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Evaluation of a new vessel system based on temporary immersion system for micropropagation

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ABSTRACT

The aim of this study was to evaluate a new culture vessel Plantform bioreactor, based on the temporary immersion system principle for micropropagation of *Digitalis lutea* × *purpurea*, *Echinacea purpurea* and *Rubus idaeus*. The multiplication ratio and shoot quality in bioreactor were either similar to or better than these on agar medium. The shoot number was similar in both systems for *Digitalis* and *Rubus*, while *Echinacea* had a significantly higher shoot number of good quality in bioreactor. *Digitalis* and *Echinacea* gained more fresh weights in bioreactor, while more for *Rubus* on agar medium. However, the dry weight was similar between the two systems for all three species. Changes in carbohydrate were evident for all species. Autoclaving media could breakdown sucrose, which is both pH- and nutrient-dependent. This study has demonstrated that the Plantform bioreactor is suitable for plant micropropagation.

1. Introduction

Large-scale micropropagation by conventional tissue culture techniques is less cost-effective due to high input of labour and a low degree of automation (Maene and Debergh, 1985; Sluis and Walker, 1985; Simonton et al., 1991; Chu, 1995). In contrast, micropropagation in bioreactors designed based on the temporary immersion system (TIS) principle with a semi-automated control system is more cost-effective (Ziv, 2000). Such a system allows cultures only having temporary contact with a liquid nutrient medium to ensure normal growth of cultures, and thus avoiding the hyperhydricity problem. There are different types of TIS bioreactors, such as Recipient for Automated Temporary Immersion System (RITA®) and Twin Flasks (BIT®) system. The RITA® system (Vitropic, France) was first introduced by Alvard et al. (1993) and has been used in several studies (Pavlov and Bley, 2006; Zhu et al., 2005). The BIC®, system, described by Escalona et al. (1999), has also been reported in numerous studies (Escalona et al., 1999, 2003; Welander et al., 2007). However these bioreactors are either too small or too heavy to handle with small interior bottom which often leads to disorders of cultures due to high density (Welander et al., 2007). To overcome these problems, a new culture vessel named Plantform bioreactor (Fig. 1) has been recently developed (www.plantform.se.). The advantage of this bioreactor is that it has a relative greater interior bottom for cultures to grow and a suitable size for handling. Besides, such bioreactors could be placed above each other for saving culturing space, which is more attractive for large scale production. The objective of this study was to evaluate the feasibility of using the Plantform bioreactor to micropropagate plant material of good quality using three widely cultivated horticultural species, *Digitalis lutea* × *purpurea*, *Echinacea purpurea* and *Rubus idaeus*. The reason to choose these three species is that they are used not only as important ornamental and fruit, but also as medicinal purposes. Efficient production of these species in bioreactors will ensure the production of uniform plant materials for commercial cultivation and provide sufficient raw plant material for extracting bioactive compounds for the medicine industry.

2. Materials and methods

2.1. Description of the bioreactor

The new bioreactor (Fig. 1) is made of transparent polycarbonate with the size of 180 × 160 × 150 mm. In the bioreactor, a basket with holes of 1 mm in size, placed above a chamber that controls the medium flow, holds the plant material. A frame with four legs is placed above the basket to avoid the basket to rise when air pressure is applied to the bioreactor. The construction and placement

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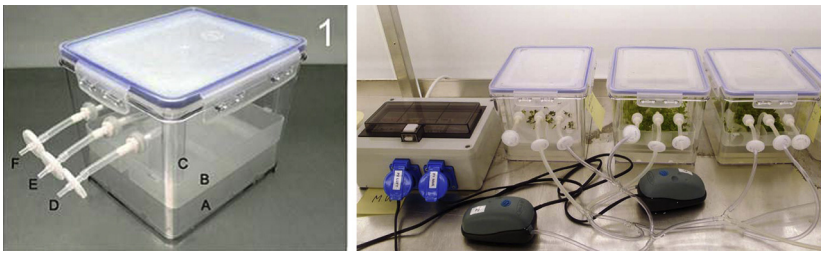

Fig. 1. Left: The Plantform bioreactor. (A) Inner container, (B) basket, (C) legs to support the basket, (D–F) inlets/outlets for gas exchange. Right: Bioreactors connected to air pumps and control unit.

of the basket is made so that the plants are only immersed into the liquid medium when air pressure is applied to the bioreactor. Furthermore, the bioreactor has three opening holes for medium supply, aeration and ventilation. Specially designed hollow 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 μm polytetrafluoroethylene (PTFE) filters to ensure that the airflow in and out of the bioreactors are sterile. The bioreactors and filters were autoclaved separately at 121 °C for 20 min. The three holes were sealed with aluminium foil and the lid was only attached at two of the four sides to counteract pressure changes during autoclaving. All further operations with the bioreactors were performed in a laminar flow hood. For more detailed information on the bioreactor see www.plantform.se.

2.2. Plant materials

In vitro grown cultures of three plant species were used in this study: *D. lutea* × *purpurea* (hereafter refers to *Digitalis*), *Echinacea purpurea* 'Magnus' (hereafter refers to *Echinacea*) and *Rubus idaeus* 'Mormorshallon' (hereafter refers to *Rubus*). Nearly uniform shoots were chosen for the experiments. Three bioreactors for each species were used with each bioreactor as one replicate. All bioreactors contained 40 shoots each for *Echinacea* and *Rubus*, but 15 shoots for *Digitalis* due to limited material. The initial explants were approximately 1 cm in length. Meanwhile, the same amount of explants grown in 8 jars on agar medium was included for comparison.

2.3. Culture media

The basal medium was either MS (Murashige and Skoog, 1962) (M0222, Duchefa, Haarlem, The Netherlands) or Lepoivre (Quoirin et al., 1977) (Q0251, Duchefa, Haarlem, The Netherlands) depending on species. Full strength of Lepoivre was used for *Digitalis*, supplemented with 0.5 mg nicotinic acid l^{-1}, 0.5 mg pyridoxine HCl l^{-1}, 1 mg 6-benzylaminopurine (BAP) l^{-1}, 0.1 mg indole-3-butyric acid (IBA) l^{-1} and 30 g sucrose l^{-1} with pH at 5.5. The same medium was used for *Echinacea* except that BAP was reduced to 0.2 mg l^{-1} without addition of IBA. For *Rubus*, full strength of MS was used, supplemented with 0.5 mg BAP l^{-1}, 0.01 mg IBA l^{-1}, and 30 g sucrose l^{-1} with pH at 5.2. For the agar media, 6 g agar l^{-1} (Bacto Agar B1000-1, Saveen Werner AB, Malmö, Sweden) was added to the corresponding liquid medium as stated above and dispensed into thermoplastic jars with a size of 8 × 9 × 9 cm. The media were autoclaved at 121 °C for 20 min after pH adjustment. The medium volume in each bioreactor was 500 ml.

2.4. Bioreactor control and growing conditions

The Plantform bioreactors were used in this study with the pneumatic pumping system controlled by an automatic timer for regulating aeration and medium supply to the explants in the bioreactor (Fig. 1). All bioreactors, regardless of plant species, were exposed to aeration and medium supply with the same regime, namely twice a day with the immersion time of 6 min. Aeration was once every hour and lasted for 4 min each. All cultures were maintained in a climate chamber with the 16/8 h photoperiod at 33 μmol m^{-2} s^{-1} and the temperature of 23 °C/18 °C (day/night).

2.5. Evaluation of culture growth

Fresh weights of the explants were measured at both start and end of the experiments. Dry weights were measured at the end of the experiments after drying the cultures at 70 °C for 24 h. The total number of shoots was counted after 4 weeks of cultivation.

2.6. Sugar analysis in the culture media in bioreactor

Sugars (fructose, glucose, sucrose) were measured after cultivation to evaluate its changes. Before measurement, the media was first filtrated with Whatman no 1 filter paper (11 μm) to remove the possible solid residue. The concentrations of D-fructose, D-glucose and sucrose were analysed using a ready-to-use kit from Megazyme, Ireland (www.megazyme.com).

2.7. Acclimatization of plantlets

In order to evaluate if the plantlets from the bioreactors could acclimatize as good as those from agar medium, shoots produced in both systems were rooted in the rooting medium for two (*Digitalis*) and four (*Rubus*) weeks, respectively. The rooting medium for *Digitalis* consisted of full strength of Lepoivre, supplemented with 0.5 mg nicotinic acid l^{-1}, 0.5 mg pyridoxine HCl l^{-1}, 5 mg IBA l^{-1} and 30 g sucrose l^{-1}, 6 g agar l^{-1} with pH at 5.5. For *Rubus*, full strength of MS was used, supplemented with 0.1 mg IBA l^{-1}, and 30 g sucrose l^{-1}, 6 g agar l^{-1} with pH at 5.2. *Echinacea* was not investigated as the shoots had been already rooted in the multiplication medium. At least 30 plantlets from each species were planted in pots containing 70% planting soil and 30% perlite in greenhouse with natural light (April–May). The plantlets were covered with plastic film to avoid desiccation. The survival was recorded after 4 weeks.

2.8. Statistical analyses

The results were analysed with statistical software Minitab 16 and two sample *t*-tests was used to make pair wise comparisons between the two systems with the significance level $P = 0.05$.

3. Results and discussion

3.1. Fresh and dry weight

Fresh weight increase of the cultures differed between bioreactor (hereafter refers to TIS) and agar medium (Fig. 2). *Digitalis* and *Echinacea* gained significantly more weight during cultivation

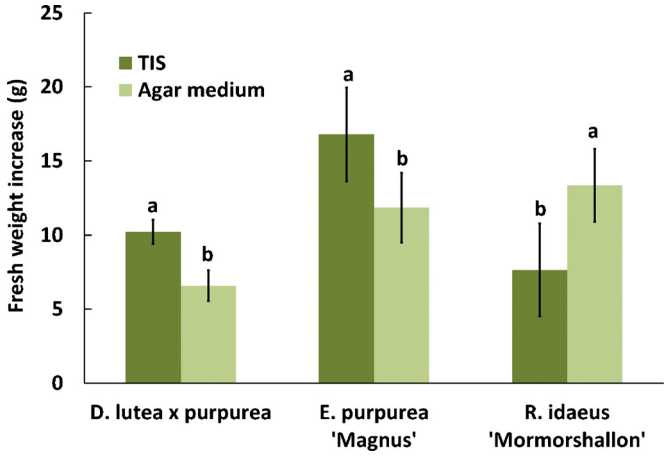

Fig. 2. Comparison of fresh weight increase in *Digitalis*, *Echinacea* and *Rubus* between TIS and agar medium. Error bars indicate standard deviation. Different letters above the bars within the same species indicate a significant difference at $P = 0.05$.

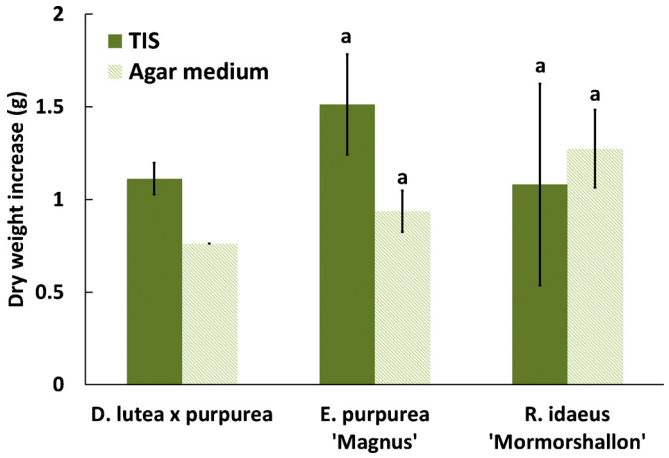

Fig. 3. Comparison of dry weight of *Digitalis*, *Echinacea* and *Rubus* between TIS and agar medium. Error bars indicate standard deviation. Different letters above the bars within the same species indicate a significant difference at $P = 0.05$.

in TIS compared to agar medium, while *Rubus* showed the opposite result.

Neither *Echinacea* nor *Rubus* showed any significant difference in dry weight between TIS and agar medium. No statistical analysis was carried out on the dry weight for *Digitalis* due to limited material (Fig. 3). The dry weight ranged between 9.0–13.7% for TIS and 8.7–11.6% for agar medium.

Previous studies on different cultivation methods have also shown a clear variation in changes in fresh and dry weight. For *Siraitia grosvenorii*, both fresh and dry weights of shoots were significantly higher in TIS than on solid or other liquid media (Yan et al., 2010). However, the fresh weight of pineapple (*Ananas comosus* L. Merr) was higher in TIS than on solid or other liquid media, while no difference in dry weight was found (Escalona et al., 1999). These results may suggest that the biomass production is species and culturing condition-dependent.

3.2. Shoot multiplication

No significant differences were observed between TIS and agar medium for either total number of shoots or number of ready for rooting shoots for *Digitalis* and *Rubus*. However, TIS resulted in significantly more ready for rooting shoots than agar medium for *Echinacea* (Fig. 4). Studies on sugarcane grown on solid,

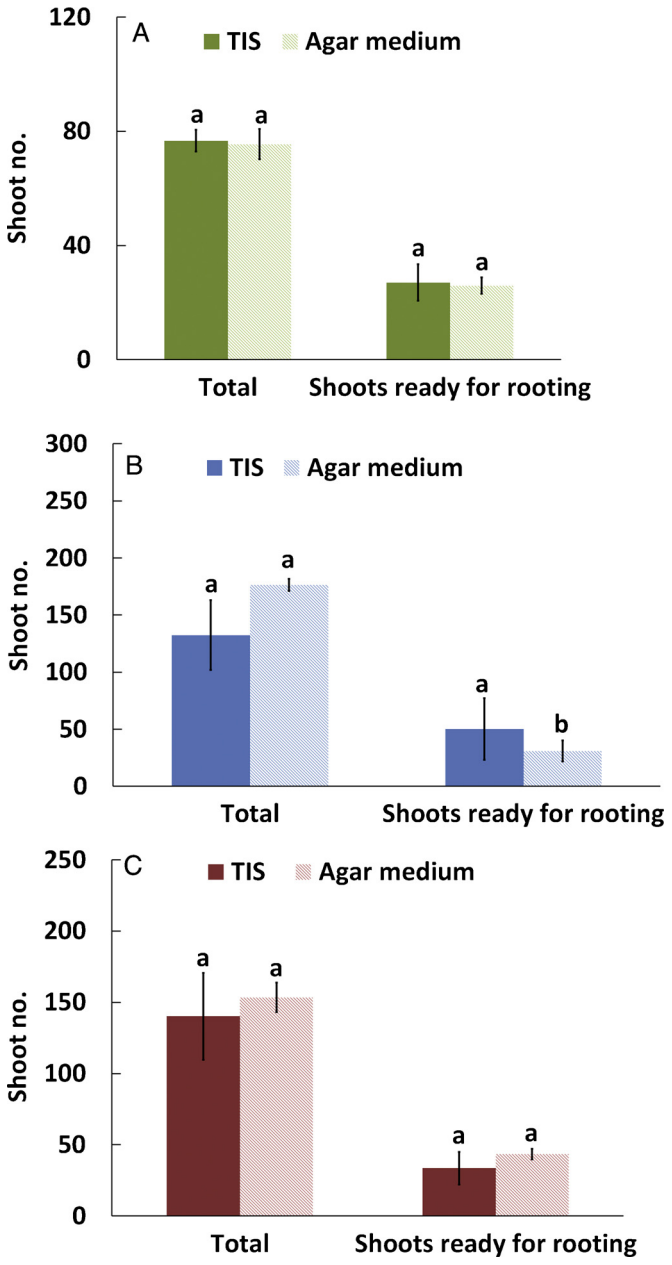

Fig. 4. Comparison of shoot multiplication ratio (total) and shoots ready for rooting of (A) *Digitalis*, (B) *Echinacea* and (C) *Rubus* in TIS and agar medium. Error bars indicate standard deviation. Different letters above the bars within the same species indicate a significant difference at $P = 0.05$.

liquid and TIS media have shown that better shoot multiplication ratio and shoot height were obtained in TIS compared to other systems (Lorenzo et al., 1998). A similar result was obtained in *Siraitia grosvenorii* (Yan et al., 2010). Zhu et al. (2005) have reported a higher multiplication ratio of apple rootstock M26 in TIS than on solid medium and a similar result was reported in pineapple (*Ananas comosus* L. Merr) (Escalona et al., 1999). However, Damiano et al. (2005) compared TIS and solid medium on apple, peach, cherry and plum, and found no difference in multiplication ratio between TIS and solid medium. Overall, the abovementioned studies on different plant species including the current study have shown that 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

Table 1
Sugar concentration in the TIS medium presented either as individual compounds (sucrose, D-glucose and D-fructose) or as total sugars for each species. Week 0 represents the start medium after autoclaving and 4 represents 4 weeks after cultivation.

Species	Week (no)	Sucrose ($g\,l^{-1}$)	D-Glucose ($g\,l^{-1}$)	D-Fructose ($g\,l^{-1}$)	Total sugars ($g\,l^{-1}$)
Digitalis	0	26.5	2.3	2.2	31.0 ± 0.564
Digitalis	4	21.5	3.7	5.6	30.9 ± 1.631
Echinacea	0	25.2	2.2	2.5	30.0 ± 0.089
Echinacea	4	21.7	3.2	3.6	28.4 ± 0.754
Rubus	0	17.5	6.1	5.8	29.4 ± 0.859
Rubus	4	12.5	7.2	7.2	26.9 ± 0.389

Table 2
Sucrose (Suc) breakdown into D-glucose (D-gluc) and D-fructose (D-fruc) during autoclaving at different pH and nutrient media.

Medium	pH BA*	pH AA**	Suc ($g\,l^{-1}$) BA*	Suc ($g\,l^{-1}$) AA**	D-gluc ($g\,l^{-1}$)	D-fruc ($g\,l^{-1}$)	Total sugar
Suc in water			30	30			
Suc in ascorbic acid	5.0	4.29	30	26.3	0.8	2.4	29.5
Suc in MS	5.2	4.28	30	17.5	6.1	5.8	29.4
Suc in MS	7.0	6.05	30	27	0.6	1.7	29.3
Suc in MS + MES	5.5	5.21	30	23	4.0	5.5	32.5
Suc in Lepoivre	5.5	4.91	30	26.5	2.3	2.2	31

* BA = before autoclaving.
** AA = after autoclaving.

to be the explanations for the better growth and higher shoot multiplication ratio for TIS (Etienne and Berthouly, 2002).

3.3. In vitro growth and development

Shoot cultures from TIS and agar medium showed similar growth for all three species (Fig. 5). The majority of the cultures for *Digitalis* and *Echinacea* were healthy and grew normally. Some of the *Rubus* cultures looked strange *in vitro* with shrivelled or curled leaf edges both in TIS and agar medium, probably an indication of hyperhydricity, but this was disappeared after acclimatization in greenhouse. The hyperhydricity problem has been observed in liquid cultivation (Kevers et al., 2004; Welander et al., 2007). Plants cultivated in TIS were supposed to be less prone to hyperhydricity and necrosis compared to liquid and solid media, probably due to the repeated air replenishment in the system (Damiano et al., 2005). Studies on *Musa* AAB confirmed that the forced air supply in TIS results in better shoot quality compared to solid media (Roels et al., 2006). Length and frequency of medium immersion are of great importance for normal culture growth in TIS (Etienne and Berthouly, 2002). A bigger space in the bioreactor helps to prevent hyperhydricity. In the study on *Pinus radiata* D. Don, Aitken-Christie and Davies (1988) initially used a TIS bioreactor with the size of 390 × 390 × 120 mm. However, this large size was proved to be detrimental due to high risk of contamination. In comparison, the size of the Plantform bioreactor (180 × 160 × 150 mm) used in this study is much smaller and has proved to be adequate since it is large enough to room numerous explants and easy to handle with low risk of contamination.

3.4. Carbohydrate consumption

The concentration of sucrose was decreased after cultivation in TIS for all three species and this reduction will be greater if about 15% water loss is taken into consideration (Table 1). It should be pointed that, although sucrose ($C_{12}H_{22}O_{11}$, 342.30 $g\,mol^{-1}$) is composed of glucose ($C_6H_{12}O_6$, 180.16 $g\,mol^{-1}$) and fructose ($C_6H_{12}O_6$, 180.16 $g\,mol^{-1}$), the molar mass of one sucrose molecule is not equal to the total molar mass of one glucose molecule plus one fructose molecule (360.32 $g\,mol^{-1}$) since an additional water molecule is added during hydrolysis of the sucrose molecule. This means when the disaccharide sucrose decomposes into D-fructose and D-glucose the entire molar mass increases. This will explain why the total amount of sugar is slightly higher than the initially added amount after hydrolysis and why the amount of D-glucose and D-fructose increased after cultivation (Table 1).

Our data showed that the hydrolysis of sucrose is influenced by autoclaving and pH as well as mineral composition of medium (Table 2). Autoclaving of sucrose in water results in no breakdown of the sugar. The highest breakdown of sucrose was found in MS medium which has higher nutrient concentration and lower pH (Table 2). Chakrabarty et al. (2007) reported that quantities of sucrose in the medium had been hydrolysed directly after autoclaving and the breakdown of sucrose appeared to be medium-dependent. They also reported in apple rootstock 'M9 EMLA' that the concentrations of glucose and fructose in the analysed medium were equal, and thereby concluded that the plants did not prefer any of the monosaccharaides to the other. In this study, *Echinacea* and *Rubus* had similar values for glucose and fructose, while a higher fructose concentration was maintained in the medium for *Digitalis*, indicating that glucose is probably preferred by this species (Table 1). The metal salts could catalyse the reaction since hydrolysis is much less when sucrose is only dissolved in ascorbic acid solution without presence of other nutrients.

3.5. In vitro rooting and acclimatization in greenhouse

Fifty shoots from *Digitalis* and *Rubus* were rooted in PlantForm bioreactors or on agar medium and the rooting percentage was 100% for all cases. For *Echinacea*, the shoots rooted already in PlantForm bioreactors, so no separate rooting test was carried out. Acclimatization in greenhouse showed normal growth and appearance for plantlets for all three species regardless the system, of which the majority (95%) of the plantlets survived after one month of acclimatization (Table 3). Similar results were obtained by Lorenzo et al. (1998). However, Etienne and Berthouly (2002) stated that acclimatization and the growth vigour as well as the number of surviving individuals were usually better if the explants had been cultivated in TIS. Zhu et al. (2005) concluded normal rooting and acclimatization of the apple rootstock M26 in TIS.

In conclusion, the results have shown that the quality of plantlets proliferated in the Plantform bioreactors are as good as on solid medium or even better, demonstrating this new type of bioreactors is suitable for producing good quality plants.

Fig. 5. The appearance of shoot cultures of different plant species after 4 weeks in TIS (left column) and on agar medium (right column). (A) and (B) *Digitalis*; (C) and (D) *Echinacea*; (E) and (F) *Rubus*; (A), (C), (E) TIS; (B), (D), (F) agar medium.

Table 3
Acclimation of plantlets from either Plantform bioreactor (TIS) or agar medium in normal jars in the greenhouse.

Species	Medium	Plantlet no.	Plantlets survived*	Survival (%)
Digitalis	TIS	33	33	100
Digitalis	agar	24	24	100
Rubus	TIS	30	30	100
Rubus	agar	44	42	95
*Echinacea***	TIS	30	30	100

* The survival rate was calculated after 4 weeks.
** No rooting test was carried out since the shoots rooted already in the multiplication medium in TIS.

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