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Original Article

Possibility of Using Glass Beads as a Support Matrix for Plant Micropropagation in Temporary Immersion Bioreactors

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Abstract

Introduction: Micropropagation of plants *in vitro* using agar-free medium and bioreactors can reduce costs and make propagation by tissue culture economically feasible. The study aims to investigate the effectiveness of glass beads as a support matrix to the micro propagation of *Hibiscus rosa-sinensis* under both conventional and bioreactor culture systems.

Materials and Methods: In this study, at first, the effect of two support matrices including glass beads and agar on the micropropagation of *H. rosa-sinensis* was evaluated under a conventional culture system with Murashige and Skoog (MS) basal salt mixture including 0.5 mg/L 6-benzylaminopurine and 30 g/L sucrose. In another experiment, we assessed the application of glass beads as a support matrix in a temporary immersion bioreactor (GB-TIB), compared with the standard TIB and conventional culture systems (agar and glass beads containing vessels). For root induction, the shoots were subcultured on an MS medium containing 0.2 mg/L indole-3-butyric acids.

Results: The results indicated that the plantlets grown on the glass beads either in a conventional culture system or in TIB showed more growth in the measured parameters including shoot length, leaf number, shoot fresh and dry weight, leaf area, chlorophyll content, root length, and root fresh and dry weight compared to plantlets grown in standard TIB and agar-containing cultures.

Conclusions: The glass beads have a high potential for use in *in vitro* conditions as a good support matrix instead of gelling agents and into temporary immersion bioreactor.

Keywords: *Hibiscus rosa-sinensis*, Glass Beads, Temporary Immersion Bioreactor, GB-TIB

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Introduction

Plant tissue culture is an aseptic technique for rapid micropropagation of healthy, pathogen-free, and true to-type plants.[1] At present, several economically important plants are routinely mass-propagated in commercial plant tissue culture laboratories and their protocols are set up either by organogenesis or embryogenesis. However, this method still faces some limitations such as the time-consuming nature of the micropropagation process and high costs per produced plantlet. The major factors that lead to increased costs are labour, materials, and chemicals.[2] In addition, the cleaning, filing, and handling of many small culture vessels need more time and labour. Furthermore, some plantlets may be lost during acclimatization and transfer to soil. Efforts have been made to reduce costs and increase the quality and quantity of regenerated plantlets.[3] The two most hopeful methods are photoautotrophic micropropagation (with agar-free medium) and bioreactors. Agar is one of the expensive components, which is added as a gelling agent for solidifying the medium and preventing submerging of the explant. Different support matrices were tested as alternatives to agar in plant tissue culture, such as cassava powder, corn flour, boiled potato, and starches,[4] vermiculite and perlite,[5] plastic nets,[6] Isobgol,[7] paper pulp,[8] rice flour,[9] natural exudate gum,[10] and cast polypropylene.[11] Many of them had little success due to negative effects on explant growth, non-reusability, and other problems such as disinfection and contamination control. Hoang et al. tested four different support materials (agar, perlite, rock wool, and vermiculite) at the acclimatization phase to adapt the tissue culture plantlets of Wasabi (*Wasabia japonica* Matsumura) and found that agar and vermiculite provided the best results.[5] Another attempt to reduce costs in tissue culture is mechanization or semi-automation of the micropropagation process through bioreactors.[12-16] On this basis, further attention has been focused on the automation of different proliferation stages from explant preparation to transfer to a free environment. Plantlets propagated in a bioreactor exhibit better growth with a high survival rate during the acclimatization and transfer to soil.[17,18] A bioreactor is a modified sterile vessel that provides a controlled condition for the optimum growth of organisms like plants in a liquid medium. This system has been set up for the massive proliferation of cells, tissues, and

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somatic embryos,[19-22,14] shoots and nodal explants,[23,24,2,18] tubers,[25-28] corm,[29] and production of virus-free plants.[30] Bioreactors provide optimal conditions for dense cultures and a precisely controlled environment through regulation and enhance the performance of agitation, aeration, temperature, oxygen or carbon dioxide supply, pH, and nutrient uptake capacity.[26,31,32] A model type of bioreactor that is more competent for plant growth under *in vitro* conditions is the temporary immersion bioreactor (TIB), which is a periodic semi- or fully-automated cultivation system. The use of TIB for plant micropropagation was first reported by Takayama and Misawa (1981).[33] At present, several types of bioreactors with different designs and modifications have been introduced based on the TIB and have been used for micropropagation of several plants species such as medicinal plants,[34-37] forest trees,[38,14] fruit trees,[22,26,39-43] and ornamental plants.[44-50] The constant medium movement supplies further oxygen and nutrients to all tissues and provides optimized conditions for the rapid growth of plantlets.

Liquid culture systems provide a uniform environmental condition for growth and facilitate nutrient uptake by plant tissues. However, we sometimes need to set up a special condition to further obtain plant chemical compounds, e.g. phenolics, proteases, cardiotonic glycosides, proteins, steroids, alkaloids, and steviol glycosides.[35,51,52] Despite the advantages of bioreactors, some limitations may appear during the micropropagation process, such as hyperhydricity (with turgid, hypo-lignified, and watery tissues or vitrification), pulling the explants into the nutritional reservoir, the problem with roots growth, and non-upright growing of plantlets. Plantlets that suffer from hyperhydricity, often, produce curly, fragile, or wrinkled leaves.[1,53] One problem, which usually happens during micropropagation in temporary immersion bioreactors is pulling the explants through hoses into the nutritional reservoir, especially with callus tissues and somatic embryos. Another disadvantage of bioreactors is root and shoot growth in a horizontal position which increases abnormality and shear stress. Glass beads can be used as a semisolid bed for standing different explants and reducing the above-mentioned problems. Glass beads do not interfere with the explant and medium components, and they are easily handled, reusable, applicable to different explants, facilitate root growth, and are adaptable for use in bioreactors.

The study aims to investigate the effectiveness of glass beads as a support matrix to the micropropagation of *H. rosa-sinensis* under both conventional and bioreactor culture systems.

Materials and Methods

Plant Material and Disinfection

The nodal segments with 3 cm lengths each including one node were taken from adult field-grown Chinese hibiscus shrubs and surface sterilized using ethanol 70% for 30 sec followed by sodium hypochlorite (2.5%) for 5 min, and were then rinsed three times with sterile distilled water.

Medium Composition for Conventional Cultures

The medium was composed of the MS basal salt mixture and vitamins (Zist Arman Sabz Co. Iran) with 30 g/L sucrose containing 0.5 mg/L BAP for shoot multiplication, and 0.2 mg/L IBA for root production. The medium was dispensed into 8×12 cm glass jars with caps equipped with 0.22-µm polypropylene filters. The half of medium was solidified with 7 g/L plant agar (Duchefa Co. Netherlands) and the other half contained a liquid filled with 75 gr glass beads (3 mm in diameter) instead of agar. The glass beads had been poured into the bottom of the jars in a state that they were constantly immersed in the nutrient medium.

Bioreactor System

The bioreactor used in this study was designed based on temporary immersion bioreactors and constructed from glass materials at the Tissue Culture Laboratory of the Shahid Chamran University of Ahvaz, Iran.

The bioreactor consisted of two pumps, a digital control panel, silicone hoses (0.5 and 0.8 cm in diameter), disposable microfilters (0.22 µ), a medium reservoir (the nutrition vessel), and a culture vessel (the culture chamber). In another bioreactor, the culture vessel was filled with glass beads (3 mm in diameter) to about half its capacity, acting as a soil-like drainable matrix (Figure 1f and 2b). The liquid medium could easily flow up through the glass beads toward the explants standing upright on the surface of the glass beads. We named this type of bioreactor Glass Beads containing Temporary Immersion Bioreactor (GB-TIB). A sparger-like punched pipe was embedded in the bottom of the culture vessel inwardly opposite to the hose junction (Figures 1e, 2c). The culture vessel also had two other ports at the opposite position in the upper space near the lid for ventilation (Figure 2b). Micro pore filters (0.22 µ) were installed at each air entrance and air exist ports (Figure 1i).

After assembling the bioreactor system, it was sterilized with an autoclave and was then transferred to a sterile hood to culture the explants inside the culture vessel on the surface of the glass beads. One litter of sterile liquid culture medium containing MS medium with 30 g/L sucrose containing 0.5 mg/L BAP was added inside the medium vessel at the shoot multiplication stage, while for root production, the medium was replaced with the same medium but with a growth regulator containing 0.2 mg/L IBA.

Incubation Condition and Growth Measurement Indices

The bioreactor and all other cultures were incubated in a

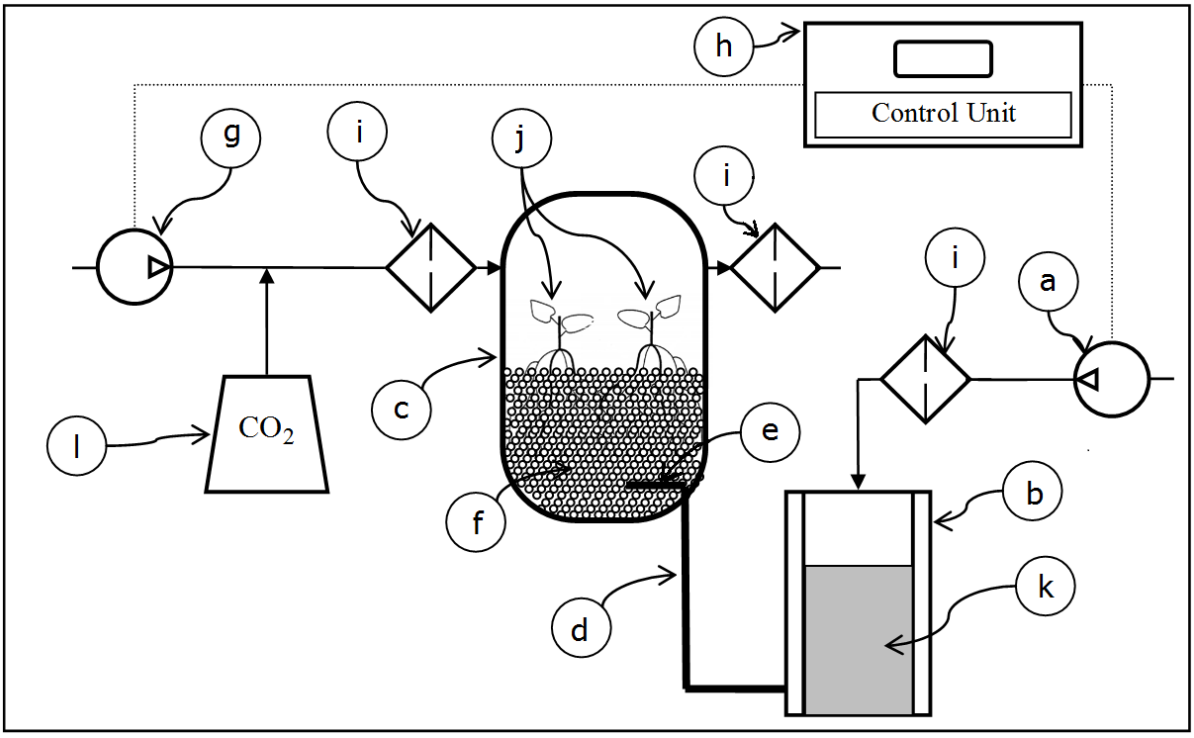

Figure 1. Schematic Diagram of the Temporary Immersion Bioreactor Containing Glass Bead (GB-TIB): **a)** nutritional pump; **b)** medium reservoir; **c)** culture vessel; **d)** silicone hose; **e)** sparger-like pipe; **f)** glass beads; **g)** air pump; **h)** control panel; **i)** 0.22 µm filter; **j)** explants; **k)** liquid medium and **l)** CO_2 generator (in this study, the bioreactor was force ventilated with ambient air, with air change rate equal to one time per each 13 minutes).

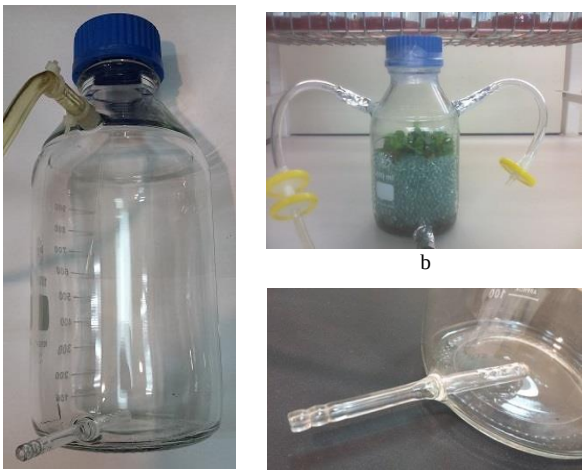

Figure 2. The Modified TIB: medium reservoir **(a),** culture vessel with plantlets grown on glass beads **(b);** sparger-like pipe of the culture vessel **(c)**.

growth room under lighting with 5,000 lux intensity for 16-hour photoperiod at 25 ± 1 °C. At the end of the experiment, some growth indices including leaf number, shoot and root length, shoot and root fresh/dry weight, leaf area, and chlorophyll content were measured and the SAS software was used for data analysis.

Results and Discussion

Effect of Glass Beads on Conventional Culture

The results of this experiment suggested that the two support matrices had significant effects on the shoot growth parameters of Chinese hibiscus. The use of glass beads as support material in the medium enhanced shoot length (207%), number leaf (117%), shoot fresh weight (178%), and shoot dry weight (147%) as compared to agar. The plantlets grown in the glass beads were more vigour and healthy compared to plantlets grown on agar solidify agent (Table 1; Figure 3).

Effect Glass Beads in TIB

The results indicate that using glass beads in the temporary immersion bioreactor significantly enhanced all the measured growth parameters compared to the plantlets grown in the standard TIB (without glass beads) and conventional agar and glass beads containing cultures. Thus, the length of the shoots in GB-TIB increased by 136%, 226%, and 381% compared to TIB without glass, agar and glass beads containing cultures, respectively. Likewise, other measured traits of the plantlets in GB-TIB, including leaf number (125%, 154%, and 300%), leaf area (114%, 150%, and 246%), shoot fresh weight (118%, 136%, and 161%), shoot dry weight (118%, 156%, and 169%), root length (138%, 202%, and 208%), root fresh weight (127%, 142%, and 101%), root dry weight (126%, 149%, and 244%) and chlorophyll content by (155%, 331%, and 369%), showed improvements compared to the plantlets in TIB without

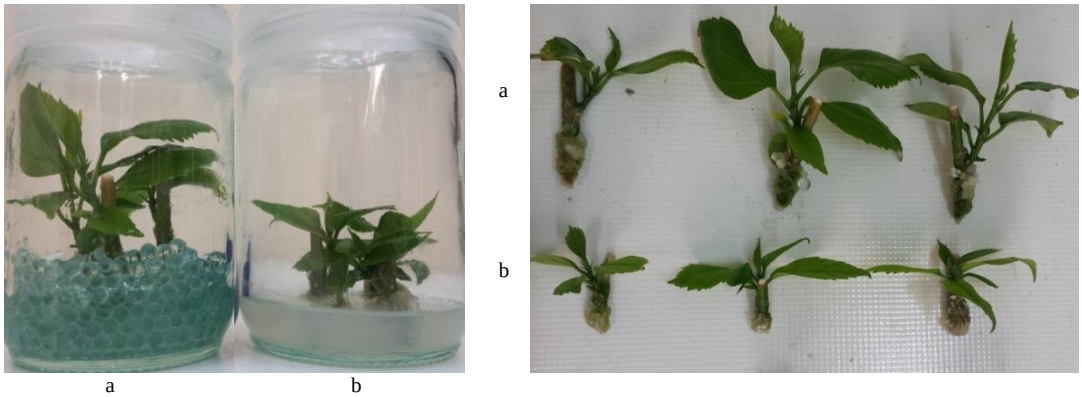

Figure 3. Shoots of *Hibiscus rosa-sinensis* produced *in vitro* after 35 days on: **a)** glass beads and **b)** agar support matrices.

Table 1. Effect of Support Matrix Type on *in vitro* Shoot Proliferation of *Hibiscus rosa-sinensis* in the Conventional Culture System

Support Matrix Type	Shoot Length (cm)	Number Leaf	Shoot Fresh Weight (g)	Shoot dry Weight (g)
Glass beads containing medium	3.21[a]	7.44[a]	0.302[a]	0.0367[a]
Agar containing medium	1.55[b]	6.33[a]	0.170[b]	0.0250[b]

Different letters indicate values are significantly different at p <0.05.

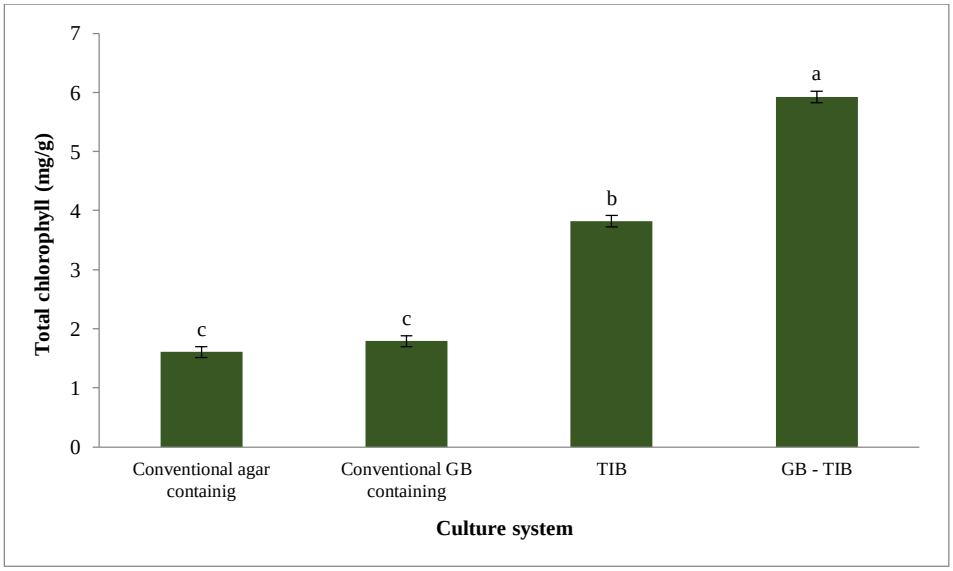

Figure 4. Effect of Culture System Type on Chlorophyll Content of *Hibiscus rosa-sinensis* Plantlets. TIB: Temporary Immersion Bioreactor; GB-TIB: Glass Bead Temporary Immersion Bioreactor.

Table 2. Effect of culture system type on *Hibiscus rosa-sinensis* plantlets growth *in vitro*.

Culture System*	Shoot Length (cm)	Root Length (cm)	Leaf Number	Leaf Area (cm^2)	Shoot Fresh weight (g)	Root Fresh weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)
Conventional agar containing culture	1.54[d]	1.33[d]	5.5[d]	354.37[d]	0.3028[d]	0.4803[c]	0.04706[c]	0.04749[c]
Conventional glass beads containing culture	2.6[c]	1.85[c]	10.7[c]	580.92[c]	0.3595[c]	0.6991[b]	0.05103[c]	0.07768[b]
TIB	4.32[b]	2.7[b]	13.2[b]	761.9[b]	0.4153[b]	0.7827[b]	0.06733[b]	0.09246[b]
TIS glass beads containing	5.87[a]	3.73[a]	16.5[a]	871.51[a]	0.4886[a]	0.9917[a]	0.07953[a]	0.11613[a]

*Agar-containing and glass beads containing culture vessels were naturally ventilated but the TIB and GB-TIB was forced ventilated. Different letters indicate values are significantly different at p <0.05.

glass beads and the plantlets in conventional agar and glass beads cultures, respectively. However, no significant differences were observed between the root fresh weight and root dry weight of the plantlets grown in TIB without glass beads and grown on conventional glass beads containing cultures, and shoot dry weight, and chlorophyll content of the plantlets grown in the agar and glass beads containing conventional cultures (Table 2; Figure 4). The plantlets grown in the GB-TIB were more vigour and healthy and successfully transferred to the soil (Figures 5 and 6). The results indicated

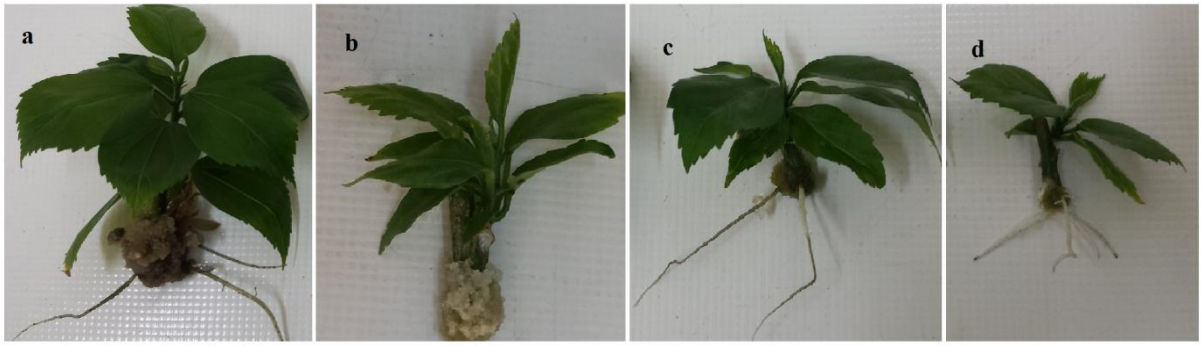

Figure 5. Plantlets of *Hibiscus rosa-sinensis In Vitro* Grown after 30 Days in Different Culture Systems: **a)** Glass bead containing temporary immersion bioreactor; **b)** Temporary immersion bioreactor; **c)** Conventional glass beads containing culture; **d)** Conventional agar containing culture.

Figure 6. Adaptation of the *Hibiscus rosa-sinensis* to *ex vitro* Condition.

that glass beads either in TIB or in the conventional cultures vessels had a positive effect on all growth parameters of the Chinese hibiscus plantlets compared to agar containing semi-solid cultures possibly due to neutralization of negative effects of agar, increased availability of nutrients for explants, and easy diffusion of the liquid medium through glass beads to the plantlet tissues. It was reported that different brands of agar caused various effects on *in vitro* plantlets growth parameters which may give different results. This is mainly due to limited diffusion of the medium components, and water, impurities, and gel firmness. Different agars have different water content (water availability), ionic composition, mineral elements (Ca, Mg, S, Mn, Fe, and Al are tightly bound to the agar), Na and Cl concentration (from seawater), sulphate content and biologically active organic compounds. Moreover, more than 30% of medium salts might be immobilized in the agar gel.[54] Therefore adding agar to a culture medium may be inducing growth disorders like chlorosis or necrosis. Furthermore, the cost of gelling agents such as agar per unit of medium is reported approximately three-fourths of the overall nutritional medium expense.[55] For these reasons a gelling agent must be selected by taking into account its clarity, texture, and toxicity.[56] Another problem with agar in the medium its possible digestion by explant exude enzymes.[57,58] The efforts made to find a suitable and cheap substitute for agar have not been successful. Guar gum even at 60 g/L could not jellify the medium and it was too soft to support the explants in the right position. Some other gelling agents such as mung bean starch (80 g/L), sago starch (30 g/L), isabgol (20 g/L), pear sago (60 g/L), cassava starch (40 g/L) and tapioca starch (80 g/L) were not viscous like agar and after increasing their concentration, they became gluey and sticky. Another probable problem with using these alternative gelling agents is increasing the phenolic compound and enzymatic browning.[59] In some cases, mixing agar with xanthan gum[60] or starch with gel rite[61] has been tested to reduce the use of agar. Ions could migrate in gels with a speed of at least 2.5 cm/81 min.[54] The porosity of agar after gelling is nearly zero.[62] Therefore, the diffusion of solutes and gaseous exchange like O_2 supply is very low compared to glass beads support matrices.

Glass beads can facilitate water and nutrient uptake by plantlets compared to semi-solid cultures.[63] Uniform particles cause the distribution of the water and solute more homogeneous with a relatively constant flowing velocity. Furthermore, the solutes flow through uniform particles decrease the turbulent and shear stress.[64] Shear stress is generated after mechanical agitation or pneumatically aeration in some bioreactors adversely affecting cell membrane integrity, growth rate, and protein and phonic bioactive compound profiles.[31] Shear stress can also reduce cell viability,[65] decrease the regeneration rate of explants, and increase oxidative stresses.[66] Glass beads in culture vessels facilitate the absorption of nutritional elements by explants in different stages of micro propagation and allow and quit air flow in the root zone. In addition, glass beads act as an artificial sterile soil so they can hold the plant in the right position and allow efficient control of the nutritional level (only in the root zone or whole parts of the plantlets).

Therefore, glass beads provide good conditions for the root system growth in terms of nutrition and aeration and also lower the light intensity without toxic effects. Moreover, much aeration leads to the quick removal of some harmful materials such as ethylene and ethanol. Growing plantlets in such conditions increase their survival after transfer to *ex vitro*. The data in this study demonstrated the superiority of the glass beads for providing suitable conditions for explant growth *in vitro* compared to agar. On the other hand, the Chinese hibiscus explants show better responses when cultured in a glass bead containing temporary immersion bioreactor (GB-TIB) compared to standard TIB and glass bead containing conventional vessels.

Generally, the increase in shoot fresh and dry weight in bioreactors can be attributed to the absorption of nutrients and hormones from different parts of the plantlet and constant air exchange through forced ventilation. Air exchange also increases the chlorophyll content in plantlets grown under TIB conditions due to the production of more mesophyll cells.[69,70] The TIB design exposes the explants to effective transmission of light and increases biosynthesis in chlorophyll and other photoreceptor pigments.[18] Low light intensity in conventional systems reduces ATP and NADPH production, thus limiting the photosynthetic mechanism and affecting numerous processes such as chloroplast biosynthesis and normal development.[71]

Enhancing plantlet quality and quantity growing traits by TIB has been reported for various plant species, such as date palm,[72,73] banana and plantain,[18,74,75] coffee,[66,67,76] pineapple,[77] eucalyptus,[53,23] strawberry and grapevine,[43,70,78] *Colocasia esculenta* L.,[79] *Stevia rebaudiana*,[80] *Corema album*,[81] *Chrysanthemum morifolium*, and *Cnidium officinale*,[70] apple,[30] plum,[82] and potato.[28]

A high survival rate was also found in plantlets grown in TIB after being transferred to *ex vitro* conditions.[41,67,84,85] The secondary xylem of the chrysanthemum stem grown under TIB conditions increased. The secondary xylem is responsible for the thicker diameter and withstanding acclimatization and transfer to soil.[70] The physiological changes during forced ventilation in leaves cause the stomata to maintain their activity.[86] Stomata of plantlets grown under conventional *in vitro* cultures on a semi-solid medium usually malfunction[87] or partially function[18] and cannot regulate water loss when transferred to *ex vitro*, whereas stomata of the plantlets grown in TIB properly function and tend to close to prevent water loss.

Moisture maintenance leads to the accumulation of more starch in the plantlet leaves during the acclimatization process and provides more energy sources during the initial adaptation days.[70] Another advantage of the TIB system that increases plantlets' survival after transfer to *ex vitro* is that the roots do not need cleaning from agar or other gelling agents. Despite all the above-mentioned morphological and physiological changes, the plants produced by TIB have high genetic stability.[18]

Frequent air exchange in GB-TIB through forced aeration reduces accumulated toxic volatiles, enhances photosynthetic capacity,[23,67] and reduces hyperhydration.[68] The advantage of the GB-TIB is its ability to induce and proliferate somatic embryos. Glass beads can act as a suitable bed for explants, even in the form of callus clumps. It was reported that TIB enhanced embryogenesis by increasing total proteins, starch contents, and alcohol dehydrogenase activity.[83] However, callus and embryo culture in standard TIB are very difficult and may not be possible due to the absence of appropriate support matrices. GB-TIB provides a suitable and neutral support that is more adapted to callus and embryo culture. The supply of adequate nutrients and frequent aeration in GB-TIB with low light intensity provide optimum conditions for root growth. The expanded root system in GB-TIB enhances the survival of the plantlets during acclimatization and the transfer to soil. Uma et al. demonstrated that TIB can enhance the growth of both primary and lateral roots. Due to the absence of a suitable support for plantlets in standard TIB and their dispersion in the liquid medium, their root systems may twist with each other, causing increased root damage and loss of plantlets after transfer to *ex vitro*. In GB-TIB, root growth and distribution will continue without the twisting problem. Furthermore, the root systems grown in GB-TIB are more similar to their natural habitat with good morphology and a high uptake rate. On the other hand, pulling out the plantlets from GB-TIB to transfer to *ex vitro* conditions for adaptation is easier, with less stress and damage. Simplifying the regulation of culture conditions at the time of operation is another advantage of TIBs, mainly with GB-TIB.[32] Furthermore, plant regeneration can be easily scaled up using GB-TIB even when the plants are less responsive to somatic embryogenesis.[67,88] GB-TIB, with new modifications compared to TIB, potentially has high performance for plant micro-propagation and makes automation of plant tissue culture more feasible. The micro propagation of plants by GB-TIB has the advantages of other bioreactors due to the suitable properties of glass beads, such as not adversely affecting the explant, reusability, good permeability for liquid culture medium, facilitating aeration and gas exchange, and the possibility of automation. Thus, GB-TIB can be used on a large scale as an important and easy method.

Conclusion

Following the findings of this study, glass beads have a high potential for use in *in vitro* condition as a good support matrix instead of gelling agents. Therefore, we recommend using glass beads in various culture systems such as conventional culture vessels, bioreactors, and photoautotrophic micro propagation. The glass beads temporary immersion

bioreactor (GB-TIB) has more advantages such as no hyperhydricity, low shear stress and low cost. Therefore, this type of bioreactor can be used not only for micro propagation of Chinese hibiscus but also for tissue culture automation.

Authors' Contributions

MM conceptualized the research and designed the experiments. MM and FF conducted the research procedure. MM, FF, and MC conducted the statistical analysis and drafted the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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