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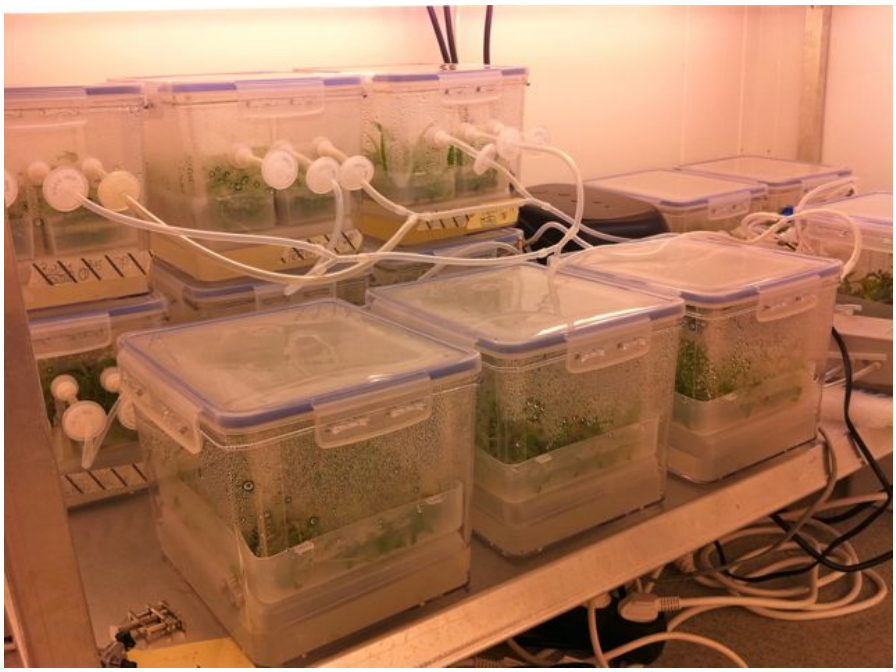

# Evaluation of a new type of temporary immersion system (TIS) bioreactors for plant micropropagation

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# Evaluation of a new type of temporary immersion system (TIS) bioreactors for plant micropropagation

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**Swedish title:** Utvärdering av en ny typ av bioreaktorsystem baserat på temporär nedsänkning i näringslösning för mikroförökning av växter

**Supervisor:** Professor Margareta Welander, Swedish University of Agricultural Sciences, Department of Plant Breeding and Biotechnology

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**Key Words:** bioreactor, conductivity, *Digitalis*, *Echinacea*, liquid medium, mass propagation, medium analyzes, micropropagation, nutrients, pH, *Rubus*, semi-automatic, sugars, temporary immersion system


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## ABSTRACT

Micropropagation has been revealed to be an efficient propagation method for production of large quantities of true to type and disease free plants, while traditional methods of micropropagation on solid medium are limited in commercial production due to high labor costs. Bioreactor production in liquid medium has therefore been studied and tested in recent years, but there are still no appropriate types of bioreactors until now. A new type of bioreactors based on the temporary immersion system (TIS) principle has been developed by Professor Margareta Welander at the Department of Plant Breeding and Biotechnology, the Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden, and by Dr. AJ Sayegh, TC propagation Ltd, Bree, Enniscorthy, Co Wexford, Ireland.

The aim of this study is to evaluate this new bioreactor for micropropagation using the three widely cultivated and important, horticultural species *Digitalis lutea* x *purpurea*, *Echinacea purpurea* 'Magnus' and *Rubus idaeus* 'Mormorshallon' in comparison with micropropagation on solid medium. Fresh and dry weights, the shoot production rate as well as survival and general plant appearance were evaluated. Further on, pH, conductivity, and composition of mineral nutrients and carbohydrates were measured regularly during the culture period. The acclimatization capability of the plantlets in greenhouse was also evaluated.

The results show that this newly developed bioreactor is suitable for mass production of target plant species with a similar survival rate and plant quality to those from solid medium. The multiplication rate was either similar or better than that from solid medium. Plant analyses revealed a significant difference ($p \leq 0.05$) in fresh weight between TIS and the solid medium. However, the optimal cultivation medium differed between the species, where *D. lutea* x *purpurea* and *E. purpurea* 'Magnus' gained more weight with TIS while *R. idaeus* 'Mormorshallon' gained more weight with solid medium. No significant differences were found when comparing dry weights and the multiplication rate except for *E. purpurea* 'Magnus' where TIS resulted in a significantly ($p \leq 0.05$) higher number of shoots of an adequate size for ex vitro acclimatization. Except for almost full depletion of iron and a 50% decrease in $NH_4^+$ concentration in the media of *D. lutea* x *purpurea* and *E. purpurea* 'Magnus', only minor changes in pH, conductivity, carbohydrate and mineral nutrient concentrations were observed over time for all species. Overall, the results of the medium analyses show that the bioreactor system is stable and reliable.

**Key words:** bioreactor, conductivity, *Digitalis*, *Echinacea*, micropropagation, nutrients, pH, *Rubus*, temporary immersion system

## SAMMANFATTNING

Mikroförökning har visat sig vara en effektiv förökningsmetod för produktion av stora kvantiteter av sortäkta och friska plantor, medan traditionella mikroförökningsmetoder på fast medium är begränsade inom den kommersiella produktionen på grund av höga arbetskostnader. Bioreaktorproduktion i flytande medium har därför studerats och undersökts under de senaste åren, men det finns tills nu ännu inga tillämpliga sorters bioreaktorer. Ett nyligen utvecklat bioreaktorsystem baserat på temporär nedsänkning i näringslösning (TIS) har blivit framtaget av Professor Margareta Welander vid institutionen för Växtförädling och bioteknologi, Sveriges Lantbruksuniversitet (SLU), Alnarp, Sverige, och av Dr. AJ Sayegh, TC propagation Ltd, Bree, Enniscorthy, Co Wexford, Irland.

Syftet med denna studie är att utvärdera denna nya bioreaktor genom mikroförökning av de tre välkända och betydelsefulla hortikulturella arterna *Digitalis lutea x purpurea*, *Echinacea purpurea* 'Magnus' och *Rubus idaeus* 'Mormorshallon' i jämförelser med kulturer på fast medium. Frisk- och torrvikter, skottproduktion samt överlevnad och allmänt plantutseende utvärderades. Vidare mättes pH, ledningstal samt sammansättning av näringsämnen och kolhydrater regelbundet under försökstiden. Acklimatiseringsförmågan hos mikroplantorna utvärderades också i växthus.

Resultaten visar att denna nyutvecklade bioreaktor är lämplig för massförökning av de undersökta växtarterna med liknande överlevnad och kvalitet hos plantorna. Växtmätningarna visade antingen likvärdiga eller bättre förökningsresultat i jämförelse med det fasta mediet. Växtmätningarna gav signifikanta skillnader ($p \leq 0.05$) i friskvikt mellan TIS och det fasta mediet. Vilket odlingsmedium som var mest optimalt skiljde sig dock mellan de olika växtslagen, där *D. lutea* x *purpurea* och *E. purpurea* 'Magnus' gav större viktökning med TIS medan *R. idaeus* 'Mormorshallon' gav större viktökning med fast medium. Det fanns inga signifikanta skillnader mellan torrvikt och antalet skott. Undantaget var *E. purpurea* 'Magnus' där TIS observerades ha ett signifikant ($p \leq 0.05$) högre antal skott av tillräcklig storlek för acklimatisering. Bortsett från att järnet nästan förbrukades helt och att $NH_4^+$-koncentrationen i medierna för *D. lutea* x *purpurea* och *E. purpurea* 'Magnus' minskade med 50 %, skedde enbart små förändringar av pH, ledningstal samt mineralnärings- och kolhydratkoncentrationer över tiden. Resultaten från mediumanalyserna kan övergripande tolkas som att bioreaktorsystemet är stabilt och tillförlitligt.

**Nyckelord:** bioreaktor, *Digitalis*, *Echinacea*, mikroförökning, näringsämnen, pH, *Rubus*, temporary immersion system

# INDEX

# 1 INTRODUCTION

## 1.1 Objectives of the study

Micropropagation has been revealed to be a highly efficient propagation method for production of large quantities of true to type and disease free plants with minimum use of space and less mother plant material. However, application of traditional micropropagation using solid medium is today still limited in commercial production mainly due to its intensive labor input and thereby expensive.

Unlike traditional micropropagation, semi-automatic bioreactors use liquid media based on the temporary immersion principle. In this system, plant cultures allow to have temporary access to the liquid medium to avoid the hyperhydricity problem. The semi-automatic bioreactor system has been considered as a promising alternative to the solid culture system. However, there are no cost effective bioreactors available for commercial production.

Very recently, a new type of TIS bioreactors has been developed by Professor Margareta Welander at the Department of Plant Breeding and Biotechnology, the Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden, and by Dr. AJ Sayegh, TC propagation Ltd, Bree, Enniscorthy, Co Wexford, Ireland. Compared with the other existing ones, this bioreactor is made of plastic and has a large bottom that allows more plant material to grow in it, thus increasing the production efficiency.

The focus of this study is on culture growth in relation to the medium usage. The following research questions are dealt with during the study:

- Is this new bioreactor more efficient than solid medium in terms of handling, mass production, survival and quality of the plants?
- How are the mineral nutrients and sugars in the liquid medium used during the cultivation in the bioreactor?

## 1.2 Strategy

The project was executed through micropropagating the three different plant species *Digitalis lutea* x *purpurea*, *Echinacea purpurea* 'Magnus' and *Rubus idaeus* 'Mormorshallon' in the bioreactors. The effectiveness of the bioreactor in micropropagation was examined through measurements of biomass production and shoot number. Meanwhile, pH, conductivity and the uses of mineral nutrients and sugars were measured. Except for laboratory work, literature studies are also included.

## 1.3 Limitations

Due to limited time for practical work, only *R. idaeus* 'Mormorshallon' has been fully investigated in terms of utilization of macronutrients, and only *E. purpurea* 'Magnus' has been fully analyzed for micronutrients. In the remaining bioreactors only nitrogen (N), phosphorus (P) and carbohydrate usage in the medium have been analyzed.

# 2 BACKGROUND

## 2.1 An introduction to micropropagation

Micropropagation is one of the existing plant tissue culture methods (Preece & Read, 2005; Raven et al., 2005). The name tissue culture itself refers to cell cultivation under controlled and sterile conditions, where clones of plants can be formed based on the totipotency theory of plants; i.e. one competent mature plant cell has capability of developing into a complete plant (Raven et al., 2005).

### *2.1.1 Performance*

Micropropagation is performed through placement of small-sized surface sterilized explants (pieces of plant tissues and/or organs) on specially designed plant specific media (Preece & Read, 2005). Surface sterilization is often performed with sodium or calcium hypochlorite, commercial bleach, or other sterilization agents (Welander et al., 2007). Plant media are composed of a blend of different inorganic compounds (macro- and micronutrients), organic substances (carbohydrates, hormones, vitamins etc.) and also a gelling substance (e.g. agar) (Preece & Read, 2005) in case of solid medium culture.

Micropropagation can be divided into different phases: establishment, shoot multiplication, rooting and acclimatization (Preece & Read, 2005). Medium transfers may be needed more than once during the shoot multiplication time before the size and number of plants have been obtained. Shoot production can occur in two different ways, from axillary or adventitious shoots (Preece & Read, 2005; Welander et al., 2007). However, axillary shoot formation is formed from already existing meristems and regarded as a more secure method in terms of avoidance of production of off-type plants. The rooting phase involves four different stages: induction, initiation, organization and growth (Welander et al., 2007). The phytohormone auxin promotes root induction, but inhibit root growth. Exogenous application of auxin is normally required during root induction, while explants need to be transferred to hormone free medium after root induction for most species.

### *2.1.2 Sugars and nutrients*

Sugars, such as sucrose, glucose and fructose are essential energy sources that need to be included in the micropropagation media during cultivation. It is common to add sucrose as the sugar source in most cases (Ziv, 2000). Sucrose is a disaccharide that is composed of the monosaccharaides fructose and glucose, which can be detected in several different plants (Raven et al., 2005). Glucose is the sugar produced during photosynthesis, but sucrose is the major transport sugar in plants. The usage of disaccharides, such as sucrose, for energy purposes leads to a hydrolysis reaction when the disaccharide is broken down into its monomers. This hydrolysis reaction also includes addition of a water molecule. Sucrose can be fully or partly hydrolyzed into glucose and fructose within a couple of weeks (Ziv, 2000).

Nutrients are essential for plant growth and development. Raven et al. (2005) present 17 elements as essential for all vascular plants including macronutrients (≥1000mg/kg dry weight) and micronutrients (≤100mg/kg dry weight). Macronutrients are sulfur (S), phosphorus (P), magnesium (Mg), calcium (Ca), potassium (K), nitrogen (N), oxygen (O), carbon (C) and hydrogen (H) and micronutrients include molybdenum (Mo), nickel (Ni), copper (Co), zinc (Zn), manganese (Mn), boron (B), iron (Fe) and chlorine (Cl). All of the above mentioned elements except for hydrogen, carbon and oxygen are defined as mineral nutrients (Taiz & Zeiger, 2006). The sources for H, C and O are instead normally retrieved from water ($H_2O$) and carbon dioxide ($CO_2$).

## 2.2 Different micropropagation systems

### *2.2.1 Traditional micropropagation*

Common descriptions on micropropagation in the literature are in general referring to traditional micropropagation systems in which explants are cultured on a solid medium with a gelling agent such as agar in small jars. With this propagation system, the explants are positioned one by one into the medium, with the basal end of the cutting down into the medium and the apical end above the medium, pointing upwards. The performance is therefore similar to how cuttings are put into the substrate (e.g. planting soil) in traditional propagation. This process is labor-intensive and needs automation to become economically viable for large-scale production. Escalona et al. (1999) state that common existing hinders to the commercialization of traditional micropropagation are high labor costs, low multiplication rates, and a low survival rate of plantlets after

acclimatization in general. In fact, the need for division and placement of cultured shoots has lead to labor costs being the largest financial post in traditional micropropagation with small jars and solid medium (Welander et al., 2007).

### *2.2.2 Introduction to liquid cultures*

In contrast to traditional micropropagation on solid medium, cultures in liquid medium using a bigger container is another micropropagation method. Compared with solid medium, cultivation in liquid medium has been experienced to be easier and less time-consuming. For example, positioning of explants and agar are not needed, thus leading to a reduced cost (both labor and agar accounts for high production cost in micropropagation).

Liquid medium culturing can be further divided into two different kinds of systems. The first one is continuous immersion of explants in liquid medium. In this system, the explants are constantly in contact with the liquid medium, which often leads to increased nutrient uptake and assimilation (Etienne & Berthouly, 2002). However, the constant medium contact can also lead to hyperhydricity. To avoid full immersion of the explants into the medium, both net (Chakrabarty et al., 2007) and filter paper (Escalona et al., 1999) have been tested as supports for keeping the explants away from constant immersion in the liquid medium to certain extent. In addition, Chakrabarty et al. (2007) describes the extra input of air. The second liquid culture system used is temporary immersion system (TIS), a semi-automatic cultivation system based on the ebb and flood culturing system. This system temporarily immerses the explants into the liquid medium instead of allowing them to stay in constant contact with the medium.

Cultivation in liquid medium with TIS is considered to be a technique that has great capability in terms of automation, and thereby lowering of labor costs. Lorenzo et al. (1998) also stated that the costs were reduced due to less manual labor with the TIS in comparison with traditional solid and liquid media. Zhu et al. (2005) came to a similar conclusion when comparing the TIS and solid medium systems. The cost reduction is due to less manual labor, since no positioning of the explants is needed and the medium composition can be more easily changed (Alvard et al., 1993). When comparing solid and liquid medium, labor input for rooting were also found to be less with liquid medium cultivation due to no need for shoot orientation (Welander et al., 2007).

### 2.2.3 Micropropagation in bioreactor

Micropropagation in liquid system in big containers is also called bioreactor production. A clear definition for the word bioreactor does not seem to exist. An explanation has however been given by Preil (2005) in that a bioreactor is often considered as a container designed for large-scale production of plant material. Plant propagation in liquid bioreactor systems has a wide field of application. According to Paek et al. (2005), liquid medium bioreactor systems for mass propagation have been set up for cultivation of cells, tissues, somatic embryos and organogenic propagules (e.g. bulblets and shoot clusters). They also suggest, based on production purposes, that bioreactor cultivation can be classified into the following three groups: production of biomass; metabolites and enzymes; or those where metabolites have been exogenously applied to achieve biotransformation.

### 2.2.4 Development of temporary immersion systems (TIS)

Frequently cited examples of previously developed TIS bioreactors include the Recipient for Automated Temporary Immersion System (RITA®) and the Twin Flasks (BIT®) systems. The RITA® system (Vitropic, France) was firstly introduced by Alvard et al. (1993) and has since then been used in several scientific studies (Pavlov & Bley, 2006; Zhu et al., 2005). This system is composed of a container with two compartments that are placed on top of each other (Etienne & Berthouly, 2002). The plant material is located in the top compartment and the medium in the bottom compartment. Medium immersion over explants is then performed through application of an air pressure to the lower compartment so that the medium level rises and reaches the plant culture level. The air pressure is also functioning as ventilation due to bubbles that are created during this process. Excessive air can escape from the container through a ventilation tube connected with a filter. Another type of TIS is the BIC®, which has also been reported in numerous scientific studies (e.g. Escalona et al., 1999; Escalona et al., 2003; Welander et al., 2007). The BIC system, described by Escalona et al. (1999), consists of two containers of transparent glass flasks connected with tubes of either silicone or glass. The tubes were equipped with filters to avoid contamination and were connected to air compressors, with the purpose to force the medium moving from the medium flask to the explant flask for temporary immersion. A third TIS type named Plantima® (A-tech Bioscientific Co., Taiwan) described by Yan et al. (2010) is nearly analogous to the RITA system in its construction.

The problem with the abovementioned bioreactors are that they are either too small, or too small bottom or too heavy to handle. Welander et al. (2007) used the twin flask system in their research and found out that the small round bottom area of the flasks could become a limitation to growth. As the bottom area had been filled with developing shoots and no further space was present, further development could lead to too densely packed plants and thereby also disorders. The newly developed bioreactor has overcome these problems through both a larger bottom area and at the same time reduced height for allowing more shoots to grow in the same bioreactor. In addition, TIS is often made of glass and thereby heavy to work with. The newly developed bioreactor is made of plastic and easier to handle.

Several studies have been performed on comparisons between TIS and other types of in vitro propagation systems (e.g. Chakrabarty et al., 2007; Damiano et al., 2005; Escalona et al., 1999; Escalona et al., 2003; Etienne & Berthouly, 2002; Roels et al., 2006; Welander et al., 2007; Yan et al., 2010). Altogether, the results have shown some common advantages of TIS bioreactors over traditional plant micropropagation systems regarding culture performance. Examples of advantages with TIS are higher multiplication rates (Escalona et al., 1999; Etienne & Berthouly, 2002; Zhu et al., 2005; Yan et al., 2010), higher fresh weights (Escalona et al., 1999; Yan et al., 2010) and dry weights (Chakrabarty et al., 2007; Escalona et al., 1999, Yan et al., 2010), better quality of shoots (Chakrabarty et al., 2007; Etienne & Berthouly, 2002; Roels et al., 2006) as well as higher assimilation (Escalona et al., 2003; Etienne & Berthouly, 2002). Furthermore, hyperhydricity was absent (Damiano et al., 2005). TIS is also described to most likely be effective due to the availability of ventilation, while offering necessary contact between explants and medium (Escalona et al., 1999; Etienne & Berthouly, 2002; Yan et al., 2010). Finally, one important advantage is the decreased production costs due to less manual labor (Etienne & Berthouly, 2002; Welander et al., 2007) and less spacing needed (Etienne & Berthouly, 2002).

The TIS bioreactors are believed to become a leading future micropropagation method, even though there still exist some problems with the technique (Welander et al., 2007). Ziv (2000) asserts that simple bioreactor systems with mechanized systems for cutting, sorting and delivery is the prospect of micropropagation if expenses should be able to be decreased. However, in accordance to developing commercial systems for TIS that are highly efficient, more knowledge is

needed on how the explants are utilizing the medium. Chakrabarty et al. (2007) discussed the fact that research on TIS actually is constituted of comparisons with traditional types of propagation systems, and therefore do not offer much information on physiology and morphogenesis of the plants themselves. To achieve optimal propagation systems, it is important to gain knowledge about how factors such as aeration, mixing and depletion of substances occur within the cultivation media during cultivation (Ziv, 2000). Chakrabarty et al. (2007) stated that the development of commercial apple micropropagation systems could probably be obtained by improved medium composition, which could be performed through a better understanding on how different nutrients of the medium are used by the plants during cultivation. A similar opinion has been stated by Escalona et al. (2003), who discuss that higher plant quality can be obtained through better knowledge about and control of the ecosystem within the TIS and the physiological requirements of the plant material that is growing within it. Escalona et al. (2007) also stated that this kind of knowledge has already been revealed to a much larger magnitude for solid medium.

# 3 MATERIAL & METHODS

## 3.1 A short description of the new bioreactor

The new bioreactor is shown in Figure 1A. It is made of transparent polycarbonate with the size of 180x160x150 mm. In the bioreactor a basket with holes of 1 mm in size is placed above a chamber that controls the medium flow. This basket 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 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 (Figure 1B). The filters make sure that the airflow in and out of the bioreactors is sterile. For more detailed information on the bioreactor see the report on the bioreactor's technical development (Welander, 2011).

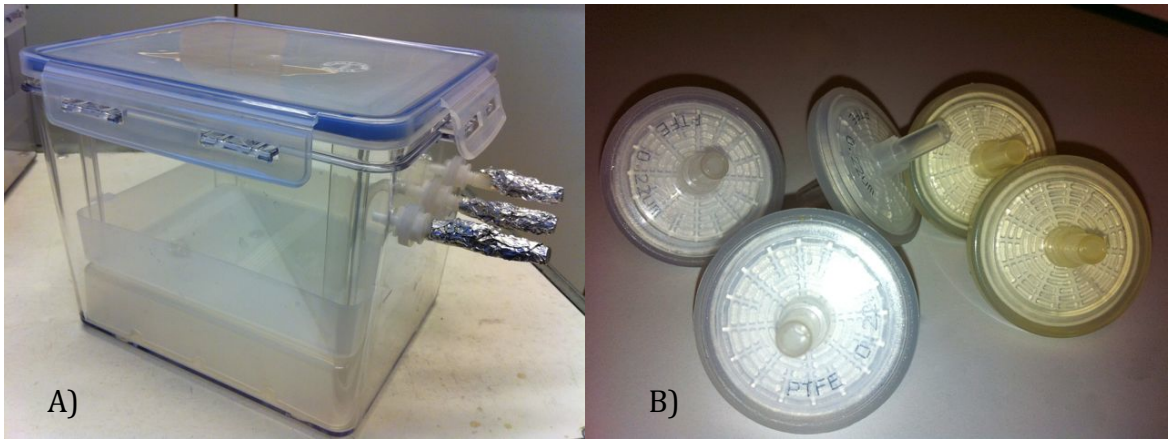



**Figure 1.** The newly developed TIS bioreactor; A) the bioreactor prepared for autoclaving by coverage with aluminum foil and enclosing of two sides of the lid, B) the PTFE filters (0,22 μm) used on the bioreactor.

The bioreactors and filters were autoclaved at 121°C for 20 minutes. The three holes were sealed with aluminum 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.

In addition, small transparent jars with solid media were used for comparing culture growth in the liquid bioreactor system and solid media (Figure 2A-B). These small jars were made of Styrolux plastic with an approximate size of 8x9x9cm. One TIS bioreactor was equivalent to eight such plastic jars since five explants were placed in each jar and one bioreactor contained 40 shoots. However, one bioreactor of *D. lutea* x *purpurea* was equivalent to three jars since the total number of explants in a bioreactor was only 15 for this species.

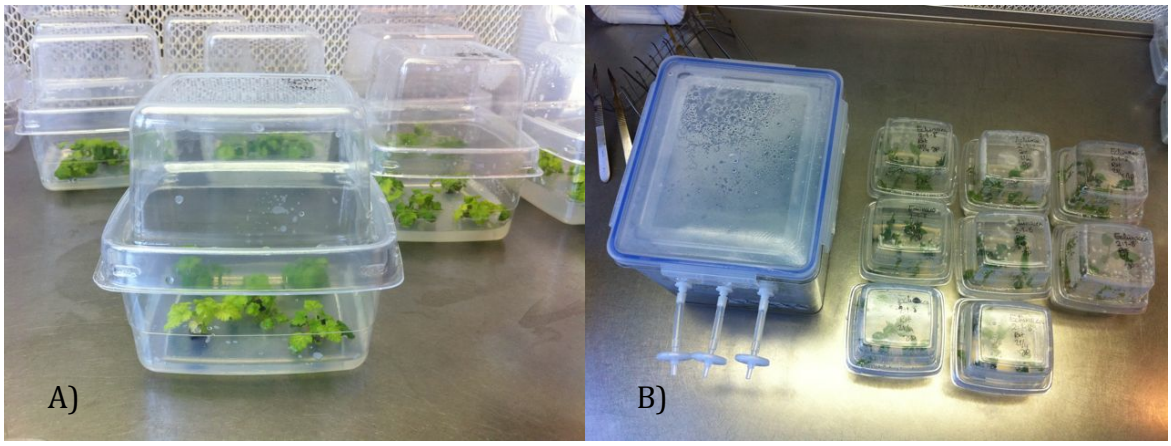



**Figure 2.** The small plastic jars and bioreactors used in this study. A) Side view of the small jars and B) a size comparison between the small jars and the TIS bioreactor.

## 3.2 Plant material

Three different, widely cultivated and important, horticultural plant species grown in vitro have been used in this study: *Digitalis lutea x purpurea, Echinacea purpurea* 'Magnus' and *Rubus idaeus* 'Mormorshallon'. All plant materials were first cultivated in liquid media in the bioreactors to get sufficient plant material for performing the experiments. As uniform shoots as possible were chosen for the experiments. Before transfer to the bioreactors, the explants were prepared by division into individual shoots and by removal of

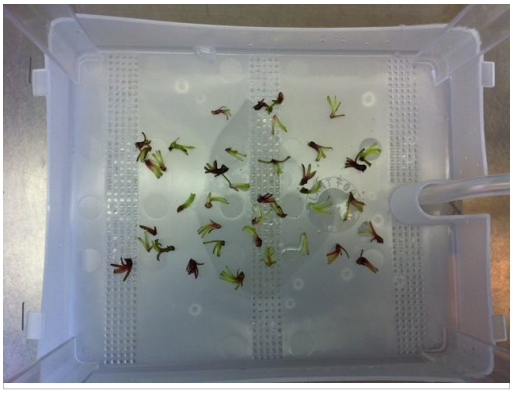


**Figure 3.** Explants that have been prepared for cultivation experiments by separation into individual shoots and by removal of leaves.

all larger/fully developed leaves (Figure 3). The explants were additionally cut into approximately 1 cm long stems.

### 3.3 Culture media

The basal media used were either Murashige & Skoog (Murashige & Skoog, 1962) or Quoirin & Lepoivre (Duchefa, 2000-2001) depending on species (Table 1).

**Table 1.** Compositions and concentrations of media used in the study (Duchefa, 2000-2001). Figures in gray in the Quorin & Lepoivre medium indicate modifications compared to the original recipe, where nicotinic acid and pyridoxine HCl are not included.

| Type of nutrients | Substance | Murashige & Skoog medium (mg/l) | Quoirin & Lepoivre medium (mg/l) |
|---|---|---|---|
| Macro elements | $CaCl_2$ | 332.02 | |
| | $Ca(NO_3)_2$ anhydrous | | 578.92 |
| | $KH_2PO_4$ | 170.00 | 270.00 |
| | $KNO_3$ | 1900.00 | 1800.00 |
| | $MgSO_4$ | 180.54 | 175.79 |
| | $NH_4NO_3$ | 1650.00 | 400.00 |
| Micro elements | $CoCl_2 \cdot 6H_2O$ | 0.025 | 0.025 |
| | $CuSO_4 \cdot 5H_2O$ | 0.025 | 0.025 |
| | FeNaEDTA | 36.70 | 36.70 |
| | $H_3BO_3$ | 6.20 | 6.20 |
| | KI | 0.83 | 0.08 |
| | $MnSO_4 \cdot H_2O$ | 16.90 | 0.76 |
| | $Na_2MoO_4 \cdot 2H_2O$ | 0.25 | 0.25 |
| | $ZnSO_4 \cdot 7H_2O$ | 8.60 | 8.60 |
| Vitamins | Glycine | 2.00 | |
| | myo-Inositol | 100.00 | 100.00 |
| | Nicotinic acid | 0.50 | 0.50 |
| | Pyridoxine HCl | 0.50 | 0.50 |
| | Thiamine HCl | 0.10 | 0.40 |

The propagation medium for *D. lutea* x *purpurea* consisted of full strength of Quoirin & Lepoivre (Q0251, Duchefa) with an addition of 0.5 mg/l nicotinic acid and 0.5 mg/l pyridoxine HCl. The medium also contained 1mg/l BAP, 0.1mg/l IBA and 30g/l sucrose. The pH was adjusted to 5.5 before autoclaving. The medium composition and pH was the same for *E. purpurea* 'Magnus' as above except for that BAP was reduced to 0.2mg/l and no addition of IBA. For *R. idaeus* 'Mormorshallon', the propagation medium

consisted of full strength of Murashige & Skoog medium (M0222, Duchefa), 0.5mg/l BAP, 0.01mg/l IBA, and 30g/l sucrose, and the pH was adjusted to 5.2. The medium formula for liquid and solid propagation was the same except for addition of 6g/l agar (Bacto Agar B1000-1, Saveen Werner AB) to the solid media in the Styrolux jars.

All components were dissolved in Millipore water. The pH was adjusted with NaOH or HCl before addition of agar and autoclaving of the medium. Autoclaving of media was performed at 121°C and 1.2 bars for 20 minutes. All media were prepared in bottles with the volume of 1 liter, which was sufficient for cultivation in two bioreactors.

### 3.4 Micropropagation

The bioreactors were autoclaved, connected with sterile PTFE filters, filled with 500 ml of the appropriate medium and the explants in a sterile environment. Finally, plastic tubes were connected between the bioreactors and a pneumatic pumping system. The pneumatic pumping system was controlled by an automatic control unit for regulation of aeration and immersion of explants in medium. All bioreactors, regardless of plant species, were exposed to aeration and immersion with the same regime. Immersion with the liquid medium was performed twice a day, at 8 am and 4 pm. The immersion time was set to approximately six minutes, the exact time depending on how fast the pneumatic pump could raise the medium levels in the bioreactors. At every hour between 8 am and 8 pm aeration was allowed for four minutes, except at the immersion times when aeration was prolonged to seven minutes.

All bioreactors contained 40 shoots each, except for the ones with *D. lutea* x *purpurea*, which contained 15 shoots each due to a limited amount of plant material. The total number of bioreactors was 22, where 14 bioreactors where filled with explants of *R. idaeus* 'Mormorshallon', 5 with *E. purpurea* 'Magnus' and 3 with *D. lutea* x *purpurea*. The bioreactors were placed in a climate chamber with the fluorescent lamps Polylux XL™ F36W/830 with a light intensity of 33 μmol $m^{-2}$ $s^{-1}$ for 16 hours per day, at a day temperature of 23°C/18°C (day/night). Comparing cultures on solid medium included the same initial amount of shoots and were treated under the same conditions as the bioreactors.

## 3.5 Growth and biomass production

Fresh and dry weights were measured periodically and observation of explants and survival were recorded. Production of shoots was evaluated at the end of each experiment. Evaluation was carried out for both bioreactors and solid medium cultures.

### *3.5.1 Weight*

Fresh weight of the explants was determined at the start of the experiment. Both fresh and dry weight of the explants was recorded at the end of the experiment. The dry weight was examined after being dried in oven at 70°C for 24 hours. The weight increase was estimated by calculating an average of biomass from all bioreactors.

### *3.5.2 Shoot multiplication*

The total number of shoots was counted at the end of each experiment, after four weeks cultivation from both bioreactor and solid medium. The counted shoots were divided into two different classes depending on their size, where class one was assigned to those shoots of adequate size to be transferred to root induction medium before acclimatization in greenhouse. Reminding smaller shoots were assigned to class two, and these shoots were further cultivated in TIS to increase the shoot size. The class division was based on visual inspection.

The shoots of five bioreactors for the species *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon' were counted, while the corresponding bioreactor number for *D. lutea* x *purpurea* was three. This means that shoots from 13 bioreactors were counted in total. In addition, the number of shoots produced from explants cultivated on solid medium was also counted after the same time. The number of plastic jars with solid media where the shoots were counted was 32 each (equal to the plant material of four bioreactors) for the species *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon', while the corresponding number for *D. lutea* x *purpurea* was 6 jars (equal to the plant material of two bioreactors).

### *3.5.3 Survival & Appearance*

Survival and appearance of the plants was examined through visual inspection after four weeks of cultivation for all the three plant species. Plants of *R. idaeus* 'Mormorshallon' were in addition examined after one, two and three weeks of cultures. The vigor of the plants was evaluated visually and the number of surviving plants in each bioreactor was counted. Evaluations of plants in bioreactor and on solid media were examined in the same way.

## 3.6 Medium analyses

Periodic measurements were performed regarding pH, conductivity, mineral nutrients (phosphorus and nitrogen) and sugars (fructose, glucose, sucrose) for liquid medium used in bioreactors. The medium was filtrated with Whatman no 1 filter papers (11μm) before being analyzed.

### *3.6.1 Conductivity & pH*

Medium conductivity and pH were measured before and after autoclaving as well as at the end of each culture using an ECTestr11 and a Metrohm 691 pH Meter respectively.

### *3.6.2 Mineral nutrients composition and consumption*

Samples of *D. lutea x purpurea, E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon' from the different start media (i.e. the medium after autoclaving but before plant cultivation) and final medium (i.e. the medium after four weeks of cultivation) were taken and analyzed by Eurofins Food & Agro Sweden, for full analysis of concentration of all macronutrients and iron. One medium sample from two weeks of *R. idaeus* 'Mormorshallon' propagation was also included. All mineral nutrients were analyzed with ICP (inductively coupled plasma emission), except for the nitrogen, which was analyzed with FIA (flow injection analysis). To gain information on concentration changes of micronutrients in the liquid media, samples from the start and final media of *E. purpurea* 'Magnus' were also analyzed with ICP.

Additionally, all media used during the experiments were analyzed for total nitrogen (LatoN, LCK 338, 20-100 mg/l $TN_b$) and phosphate (Phosphate, LCK350, 2-20mg/l $PO_4$-P, 6-60 mg/l $PO_4$) levels using ready to use kits from Hach Lange, Germany (www.hach-lange.com).

### *3.6.3 Sugar composition and consumption*

All media used during the experiments were analyzed for the concentration of D-fructose, D-glucose and sucrose using a ready to use kit from Megazyme, Ireland (www.megazyme.com). Analyzes were carried out both at the start and the end of cultivation.

## 3.7 Acclimatization of plantlets

In order to evaluate the acclimatization capability under greenhouse conditions, plantlets produced in bioreactor or on solid medium were root induced in the root induction medium for two (D. *lutea* x *purpurea*) and four (*R. idaeus* 'Mormorshallon')

days respectively, and then planted in soil substrate. The root induction medium for *D. lutea* x *purpurea* consisted of full strength of Lepoivre (Q0251, Duchefa) with an addition of 0.5 mg/l nicotinic acid and 0.5 mg/l pyridoxine HCl. The medium also contained 5mg/l IBA and 30g/l sucrose. The pH was adjusted to 5.5 before autoclaving. The medium composition for *R. idaeus* 'Mormorshallon' was full strength of MS (M0222, Duchefa), 0.1mg/l IBA, and 30g/l sucrose, and the pH was adjusted to 5.2. The medium formula for liquid and solid propagation was the same except for addition of 6g/l agar (Bacto Agar B1000-1, Saveen Werner AB) to the solid media in the Styrolux jars. The *E. purpurea* 'Magnus' medium was the same as for propagation due to earlier observation that this plant species has the capability to form roots on the medium designed for propagation. However, these plants were never acclimatized due to the need of them in future experiments.

The plants were placed in plastic boxes with a substrate consisting of 70 % planting soil and 30% perlite (Figure 4A) and were covered with transparent plastic film to avoid desiccation (Figure 4B). They were placed in greenhouse with normal natural light (April-May) and hand irrigation. The visual inspection of survival and appearance was recorded.

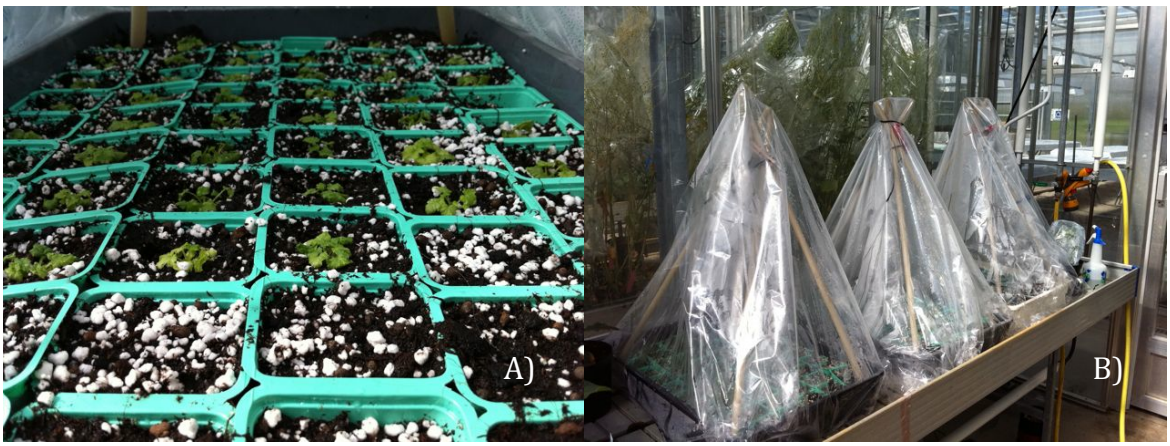



**Figure 4.** Plants grown in the greenhouse for acclimatization. A) Plants of *R. idaeus* 'Mormorshallon' planted in plastic boxes. B) Transparent plastic film for coverage of the planting boxes to avoid desiccation of the plants.

## 3.8 Statistical analyses

The results were analyzed with the commercial spreadsheet application Microsoft Excel for Mac 2011 (version 14.1.0) and the statistical software Minitab 16. The statistical method used was two sample t-tests to make pairwise comparisons between liquid and

solid media. The tests were performed on the significance level p=0.05. Due to a low number of samples the statistical analyzes should be regarded as very preliminary. However, the tendency of the results can be observed.

# 4 RESULTS & DISCUSSION

## 4.1 Plant growth and biomass production

### *4.1.1 Weight*

Fresh weight increase of the explants revealed differences between bioreactor and solid medium (Figure 5) and also depends on plant species. *D. lutea* x *purpurea* and *E. purpurea* 'Magnus' gained more weight during cultivation in bioreactor compared to solid medium, while *R. idaeus* 'Mormorshallon' showed the opposite result. There was a significant difference ($p \leq 0.05$) between the TIS and solid medium cultivation in fresh weight for all three species.

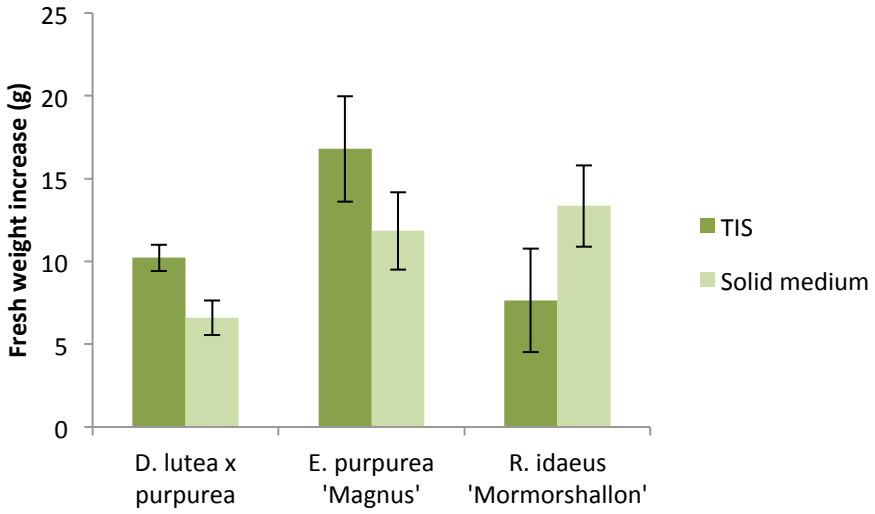



**Figure 5.** Comparison of fresh weight increase between TIS and solid media shown for *D. lutea x purpurea*, *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon'. Error bars indicate standard deviation.

When it comes to dry weight (Figure 6), there was not enough material to perform statistical analyses on *D. lutea* x *purpurea*. There were no statistical differences observed between the two cultivation methods for *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon'. The total dry mass percentage ranged 9.0-13.7% for TIS and 8.7-11.6% for solid medium for all plants, indicating that the TIS cultivation does not lead to reduction in dry-matter production. According to Preece and Read (2005) as much as 80-90% of the weight of herbaceous plants is normally made up by water. This is in line with our study. Even if plants do not seem to be affected by hyperhydricity, there is a risk that exogenous water remains on the plants, which might affect the fresh weight of the plants, while such risk does not exist for dry weight measurement.

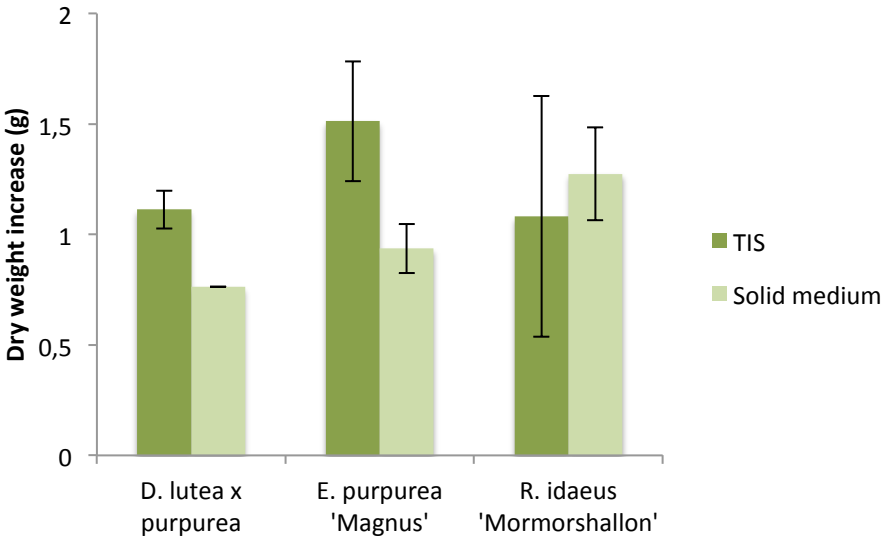



**Figure 6.** Comparison of dry weight results for TIS and solid media shown for *D. lutea* x *purpurea*, *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon'. Error bars indicate standard deviation.

The time course increase in fresh weight of TIS cultivated *R. idaeus* 'Mormorshallon' revealed a large standard deviation (Figure 7). No weight increase was observed after the first week of cultivation. This could perhaps be explained by water loss of the explants after that they had been cut, that they needed some time to adapt to the new environment, and/or that the changes were too small to be detected.

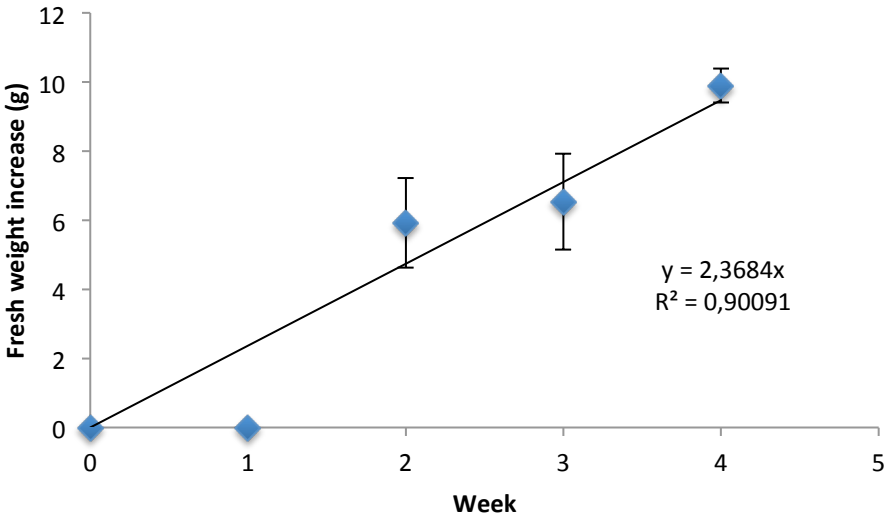



**Figure 7.** Fresh weight increase for TIS cultivated explants of *R. idaeus* 'Mormorshallon' over time. Results are averages from three replicates and error bars indicate standard deviation.

Previously performed studies on comparing different cultivation methods have revealed differing results regarding fresh and dry weights. Studies on the plant species *Siraitia grosvenorii* with TIS revealed that both fresh and dry weights of shoots displayed significantly higher values than corresponding studies on solid and liquid media (Yan et al., 2010). However, in a study on pineapple (*Ananas comosus* L. Merr) made by Escalona et al. (1999), the fresh weight was higher for TIS than for both solid and liquid medium, whereas no difference in dry weight was seen. The results may be an indication that differences occur depending on plant species and cultivation method. However, other parameters such as medium composition will also affect the plant performance.

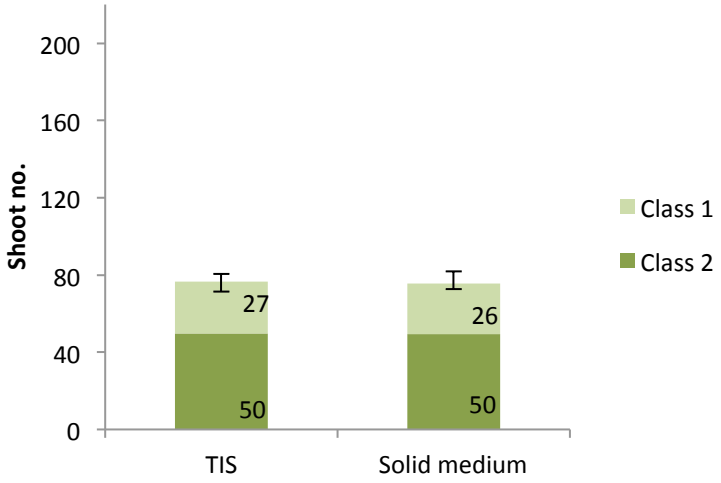
### *4.1.2 Shoot multiplication*

All fully developed shoots from each bioreactor and plastic jar were counted at start and four weeks after cultivation (Figure 8). Due to different initial shoot numbers for different species (15 for *D. lutea x purpurea*, 40 for *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon') and different growth characteristics among the species, comparisons have not been made among the species.

No significant differences were observed between TIS and solid medium for either the total number of shoots or number of shoots that were of adequate size for acclimatization (Class 1) in *D. lutea* x *purpurea* and for *R. idaeus* 'Mormorshallon'. However, for *E. purpurea* 'Magnus', TIS resulted in significantly ($p \leq 0.05$) more shoots that were of adequate size for acclimatization than solid medium (Fig 8B).

Previous studies have in general revealed a higher shoot multiplication in TIS compared to other cultivation systems. Studies on solid, liquid and TIS with sugarcane (*Saccharum* spp. cv. C-1501-73) revealed that a better shoot multiplication and height was achieved with TIS than with the other systems (Lorenzo et al., 1998). In *Siraitia grosvenorii*, it was revealed that shoot multiplication rate and shoot length were significantly better in TIS than on solid and liquid media (Yan et al., 2010). Zhu et al. (2005) also concluded higher multiplication rate (apple rootstock M26) in TIS compared with solid medium. In a study on pineapple (*Ananas comosus* L. Merr), the shoot number was higher for TIS than for solid or other liquid medium (Escalona et al., 1999). However, Damiano et al. (2005) compared TIS and solid medium on apple (Jork 9), peach (cv. Yumyeong), cherry (cv. Biggareau Burlat) and plum (cv. Adara) and found no

A)

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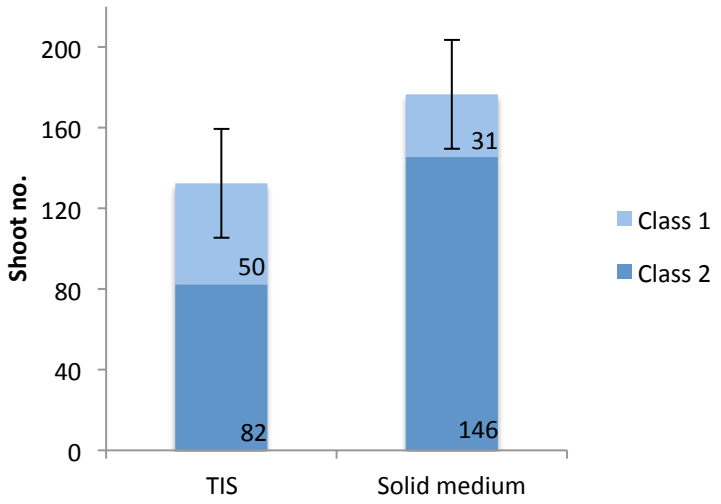


C)

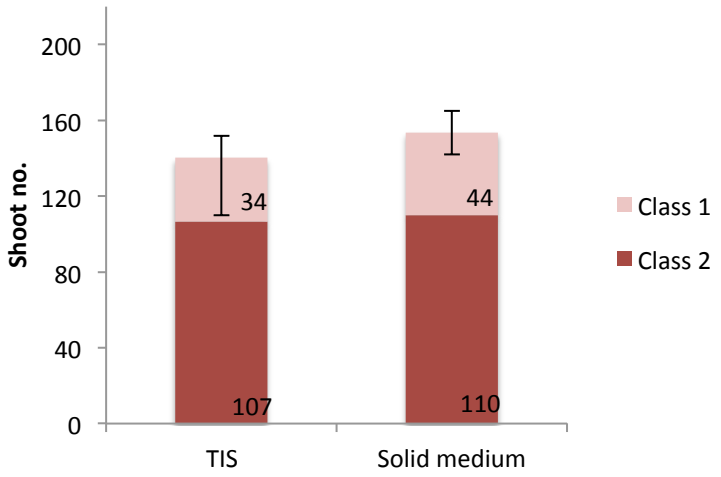


**Figure 8.** Shoot multiplication for *D. lutea* x *purpurea* A), *E. purpurea* 'Magnus' B) and *R. idaeus* 'Mormorshallon' C). The figures display shoots of adequate size for acclimatization (Class 1) and shoots of smaller size that needed further cultivation in the medium (Class 2). Error bars indicate standard deviation of total number of shoots.

difference in multiplication rate between TIS and solid medium. Chakrabarty et al. (2007) found that plants cultivated in TIS had higher photosynthetic rates with the maximum quantum yield of photosystem II. Aeration and temporary immersion of explants in the medium are among the explanations for the success of TIS (Etienne & Berthouly, 2002).

In conclusion, the above-mentioned studies on different plant species show in general that the shoot multiplication is higher in TIS than other media. This is in agreement with the results from this study. The reason why TIS generally results in a higher multiplication rate might be related to the easier access of plant growth regulators. However, this needs to be confirmed.

### *4.1.3 Survival & Appearance*

Survival and growth of cultures from both TIS and solid media showed healthy appearance (Figure 9). Differences in size and appearance could be observed among individuals and within/between treatments. The majority of the plants of both *D. lutea* x *purpurea* and *E. purpurea* 'Magnus' looked healthy regardless of cultivation method. Some of the *R. idaeus* 'Mormorshallon' cultures looked rather strange during the micropropagation period with shriveled or curled leaf edges, which could be an indication of hyperhydricity. Problems with hyperhydricity have been observed in liquid medium cultivation (Welander et al., 2007). Typical signs of hyperhydricity are that the leaves often become broader and translucent (Chakrabarty et al., 2007), with a fragile, thicker, curled and/or wrinkled appearance (Chakrabarty et al., 2007; Kevers et al., 2004) and the stems also appear broader, thicker with shorter internodes (Kevers et al., 2004). Nevertheless, in this study the observation was the same for both cultivation systems and disappeared during the acclimatization period. Hyperhydricified shoots often do not survive acclimatization well (Kevers et al., 2004), so it is therefore more likely that the strange appearance of some cultures in this study was actually not due to hyperhydricity. Earlier research results have revealed that plants cultivated in TIS were less prone to hyperhydricity and necrosis compared with liquid and solid media (Damiano et al., 2005). In comparison with TIS, cultivation in liquid medium has been proven to result in many (28.7%) shoots (apple rootstock 'M9 EMLA') with typical hyperhydricity signs (Chakrabarty et al., 2007). In addition, shoots of liquid medium cultivation had lower dry biomass, which was ascribed to be due to higher water content, compared to the plants from the TIS. Damiano et al. (2005) actually ascribes the

lower hyperhydricity problems in TIS to be due to the repeated replenishment of the bioreactor headspace in these systems.


**Figure 9.** Appearance of cultures of different plant species and media after four weeks of cultivation. *D. lutea x purpurea* cultivated A) in TIS and B) on solid medium, *E. purpurea* 'Magnus' cultivated in C) TIS and D) on solid medium and *R. idaeus* 'Mormorshallon' cultivated E) in TIS and F) on solid medium.

Studies on *Musa* AAB by Roels et al. (2006) confirmed the observation that forced air supply in TIS results in better shoot quality compared to cultivation on solid media. The improved shoot quality was probably due to increased $O_2$-level, by supplementary air whereas $CO_2$ and $C_2H_4$ at the same time were reduced. Welander et al. (2007) also reported that aeration might have a positive influence on the gas composition within the

bioreactor. Alvard et al. (1993) concluded that lack of aeration in liquid bioreactor systems hampers growth due to lack of oxygen in the small explants. Supplementation of airflow is an important factor for promotion of growth and development through aeration and mixing of the explants when cultivating in liquid media, but too strong air forces could also lead to damage of the plant material (Ziv, 2000). Length and frequency of immersion times are mentioned to be of great importance in controlling and promoting plant growth within TIS (Etienne & Berthouly, 2002). The capability of controlling these issues is also concluded to be an advantage in comparison with older bioreactor systems.

It is important to note that none of the bioreactors in the present experiments became contaminated during cultivation. In the study on *Pinus radiata* D. Don, Aitken-Christie & Davies (1988) used a TIS bioreactor with the dimensions 390x390x120mm. The large size of the bioreactor was detrimental due to high risk of contamination since it could not be securely fitted into the laminar flow hood, and the size of the container was therefore decreased (250x390x120mm). In comparison, the size of the bioreactor (160x160x150mm) used in this study is much smaller. The size of this bioreactor seems to be adequate since it is of enough size to room numerous explants, while at the same time it is not so large so that the contamination risk jeopardizes cultivation.

## 4.2 Medium analyses

### *4.2.1 Conductivity & pH*

A decrease in conductivity in the TIS medium was observed for all three species when comparing the medium before and after four weeks of cultivation (Table 2). Theoretically, it is expected to get a decrease in conductivity due to plant uptake of mineral nutrients for their growth. Measurements of pH on autoclaved starting and finishing media revealed a decrease for *D. lutea* x *purpurea* and *E. purpurea 'Magnus'*, while *R. idaeus* 'Mormorshallon' showed the opposite change (Table 2). Like in this study, Chakrabarty et al. (2007) also observed a slight decrease in the medium pH during cultivation of apple rootstock 'M9 EMLA'.

**Table 2.** Conductivity and pH in TIS media for *D. lutea* x *purpurea*, *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon'. Measurements have been performed before cultivation (start) and after four weeks of cultivation (finishing). Final results are averages from replicates with standard deviation.

| Species | Conductivity (mS/cm) | | pH | | |
|---|---|---|---|---|---|
| | Start | Final | Start (not autoclaved) | Start (autoclaved) | Final |
| *D. lutea* x *purpuea* | 3,80 | 3,27 ± 0,058 | 5,50 | 4,67 | 4,48 ± 0,078 |
| *E. purpurea* 'Magnus' | 3,55 | 2,98 ± 0,176 | 5,50 | 4,91 | 4,65 ± 0,106 |
| *R. idaeus* 'Mormorshallon' | 5,20 | 4,87 ± 0,208 | 5,20 | 4,28 | 4,63 ± 0,135 |

Before autoclaving, the pH of *D. purpurea* x *lutea* and *E. purpurea* 'Magnus' media was adjusted to 5.5 and to 5.2 for *R. idaeus* 'Mormorshallon'. Table 2 shows that the pH declines in the media after autoclaving, and this decline is much larger than changes occurred during cultivation. Perhaps, the starting pH should be set to a higher level to counteract the pH decline after autoclaving. Another solution could be to filter-sterilize the medium instead of autoclaving. Tests performed with separate autoclaving of sugar in water from the rest of the medium ingredients as wells as usage of medium with MES also indicated more stable pH.

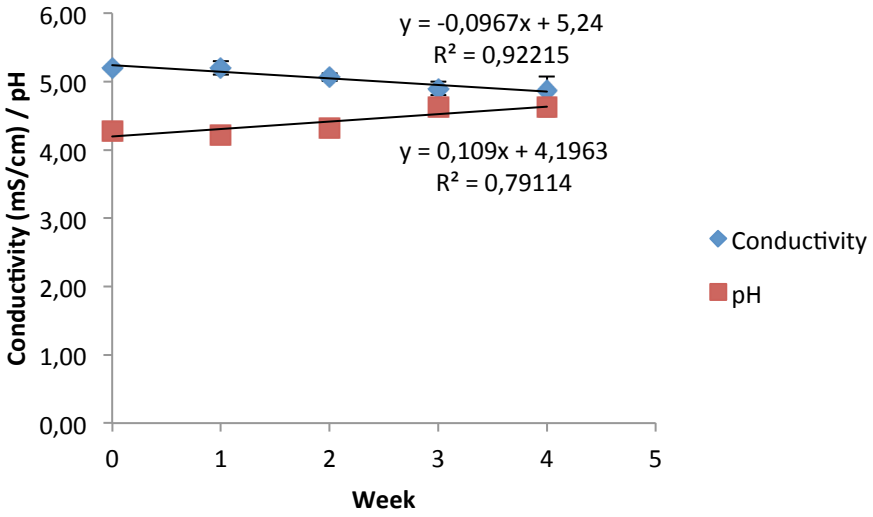


**Figure 10.** Changes in conductivity and pH in TIS media for *R. idaeus* 'Mormorshallon' over time. Error bars indicate standard deviation.

Conductivity measurements performed periodically on the TIS medium during cultivation of *R. idaeus* 'Mormorshallon' revealed a small decrease over time (Figure 10). Results from measurements of pH performed periodically on the TIS medium during cultivation of *R. idaeus* 'Mormorshallon' are also shown in Figure 10. At the beginning of the cultivation period, there was a slight decline in pH, followed by a slight increase later on. One explanation could be that pH is not only affected by the plant but also by the aeration system, which lead to carbon dioxide ($CO_2$) partial pressure changes so that $CO_2$ dissolves in the liquid medium. $CO_2$ dissolved in water reacts with a water molecule ($H_2O$) to form dihydrogen carbonate ($H_2CO_3$), which in turn is in chemical equilibrium with bicarbonate ions ($HCO_3^-$) and hydrogen ions ($H^+$). In addition, $HCO_3^-$ ions form equilibrium with carbonate ions ($CO_3^{-2}$) and $H^+$. The hydrogen ion production is affected by pH. With an initially high pH, the equilibrium reaction leads to an increase in the number of hydrogen ions ($H^+$) in the liquid, and thereby a pH decrease.

Changes in pH can also be due to nitrogenous absorption by the plants. Uptake of either nitrate ($NO_3^-$) or ammonium ($NH_4^+$) ions can affect the pH to change in different directions due to differences in proton flow. Higher uptake of $NH_4^+$ ions leads to a decreased pH of the medium due to exudation of $H^+$, while higher uptake of $NO_3^-$ ions will lead to an increased pH due to exudation of $OH^-$ (Raven & Smith, 1976). Keeping a balance between $NH4^+$ and $NO3^-$ in the medium generally stimulates plant growth. This will in turn lead to balancing of cation-anion inside the plants. The advantage of keeping a balance is also explained to be pH independent. All media within this study were based on nitrogen source that has been formed through a combination of $NH4^+$ and $NO3^-$ ions, but their mutual relations differ (Table 4.2.2A).

### *4.2.2 Mineral nutrients composition and consumption*

The full mineral nutrient analysis performed on TIS media revealed that for the majority of the nutrients no major changes in concentration occurred over time (Table 3). The nutrient changes were in general following a similar pattern for all three species, but a difference could be seen for ammonium-nitrogen. Both *D. lutea* x *purpurea* and *E. purpurea* 'Magnus' used approximately half of the ammonium-nitrogen in their media, while a much lower percentage (~15%) was used for *R. idaeus* 'Mormorshallon'. The basal medium of *R. idaeus* 'Mormorshallon' is MS, which had more than four times higher ammonium-nitrate concentration than Lepoivre used in this study.

However, the most remarkable part in the results is the great decrease in iron (Fe) concentration (Table 3 and Figure 11) for all three plant species. The micronutrient iron is important for synthesis of chlorophyll and also serves as a constituent of cytochromes (electron carriers during photosynthesis and respiration reactions) and nitrogenase (enzyme for catalyzation of nitrogen-fixing reactions) (Raven et al., 2005). Symptoms of iron deficiency are revealed through appearance of interveinal chloroses on young leaves and in the form of short and slender shoots. Chloroses on young leaves are due to that iron is immobile in the plant, and it therefore cannot be redistributed from older leaves when deficiency appears (Taiz & Zeiger, 2006). This could actually be observed on some of the plants in this study. Due to the low concentration of iron at the end of the cultivation period, it was decided to investigate if concentrations of manganese (Mn), molybdenum (Mo), boron (B), copper (Cu) and zinc (Zn) also decreased in the same way. Analyzing was performed on *E. purpurea* 'Magnus' and the results showed that only minor changes occurred.

**Table 3.** Differences in mineral nutrient concentrations (mg/L) before (start) and after four weeks of TIS cultivation (final) in *D. lutea* x *purpurea*, *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon'. For *R. idaeus* 'Mormorshallon', one measurement was also executed after two weeks of cultivation, when the cultivation had run midway. Analyzes performed by Eurofins Food & Agro Sweden AB.

| Substance | *Digitalis* (mg/l) | | *Echinacea* (mg/l) | | *Rubus* (mg/l) | | |
|---|---|---|---|---|---|---|---|
| | Start | Final | Start | Final | Start | Midway | Final |
| Nitrate-Nitrogen | 390 | 350 | 390 | 340 | 510 | 510 | 480 |
| Ammonium-Nitrogen | 62 | 30 | 62 | 38 | 270 | 260 | 230 |
| Phosphate | 65 | 62 | 65 | 58 | 38 | 38 | 33 |
| Potassium | 660 | 600 | 660 | 620 | 650 | 700 | 650 |
| Sulfate-Sulfur | 33 | 33 | 33 | 33 | 34 | 37 | 34 |
| Calcium | 130 | 130 | 130 | 130 | 110 | 110 | 110 |
| Magnesium | 24 | 25 | 24 | 23 | 23 | 25 | 24 |
| Iron | 4,7 | 1,4 | 4,7 | 0,53 | 4,6 | 1,6 | 0,85 |

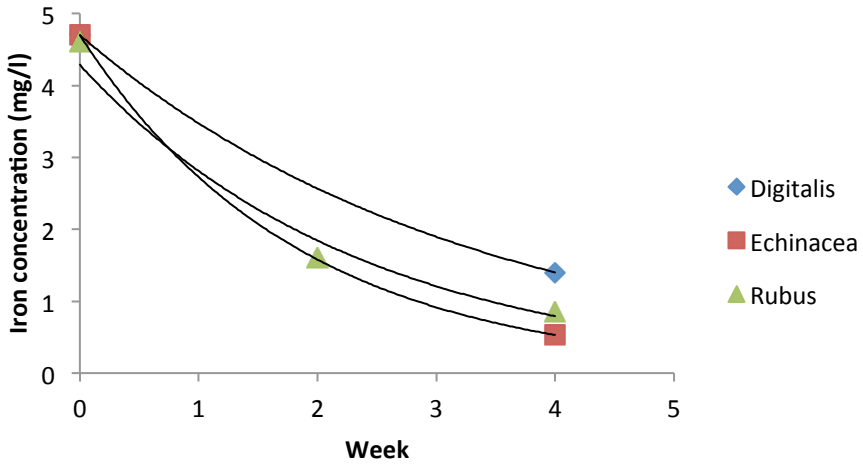


**Figure 11.** Differences in iron concentration in the TIS medium before cultivation (start) and after four weeks of cultivation (final) in *D. lutea* x *purpurea*, E. *purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon', and also after two weeks of cultivation (halfway) for *R. idaeus* 'Mormorshallon'. Analyzes performed by Eurofins Food & Agro Sweden AB.

Periodic measurements on total nitrogen concentration in *R. idaeus* 'Mormorshallon' cultivated with TIS displayed a slight decrease in total nitrogen concentration over time (Figure 12) indicating that the cultures did not consume much nitrogen supplied.

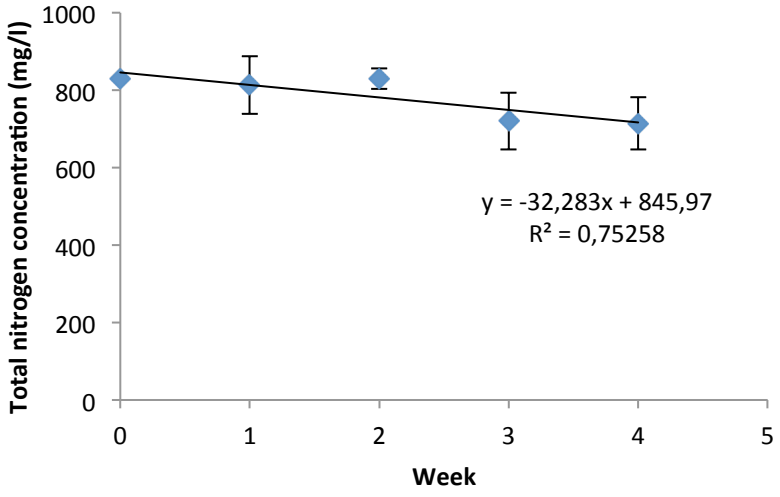


**Figure 12.** Changes in total nitrogen concentration in TIS medium for *R. idaeus* 'Mormorshallon' over time. Error bars indicate standard deviation.

Chakrabarty et al. (2007) has revealed that the time-course depletion of macronutrients in apple rootstock 'M9 EMLA' medium of TIS was much slower than that with liquid medium with constant immersion. In the case of the liquid medium both phosphate

($H2PO_4^-$) and ammonium ($NH_4^+$) ions were nearly exhausted after three weeks of cultivation, while none of the nutrients in the TIS were exhausted even after four weeks of cultivation. The later result is in line with the results from this study, where none of the nutrients were fully depleted. The only exception to this was iron, which was almost depleted at the end of the cultivation period.

Chakrabarty et al. (2007) also analyzed several mineral elements at start and at the end of the cultivation period. The concentrations of calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) did not change much over time, a similar result to ours. However they did find that sulfate ($SO_4^{2-}$), phosphate ($H_2PO_4^-$) and nitrate ($NO_3^-$) concentrations decreased considerably after four weeks of cultivation. Furthermore, the ammonium ($NH_4^+$) concentration in the media of Chakrabarty et al. (2007) was initially within the same range as ours (compared with *R. idaeus* 'Mormorshallon') or considerably lower (compared to *D. lutea* x *purpurea* and *E. purpurea* 'Magnus'), but later on decreased considerably. These differences might also be due to the uses of different plant materials.

Unfortunately, the phosphorus analysis in this study did not give any reliable results. This is probably due to the high concentration of nitrate in the TIS medium that might have been interfering during analysis.

### *4.2.3 Carbohydrate composition and consumption*

The results from sugar analyzes revealed that even after four weeks of cultivation in TIS, the majority of the initially added total sugar of 30 g/l was still left in the medium for all three species (Table 4). Periodic sugar measurements on *R. idaeus* 'Mormorshallon' showed a similar result. It is possible that the forced aeration of the bioreactors had contributed to a reduction in sugar usage by the explants due to increased photosynthesis capacity.

The analysis has shown that sucrose was hydrolyzed to D-glucose and D-fructose both after autoclaving and during cultivation. The hydrolyzing of sucrose during autoclaving is influenced by the medium pH and the medium mineral composition. Autoclaving of sucrose with only water resulted in no breakdown of the sucrose. The higher breakdown of sucrose was found in MS medium with a higher nutrient concentration and a lower pH compared to Lepoivre.

**Table 4.** Sugar concentration in the TIS medium after different weeks of cultivation.

| Species | Week after cultivation | Sucrose (g/l) | D-glucose (g/l) | D-fructose (g/l) | Total sugars (g/l) |
|---|---|---|---|---|---|
| *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* | 1 | 16,5 | 6,2 | 5,7 | 28,4 ± 0,756 |
| *Rubus* | 2 | 12,6 | 8,1 | 7,8 | 28,5 ± 0,408 |
| *Rubus* | 3 | 13,6 | 7,8 | 7,6 | 28,9 ± 1,740 |
| *Rubus* | 4 | 12,5 | 7,2 | 7,2 | 26,9 ± 0,389 |

Sucrose ($C_{12}H_{22}O_{11}$, 342.30g/mol) is a disaccharide that can be broken down into the monosaccharaides glucose ($C_6H_{12}O_6$, 180.16g/mol) and fructose ($C_6H_{12}O_6$, 180.16g/mol). The molar mass of one sucrose molecule is not equal to the molar mass of one glucose molecule plus one fructose molecule, but is instead 360.32g/mol. When looking at the molecular formulas it can be observed that an additional water molecule has been added during hydrolysis of the sucrose molecule. This means that when the disaccharide sucrose decomposes into D-fructose and D-glucose the entire molar mass increases. The sugar analysis of this study reveals that the amount of sucrose decreases while the amount of D-glucose and D-fructose increases.

The breakdown of sucrose is in fact already observed directly after autoclaving, before any cultivation has taken place. After autoclaving the color of the media become darker and browner. This is most likely caused by the so-called Maillard-reaction in which reducing sugars (e.g. glucose or fructose) and inorganic nitrogen compounds interact during heating, and thus leading to changes in taste, aroma and nutritional composition (Nationalencyklopedin, 2011). Since sucrose is not a reducing sugar, the amount of this disaccharide must have been hydrolyzed into glucose and fructose before the Maillard-reaction could occur. Chakrabarty et al. (2007) also observed that quantities of sucrose in the medium had already been hydrolyzed after autoclaving at the beginning of the experiment.

Furthermore, it was also revealed that the breakdown of sucrose depended on medium type used. The *R. idaeus* 'Mormorshallon' media composed of MS had much greater breakdown of sucrose than the other plant species that were cultivated in the

Lepoivre basal medium. The majority of the results also showed a similar amount of D-glucose and D-fructose which corresponds with the theory that one sucrose molecule is broken down into one glucose and one fructose molecule. It could also be an indication for how the monosaccharaides are used by the plant material. In the research on apple rootstock 'M9 EMLA', Chakrabarty et al. (2007) noticed that the concentrations of glucose and fructose in the medium were equal, and thereby they concluded that the plants did not have any preference to any of the monosaccharaides. In this study, *E. purpurea* 'Magnus' and *R. idaeus* 'Mormorshallon' revealed a similar concentration of glucose and fructose. *D. lutea x purpurea* however showed a higher fructose content left in the medium, indicating that glucose probably is preferred by this species (Table 4).

Possible explanations why the sugar concentration is higher than the initial one might depend on either a change in water content in the medium or the increased molecular weight of sugar after hydrolysis. Condensation of water could be seen on the walls of the bioreactors, which may indicate a reduction of water content and a sugar increase in the medium. Meanwhile, the frequent ventilation might also cause water loss from the bioreactors. It could also be that water vapor has left the bioreactor through the non-connected ventilating third PTFE filter. This filter has a membrane pore size of 0,22 μm, while the size of a water molecule is much smaller. It has been reported that some cultivation systems loose water through evaporation, leading to higher concentration of substances in the medium (Ziv, 2000).

### 4.3 Acclimatization tests

The acclimatization tests gave positive results in terms of high survival and normal growth for plantlets both from TIS and solid media and no direct differences could be observed between the two methods (Figure 13). Differences in size were observed between individuals, which was most likely due to natural variation.

Plants transferred to planting soil after root induction for two (D. *lutea* x *purpurea*) and four (*R. idaeus* 'Mormorshallon') days respectively, were growing nicely with healthy appearance. Even if some of the *R. idaeus* 'Mormorshallon' plants had signs of hyperhydricity, all these signs disappeared during the acclimatization period. According to Kevers et al. (2004), shoots that have hyperhydricity signs neither accomplish rooting or the acclimatization period well, and plants that manage to survive

appear abnormal. Since the rooted and acclimatized plants of this study survived and grew well, hyperhydricity was most likely not the case.

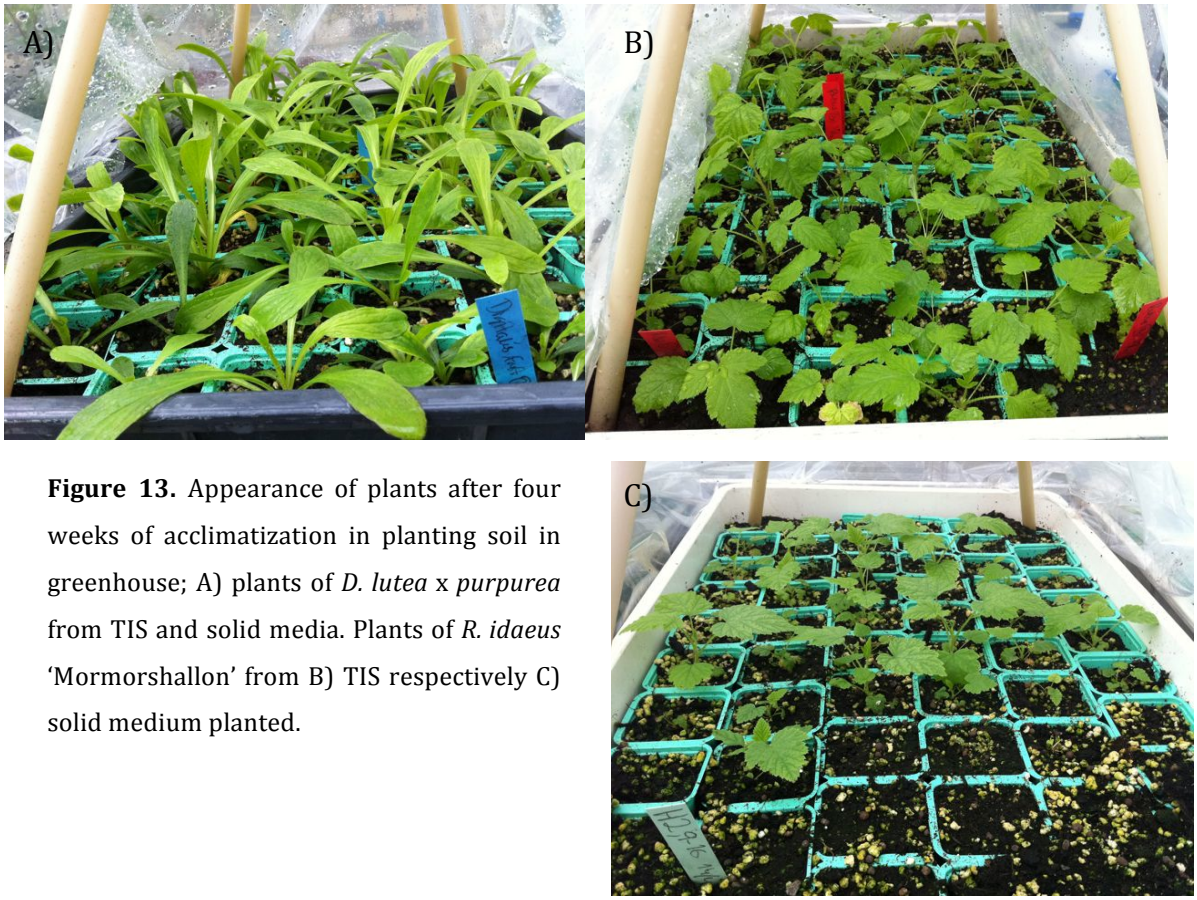


**Figure 13.** Appearance of plants after four weeks of acclimatization in planting soil in greenhouse; A) plants of *D. lutea* x *purpurea* from TIS and solid media. Plants of *R. idaeus* 'Mormorshallon' from B) TIS respectively C) solid medium planted.

The majority (98 %) of the plantlets of all species from the two systems survived after one month of acclimatization. Only five plants of *R. idaeus* 'Mormorshallon' did not survive; where one plant was originally derived from TIS medium and four plants from solid medium. These plants died in the beginning of the acclimatization period, and the death cause was actually mainly due to overwatering. In comparison with an older study dealing with acclimatization of TIS cultivated plants, similar results were obtained: No differences in plant growth were revealed for sugarcane plants (*Saccharum* spp. cv. C-1501-73) that had been propagated in either TIS or with the conventional systems (Lorenzo et al., 1998). In the review on TIS, Etienne & Berthouly (2002) also concluded that acclimatization and the following plant vigor and number of surviving individuals usually were better if the explants had been cultivated in TIS. Zhu et al. (2005) also concluded normal rooting and acclimatization of the apple rootstock M26 in TIS.

# 5 CONCLUSIONS

## 5.1 Conclusions

In comparison with solid medium, the newly developed bioreactor can be concluded to be well functioning for mass production as well as similar survival and quality of plantlets. The bioreactor is composed of only one unit, is easy to handle and occupy less space.

Medium analyzing for mineral nutrients and sugars in liquid media revealed changes that were similar for all three species. Minor changes in pH, conductivity, carbohydrate and mineral nutrient concentrations were observed over time. The only mineral nutrient that was excluded from this pattern for all three species was iron that was almost depleted in the medium. In addition, a 50% decrease in $NH_4^+$ concentration was observed in the media of *D. lutea* x *purpurea* and *E. purpurea* 'Magnus'. The slight mineral nutrient and carbohydrate decreases were confirmed accompanying a slight decrease in conductivity. Overall, the results of the medium analyses show that the bioreactor system is stable and reliable. Noteworthy is also that autoclaving of media could have great impact both on lowering of pH and breakdown of sucrose.

## 5.2 Future studies

From the revealed results, future interesting studies would be to investigate plant cultivation with lower sugar and mineral nutrient concentrations as well as higher iron concentrations with addition of $CO_2$. Expenses can be reduced if excessive medium substances can be reduced without affecting plant growth.

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