reported that developed leaves of *Quercus roburi* in TIS micro-environment had epicuticular waxes and large stomata with elliptical shape, which indicates their good functionality. These leaf features are considered to provide a good adaptability to *ex vitro* conditions. Moreover, Etienne and Berthouly (2002) reported that tissue culture in TIS improves plant

material quality resulting in an increased of shoot vigour and in the frequency of healthy plants. The accumulated reserves are used during the first days of acclimatization leading to higher survival rates and to better plant quality of TIS-derived plantlets (Aragón *et al.*, 2014).

*Effects of growing system on plant acclimatization*

Produced plantlets in both culture systems were transferred to greenhouse for their acclimatization under controlled conditions. Survival rate was recorded after two months of acclimatization for both varieties. Results showed significant differences in plantlet survival rate between GM and TIS. In fact, TIS derived plantlets showed an average survival rate of 95% while those derived from GM have a survival rate beneath 82%. Furthermore, it has been noticed that plantlets of 'Mejhool' variety were better at acclimatization compared to those of the 'Boufeggous' variety. Obtained results were in accordance with those reported by Carvalho *et al.* (2019) who stated that in addition to known TIS fundamental advantages, TIS-derived plants were more successful in surviving the *ex vitro* acclimatization stage than those produced on semi-solid media or continuous immersion systems. In fact, it has been reported that plants grown in bioreactors systems are comparable to plants grown in *ex vitro* conditions, providing a higher survival rate in acclimatization stage (Etienne and Berthouly, 2002). Accordingly, 'Calathea' plants produced by TIS presented more functional photosynthetic and respiratory apparatus, and could adapt more successfully to environmental changes during *ex vitro* acclimatisation (Yang and Yeh, 2008). In addition, Gatti *et al.* (2017) reported that developed leaves in Oak tree had large stomata with elliptical shape, which indicates good functionality, and produced epicuticular waxes under TIS culture. These leaf structures could be viewed as a functional response to the good ventilation into culture vessels and positively influence the final acclimatisation phase. Besides, forced ventilation leads to the complete renewal of the culture's atmosphere, which prevents the accumulation of carbon dioxide and ethylene that generally occurs in a semi-solid culture and have a negative effect on morphogenesis (Roels *et al.*, 2006). Moreover, apple rootstock 'M9 EMLA' plants produced in TIS showed higher photosynthetic rate, maximum quantum yield of photosystem-II and slow but steady rate of nutrient absorption, indicating a higher rate of photomixotrophic metabolism (Chakrabarty *et al.*, 2003). Consequently, plantlets from bioreactors had 100% average survival in the greenhouse for 'Samoan ma'afala' and 'Fijian koqo', two breadfruit (*Artocarpus altilis*) varieties, whilst 83% survival was observed for plantlets from GM systems (Shandil and Tuia, 2015). According to Etienne and Berthouly (2002), enhanced acclimatization of the plant material produced in bioreactors has been claimed as one of the main advantages of TIS.

## Conclusions

The effects of temporary immersion system culture on 'Mejhool' and 'Boufeggous' date palm varieties micropropagation was investigated. Results indicated that in overall, TIS promoted the growth of produced plantlets compared to gelled media. Shoot and root lengths as well as fresh and dry weights were significantly higher in TIS than in GM. Moreover, the quality of obtained shoots was better in TIS compared to GM. Shoots from TIS were dark green and their leaves were comparable to those of seedlings-derived plants. Accordingly, plant material propagated by temporary immersion system performed better during the acclimatization phase than material obtained on semi-solid media. Hence, bioreactor Plantform™ can be a valid alternative to conventional systems for date palm micropropagation, resulting in a significant reduction of *in vitro* plant cost. More research work is needed for adaptation of TIS technology to various stages of *in vitro* multiplication *of* the most important Moroccan date palm cultivars.

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**Conflict of Interests**

The author declares that there are no conflicts of interest related to this article.

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