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Temporary Immersion Bioreactor System as an Efficient Method for Mass Production of In Vitro Plants in Horticulture and Medicinal Plants

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Abstract: A temporary immersion system (TIS) bioreactor has been used as an efficient and cost-effective method for the in vitro propagation of many plant species. In the current study, the applicability of a TIS bioreactor for plantlet regeneration *Chrysanthemum morifolium* Ramat., *Fragaria* × *ananassa* Duch., and *Cnidium officinale* Makino was studied. Shoot length, a number of leaves per regenerated shoot, fresh, and dry biomass of plantlets were optimal with the TIS compared to semi-solid and liquid immersion cultures. The leaf area in cryshanthmum, strawberry, and *C. officinale* were 2.87 cm^2, 3.51 cm^2, and 1.43 cm^2, respectively, in the plants regenerated by TIS. The photosynthetic pigments were highest in strawberry plants grown in TIS bioreactor culture, and there was no significant difference between semi-solid and liquid culture while the highest values were obtained in *C. officinale* maintained in semi-solid culture. The chrysanthemum and strawberry plants showed a 100% acclimatization rate in all culture systems. *C. officinale* plants showed the highest survival rate at 96.9%, which were regenerated in the TIS. TIS bioreactor culture, thus, provides a convenient method that could be adopted for commercial in vitro propagation of chrysanthemum, strawberry and *C. officinale* plants.

Keywords: acclimatization; immersion culture; regeneration; semi-solid culture; temporary immersion system

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1. Introduction

In vitro culture of the plant is an effective technique for producing genetically homogeneous plants in horticulturally important species [1–3]. By optimizing in vitro culture conditions, millions of elite clones can theoretically be produced from the mother plant within a year [1]. It is well known that the use of a semi-solid medium using conventional agar increases production costs by the gelling agent during commercial mass propagation and limits the possibility of automation; thus, it is the main reason to increase the cost of in vitro culture products [4,5]. To overcome these bottlenecks of the conventional method of in vitro culture, a bioreactor culture method using a liquid medium was introduced. Since the bioreactor uses a liquid medium, undesirable physiological phenomena such as hyperhydricity may occur in the cultured plants depending on the species and culture methods [5]. Temporary Immersion System (TIS) was introduced to the bioreactor culture to supplement the immersion culture method of bioreactor culture. This system is a method of reducing hyperhydricity of plants and producing physiologically healthy plants by repeating the period in which the liquid medium ebb and flood the bioreactor for a certain

period of time by a timer and solenoid valve [5]. In this system, plantlets are immersed in the medium only temporarily to provide nutrients for growth, and the immersion period is followed by a drying period, thereby reducing hyperhydricity.

Since Alvard et al. [6] used TIS for the first time on banana (*Musa* AAA cv. Grand Naine) micropropagation, many studies using TIS have been reported to overcome the limitations of semi-solid culture and liquid culture [7–11]. TIS is a system in which cells, tissues, and organs of plants are immersed in a liquid medium for a specific time period, semi-automatically using a bioreactor [12]. This system increases the growth rate of plants in many species by improving the ventilation of the culture vessel [13]. In addition, because of these advantages, plants produced in TIS promote physiological processes such as photosynthesis, respiration, chlorophyll development, and stomata function so that they can adapt well to the ex vitro environment during the acclimatization [14].

TIS developed to date are Temporary Immersion (RITA®), Temporary Immersion Bioreactor (TIB®), Ebb-and-Flow bioreactor [15,16], Monobloc Advance Temporary Immersion System (MATIS® [17]), and SETIS™ [12]. Among various TIS bioreactors, SETIS ™ has the advantage that the size of the culture vessel is relatively large, about 6 L, and easy to handle. Kim et al. [5] successfully produced virus-free apple plantlets (*Malus domestica*) in TIS, and it was reported that sweet cherry (*Prunus ovatum*) and mlalanga (*Colocasia esculenta*) also showed excellent growth of plantlets in this system [18,19].

Chrysanthemum (*Chrysanthemum morifolium*) is one of the most popular potted and herbaceous landscape plants worldwide. In vitro culture of chrysanthemum is necessary for virus-free plant production and mass propagation of new varieties [20]. Strawberry (*Fragaria* × *ananassa* Duch.) is also a crop that has vegetative propagation, such as chrysanthemums, and it is necessary to mass produce excellent individuals by using in vitro culture [21]. In addition, *Cnidium officinale* (synonym: *Ligusticum officinale* (Makino) Kitag.) is a medicinal plant that grows with rhizomes (Cinidii Rhizoma). The plants produce a flower, but if the plant does not commonly produce seeds because of the incompatibility with chromosomes, it is necessary to produce healthy plantlets by using in vitro culture [22,23]. Therefore, the production of healthy plantlets of these crops is of great commercial importance. In addition to the importance of these three kinds of species in the horticultural industry, each of these plants has different growth characteristics (i.e., chrysanthemums that grow stems in nodes, strawberries that grow as runners on root leaves, and *C. officinale* that has rhizomes on root leaves); thus, these plants were suitable for the application of plant production in the TIS bioreactor. Strawberries have been studied by Hanhineva et al. [24] in the RITA® system to regenerate a shoot from leaf explants, and chrysanthemums have been studied by Paek et al. [25] for micropropagation with TIS. However, most of the research focuses only on plant growth while studies related to acclimatization are insufficient. Therefore, in this study, the efficiency of the TIS bioreactor was compared by using semi-solid and liquid cultures using chrysanthemum, strawberry, and *C. officinale*. In addition, through histological analysis and acclimatization of the plants produced from this system, the optimal system was investigated for the production of plantlets.

2. Materials and Methods

2.1. Plant Material and Culture Conditions

In the present experiment, in vitro plantlets of chrysanthemum 'Golden bel,' strawberry 'Seolhyang,' and *C. officinale* were subcultured at 4-week intervals in MS medium [26] containing 30 $g \cdot L^{-1}$ sucrose and maintained for one year before the use in the present study. Chrysanthemum was prepared as a 2 cm long explant including one node with two leaves, and strawberries and *C. officinale* were prepared as a 2 cm long plantlet including two leaves and used in the experiment. According to the in vitro culture method, explants were cultured using a semi-solid culture, a liquid stasis culture, and a temporary immersion system bioreactor, respectively. The TIS (SETIS ™ bioreactor, VERVIT, Zelzate, Belgium) was used for temporary immersion bioreactor culture.

The TIS consisted of an upper container (5.5 L) capable of cultivating plantlets and a lower container (4 L) with a liquid medium. The lower and upper containers are connected by a silicone hose, and the filter (0.22 µm pore size, VERVIT, Zelzate, Belgium) is attached to each area where air enters and exits to maintain sterilization. At this time, the air injection volume is 0.1 vvm (aeration volume/medium volume/minute), and the timer is set so that the plantlet is immersed by the liquid medium for 10 min every 3 h.

In the current experiment, all plants were cultured in MS medium to which no plant growth regulator was added, and 0.8% (w/v) of agar was added for semi-solid culture. TIS added 1 L of the medium, and semi-solid and liquid cultures were added at 100 mL medium to 500 mL in volume plastic containers (SPL, W 120 mm × H 80 mm, Pocheon, Korea). In liquid culture, a plastic net was used to fix the explants. Culture density was set to culture plantlets of 70 individuals per 1 L of the medium in all experiments. Medium pH was adjusted to 5.8 before sterilization and autoclaved at 121 °C for 15 min.

All plants were cultured under white light-emitting diodes (LED, 400–700 nm, PSLED-1203) with a light intensity of 80 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and under the 16 h photoperiod. The cultures were maintained for 6 weeks in a culture room at 25 ± 1 °C, and then the plants harvested in each culture method were examined for growth such as plant height, number of leaves, and root length and sampled for analysis.

2.2. Cytological and Histological Analysis

Cytologic analysis of the leaves of harvested plantlets was peeled 3 min after manicure was applied in the middle area between the central vein and the edge of the leaf at the front and back of the leaf. The peeled manicure section (hardened film strip) was observed with an optical microscope (Olympus BX40, Tokyo, Japan) to measure the size of the epidermal cells (version 4.5, Leica Application Suite, Wetzlar, Germany).

For histological analysis of chrysanthemum, the stem segments were embedded in 3% agar solution, then solidified, and cut thinly with a razor blade. Agar pieces containing stems were stained with Toluidine blue O (0.05% in water, pH 4.4 buffered with 10 mM Na-acetate) for 10 min. The cut surface of the dyed stem was observed with an optical microscope (Olympus BX40, Tokyo, Japan), and the diameters of cortex, pith, and secondary xylem tissue were examined according to Yeung's method [27].

2.3. Chlorophyll and Carotenoid Content Measurement

The content of chlorophyll a, b, and carotenoids of in vitro cultivated plant leaves of the three species was analyzed. Around 25 mg of the third leaf cut from the plantlet, and then immersed in 6 mL of 80% (*v/v*) acetone, and maintained for 48 h in a cool and dark state. The absorbance of the supernatant was measured at 645 and 663 nm with a spectrophotometer (Libra S22, Biochrom Ltd., Cambridge, UK). Total chlorophyll content and carotenoid content were calculated according to the method described in Lichtenthaler et al. [28].

2.4. Acclimatization of Harvested Plants from TIS

In order to evaluate the effect of the culture system on the acclimatization of chrysanthemum, strawberry, and *C. officinale* plants, 30 plantlets were collected from each culture system and then planted in 42 pore floral foams in plastic trays. The plants were watered once every 3 days during the 30 days of the acclimatization process. Acclimatization was maintained with a 300 µmol m^{-2} s^{-1} PPFD in an external environment of 25 ± 2 °C. After 4 weeks, the plant length, number of leaves, and the number and length of roots (≥0.5 cm) were evaluated.

2.5. Statistical Analysis

All experiments were repeated 3 times in a completely randomized. Data are expressed as mean ± standard error. Statistically significant differences were determined using Duncan's multiple range test with SAS (SAS10, Cary, NC, USA).

3. Results and Discussion

3.1. Plant Growth in Different Culture Systems

The comparison of plant growth showed significant differences among the three culture systems in all plant species (Figures 1–3). Chrysanthemum plants had the highest fresh weight of 2.75 g and 0.44 g of dry weight per explant in TIS culture, 2.9 times of fresh weight compared to semi-solid culture, and 2.2 times of liquid culture. Strawberry plants also showed the best growth in TIS culture with 1.10 g (semi-solid four times, liquid three times) of the fresh weight per explant and 0.17 g of the dry weight per explant, and *C. officinale* also showed the best growth with 0.85 g (semi-solid 8.1 times and liquid 2.3 times) of the fresh weight per explant. In the shoot growth, the highest shoot length was obtained in the plants grown in the TIS in all three species, chrysanthemum (10.6 cm), strawberry (10.8 cm), and *C. officinale* (8.6 cm), and the number of leaves was also the highest in TIS in all three species. In root length, the plants grown in TIS and liquid culture showed longer roots than semi-solid culture ones in chrysanthemum and *C. officinale*, but the root of strawberry plants showed the best development in TIS treatment, and the highest number of roots was obtained in TIS in all three species.

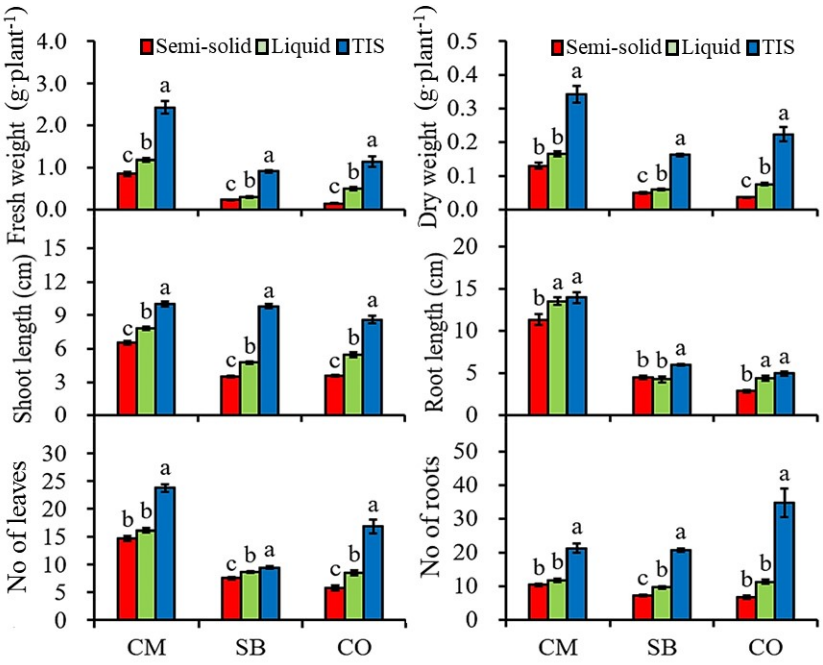

Figure 1. Fresh weigh, shoot length, and leaf number of chrysanthemum (CM), strawberry (SB), and *Cnidium officinale* (CO) plants after 4 weeks in semi-solid, liquid, and TIS cultures. Bars with different letters differ significantly from each other by Duncan's multiple-range test (DMRT; $p \leq 0.05$).

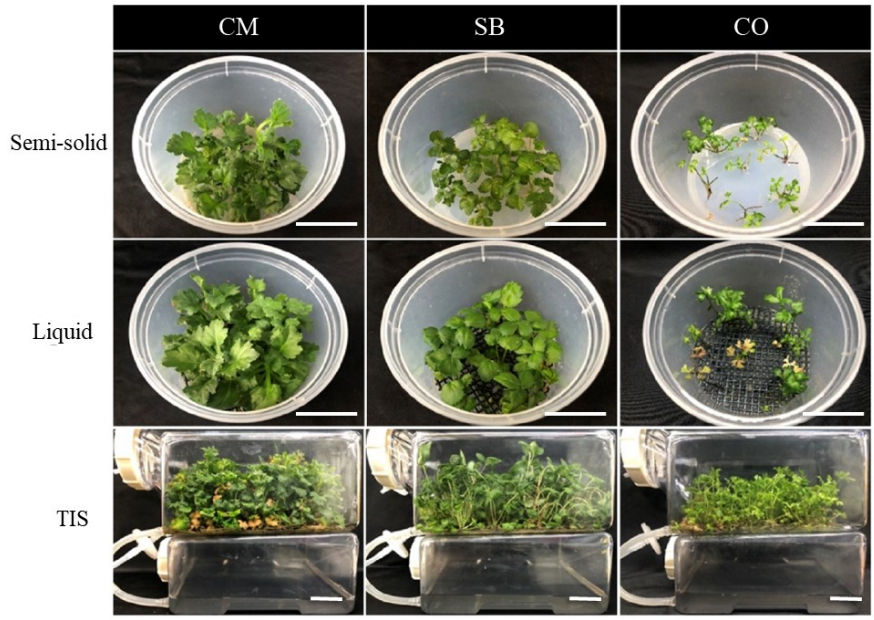

Figure 2. Chrysanthemum (CM), strawberry (SB), and *Cnidium officinale* (CO) plants after 4 weeks in semi-solid liquid and TIS cultures. Scale bars = 5 cm.

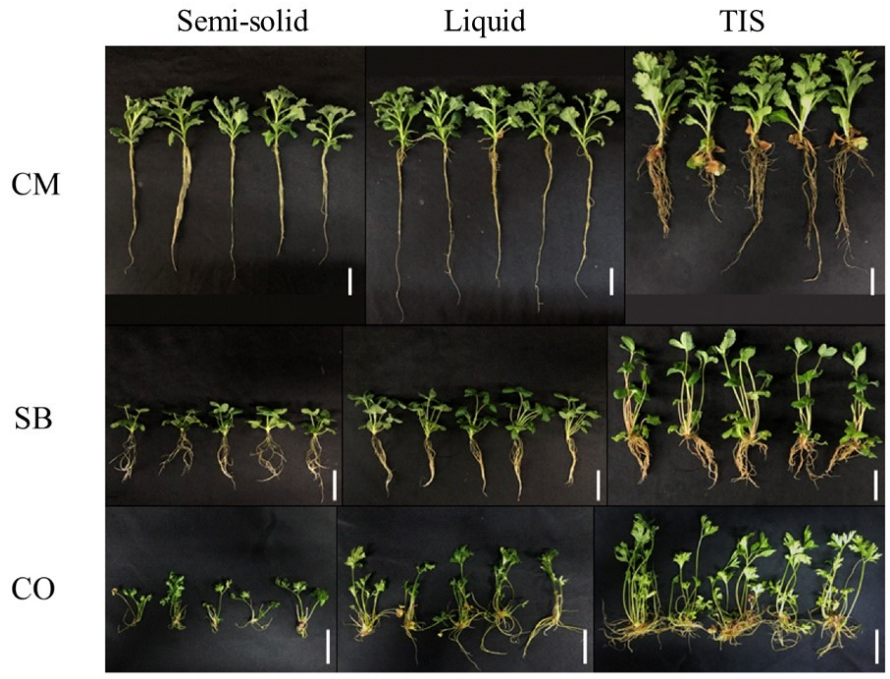

Figure 3. Harvested plants of chrysanthemum (CM), strawberry (SB), and *Cnidium officinale* (CO) plants from semi-solid, liquid, and TIS cultures. Scale bars = 3 cm.

The TIS culture combines the advantages of immersion and dry periods to maximize gas exchange and increase the absorption of nutrients [5,29]. Plants in the in vitro culture absorb nutrients not only through roots but also through leaves [30]. Under TIS, when plants are immersed in the medium, they are exposed to the medium from the entire epidermis, allowing them to absorb nutrients [5,29]. Therefore, it is thought that the growth and proliferation rate of plantlets grown in TIS was superior to that of plants grown in semi-solid or liquid culture. In addition, one of the main advantages of TIS is that volatile compounds such as ethylene are removed by promoting ventilation in the culture vessel by forcing aeration. Through this, carbon dioxide required for photosynthesis

can be resupplied [29]. Compared to TIS, semi-solid and liquid cultures are thought to have poor growth because there is no exchange of gas inside or outside the culture vessel. When pineapple (*Ananas comosus* L. Merr) was cultured in the semi-solid, liquid, and TIS bioreactor, the highest growth of plants was obtained from TIS [8]. According to studies of Thi et al. [30], when carnations (*Dianthus caryophyllus* cv. Dreambyul) were cultured in the TIS (RITA®), their fresh weight was nine times higher than in semi-solid cultures. RITA® bioreactor used in a carnation study by Thi et al. [31] consists of 1-L cylindrical vessels and two compartments: an explant culturing container and medium conservation container; thus, the volume of the container for explant culturing is about 0.5 L, which is relatively small compared to the SETIS (6 L, explant culturing container) used in this study. Therefore, the forcing aeration of the TIS bioreactor and widening the contact with the liquid medium were considered as important factors to promote the growth of the shoot and root of many horticultural crops.

3.2. Cytological and Histological Analysis

As a result of examining the area of three species of plant leaves cultured in a three-types of culture system, chrysanthemum (2.87 cm^2), strawberry (3.51 cm^2), and *C. officinale* (1.43 cm^2) all had the largest leaf area in TIS, and the leaf area of plants grown in this TIS increased by 1.4 times, 2.8 times, and 3.6 times, respectively, compared to plants grown in semi-solid culture in each plant species (Figure 4B,C). As a result of examining the leaf cell size through a microscope, the area of epidermal cells was the largest in TIS in chrysanthemum (2986.8 μm^2) and *C. officinale* (1610.8 μm^2), which were 2.2 times and 2.0 times larger, respectively, compared to plants grown in semi-solid medium. However, while the leaf area of strawberry plants increased 2.8 times in TIS compared to semi-solid culture, interestingly, the area of epidermal cells was not significantly different from that of plants grown in semi-solid medium (Figure 4A,C).

Microscopic observation of in vitro grown plants showed that the reason why the size of the leaves in chrysanthemum and *C. officinale* increased is due to the increase in epidermal cell area. In contrast, strawberry plants confirmed that the number of cells increased and not the size of epidermal cells. Histological observations were performed on the stems of chrysanthemum plants cultured in three different culture systems (Figure 5A). The lengths of the chrysanthemum stem pith (0.69 mm), secondary xylem (0.27 mm), and cortex (0.28 mm) were the longest plants regenerated in TIS (Figure 5B). The stem diameter was one of the most important features showing a significant difference in the growth of chrysanthemum plants cultured in TIS when compared to the control. The development of the secondary xylem in TIS was the best, which was responsible for the diameter of the stem being thicker than the control. Plants grown in TIS culture have a good development of secondary xylem tissue, which can withstand acclimatization stress better than those produced in semi-solid or liquid culture and, thus, increase the plant's survival rate when acclimatization occurred [32,33].

3.3. Chlorophyll and Carotenoid Contents

The contents of chlorophyll and carotenoid content in the leaves of the plantlets harvested in the three culture systems showed significant differences between the culture methods for strawberries and *C. officinale*, but there was no difference between treatments for chrysanthemums (Figure 6). The photosynthetic pigments, Chl a (18.2 $mg \cdot g^{-1}$ FW), Chl b (6.7 $mg \cdot g^{-1}$ FW), and carotenoid (4.5 $mg \cdot g^{-1}$ FW), were the highest in strawberries grown in TIS culture, and there was no significant difference between semi-solid and liquid culture while the highest values were obtained in *C. officinale* maintained in semi-solid culture (Figure 6). It was interesting that strawberries had the highest chlorophyll content per unit weight in the TIS system, which had the largest leaf area, while *C. officinale* had the highest chlorophyll content in the plants grown on semi-solid culture, which had the smallest leaf area.

The opposite results obtained from strawberry and *C. officinale* in the relationship between chlorophyll content and leaf size could be interpreted by the differences in the cell size between the two species. In the case of strawberries, plants had a similar cell size in all cultures systems regardless of the leaf area, whereas *C. officinale* had a positive correlation in cell size and leaf area and small cell size in the semi-solid culture with a small leaf area. Therefore, in the case of *C. officinale*, the cell size and area of the leaf were the smallest cultured in semi-solid medium; thus, more mesophyll cells might be contained than other two treatments when the same fresh weight of leaf samples was taken for chlorophyll content analysis. It might have resulted in high chlorophyll content as single mesophyll cells containing a similar number of chloroplasts.

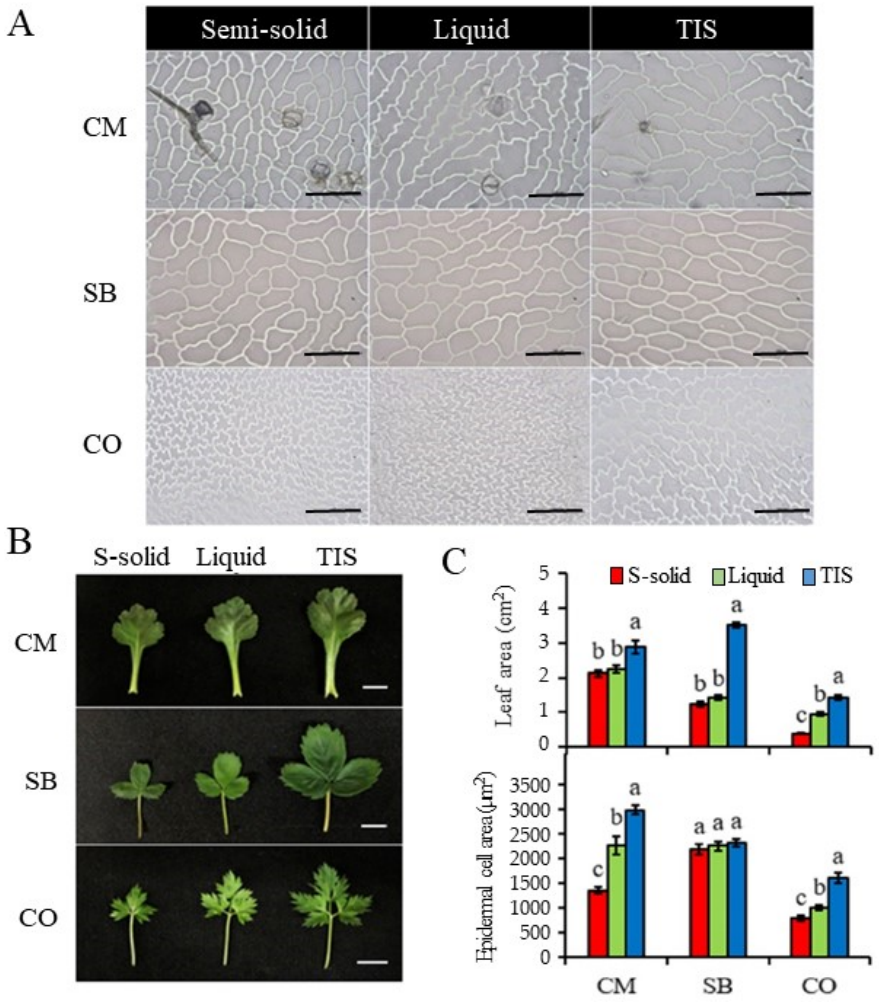

Figure 4. Cytological analysis of chrysanthemum (CM); strawberry (SB); and *Cnidium officinale* (CO) plants grown in semi-solid, liquid, and TIS cultures. (**A**) Cytological observation of adaxial leaf surface of CM, SB, and CO plants grown in semi-solid, liquid, and TIS cultures (scale bar = 100 μm). (**B**) Morphological observation of leaves of CM, SB, and CO plants grown in different culture systems (scale bar = 1 cm). (**C**) Leaf area and epidermal cell area of CM, SB, and CO plants grown in semi-solid (S-solid), liquid, and TIS cultures. Bars with different letters differ significantly from each other by Duncan's multiple-range test (DMRT; $p \leq 0.05$).

The increase in the photosynthetic pigment of strawberry leaves grown in TIS culture can be seen as an increase in photosynthesis efficiency due to gas exchange in the bioreactor [13]. A recent study of Matuszkiewicz et al. [34] using Arabidopsis showed that the plants cultured in ventilated culture sealed with permeable tape enhanced efficiency of photosynthesis and decreased ethylene production by decreasing the stress marker

compared to the ones cultured in air-tight sealed plates. The results clearly indicate that stress is greater in conventional culture.

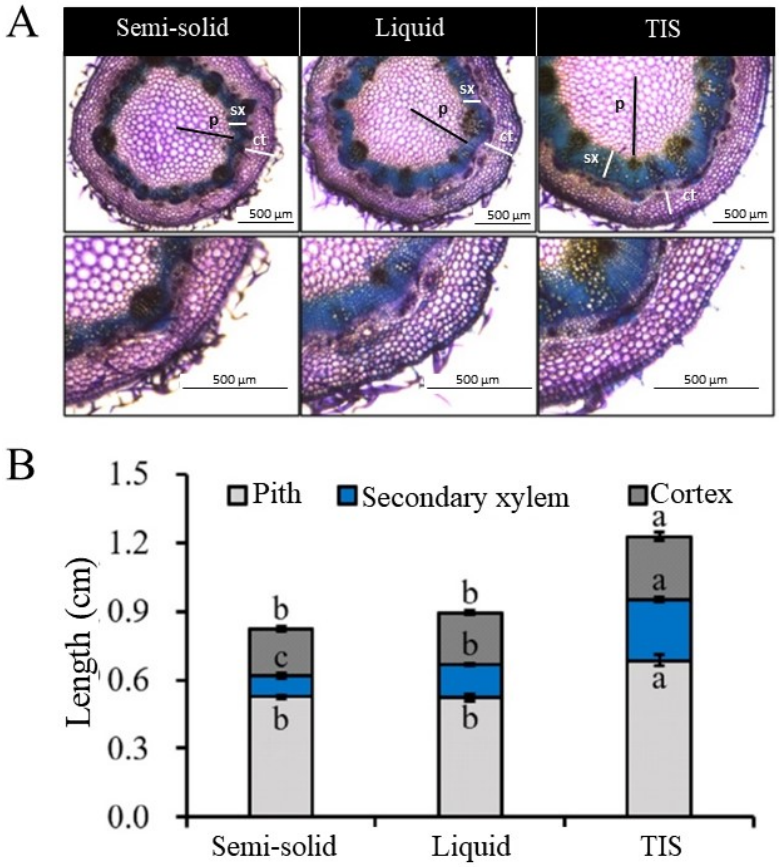

Figure 5. Histological analysis of chrysanthemum plants grown using semi-solid, liquid, and TIS cultures. (**A**) Histological observation of chrysanthemum stems in semi-solid, liquid and TIS cultures (p: pith, sx: secondary xylem, ct: cortex). (**B**) Stem diameter of chrysanthemum plants in semi-solid, liquid and TIS cultures. Different letters differ significantly from each other by Duncan's multiple-range test (DMRT; $p \leq 0.05$).

3.4. Acclimatization of Three Horticultural Species

As a result of investigating growth after 4 weeks of transplanting three types of plants cultured in three different systems into floral foam, chrysanthemum and strawberry plants showed 100% acclimatization rate in all culture systems. *C. officinale* plants showed the highest survival rate at 96.9%, which were regenerated in the TIS, whereas 89% and 86% of plants survived, which were regenerated in semi-solid and in liquid cultures, respectively (Table 1). In all three plant species, above-ground growth, such as fresh weight, dry weight, length of the above-ground part, and a number of leaves, was the best in the plants produced in TIS (Table 1). Zobayed et al. [35] reported that the histological and physiological changes in leaves, including stomata, are closer to ex vitro plants by ventilating the culture container during in vitro cultured plantlets. In this study, the TIS is ventilated culture condition; thus, the plants cultured in TIS might have received relatively less stress during the acclimatization process compared to other cultures, which is interpreted as affecting improved growth.

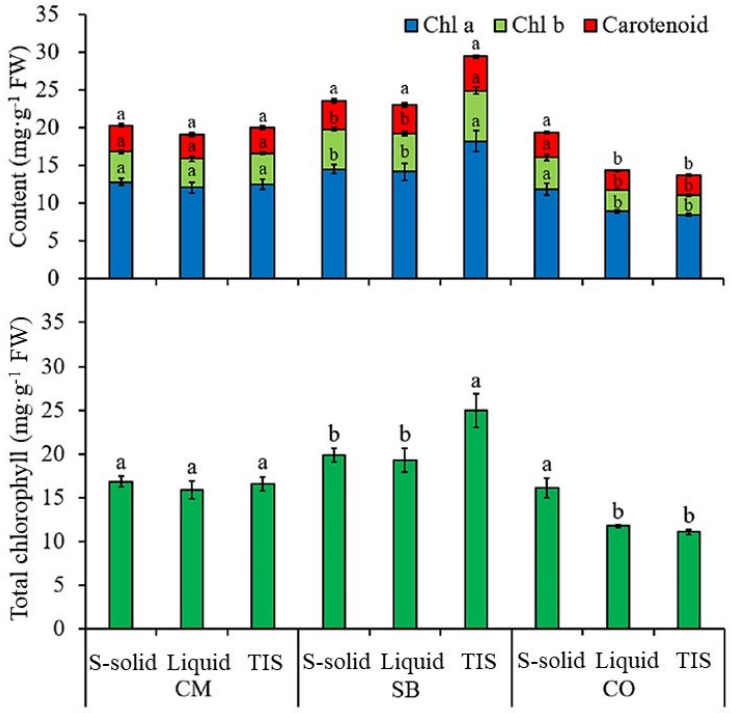

Figure 6. Chlorophyll a, Chlorophyll b, and Carotenoid content of in vitro grown chrysanthemum (CM), strawberry (SB) and *Cnidium officinale* (CO) plants grown in semi-solid (S-solid), liquid, and TIS cultures. Bars with different letters differ significantly from each other by Duncan's multiple-range test (DMRT; $p \leq 0.05$).

Table 1. Comparison of different culture systems on growth of chrysanthemum (CM), strawberry (SB) and *Cnidium officinale* (CO) plantlets after 4 weeks of acclimatization.

Species	Culture	Fresh Weight (g)	Dry Weight (g)	Shoot Length (cm)	No. of Leaves/Plantlet	Root Length (cm)	No. of Roots/Plantlet	Survival Rate (%)
CM	Semi-solid	6.82 b [z]	0.69 b	5.03 b	10.80 b	5.30 a	19.70 a	100 a
	Liquid	6.11 c	0.62 c	4.99 b	10.00 b	4.53 b	14.30 b	100 a
	TIS	7.84 a	0.91 a	5.79 a	14.20 a	5.13 a	14.10 b	100 a
SB	Semi-solid	3.01 c	0.67 c	4.87 c	8.80 a	4.62 a	8.20 b	100 a
	Liquid	4.32 b	0.79 b	6.83 b	7.50 b	3.77 a	8.60 b	100 a
	TIS	7.11 a	0.14 a	8.65 a	8.30 a	4.36 a	10.50 a	100 a
CO	Semi-solid	1.75 b	0.27 b	5.32 b	5.20 b	2.33 a	6.80 a	89.0 b
	Liquid	1.92 b	0.30 b	6.32 b	5.60 b	2.52 a	6.20 a	86.0 b
	TIS	4.42 a	0.74 a	8.14 a	7.70 a	2.56 a	8.60 a	97.0 a

[z] Different letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range test.

Unlike strawberry and *C. officinale* plants, which are radical leaf plants, chrysanthemum plants were grown by elongating nodes, and the plants were cut into 3 cm lengths (2~3 nodes) and acclimatized. Therefore, 30 individuals produced in semi-solid, liquid, and TIS increased to 44, 48, and 59, respectively, during the acclimatization process (Figure 7). These results reveal that TIS culture is more advantageous not only in the growth and development of the plants but also in their multiplication during the process of acclimatization. In particular, great effects on the mass propagation of plant species such as chrysanthemums, which grow faster and increase in their nodal length. Successful micropropagation depends not only on the number of shoots propagated from the explants but also on the morphological quality and vitality of the plants produced [36]. Therefore, acclimatization is an important step in determining the success of microreproduction [37]. According to a study by Martínez-Estrada et al. [29], *Anthurium andreanum* plants cultivated in TIS showed

more accumulation of photosynthetic products and a higher percentage of closed pores than plants cultivated in a semi-solid medium so that acclimatization could be improved. In this regard, Yang and Yeh [38] reported that the *Calathea orbifolia* plants produced in TIS had higher photosynthetic rates during the ex vitro acclimatization process and that the leaf area and fresh weight were higher than those cultured in a semi-solid medium. Similarly, Aragón et al. [14] reported that TIS-derived plants showed better growth and control of pore function in plantain (*Musa* AAB) plants. In addition, in this study, it is said that plants grown in a ventilated TIS have less moisture loss and can accumulate more starch in the leaves upon acclimatization. Since the accumulated starch can be used as an energy source during the first few days of ex vitro adaptation, it can be seen that plants produced in TIS are advantageous for acclimatization.

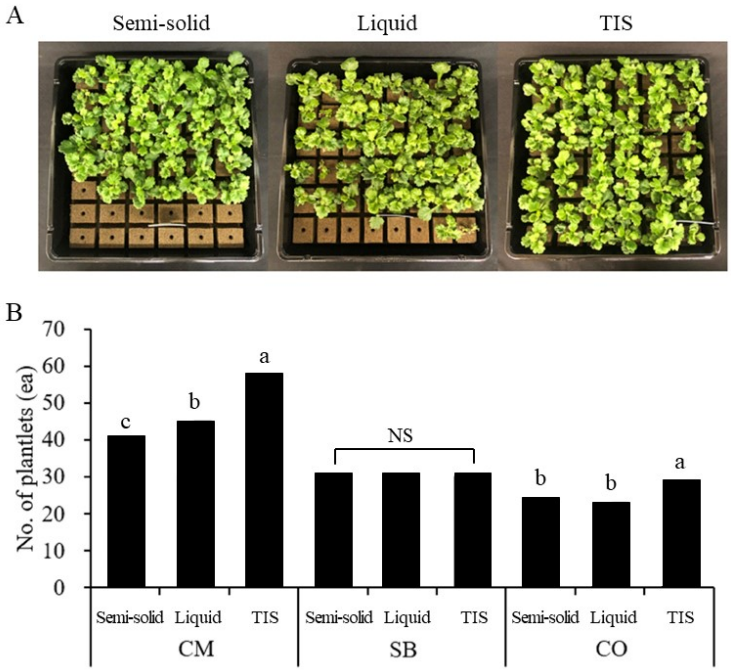

Figure 7. Acclimatization of in vitro grown chrysanthemum (CM), strawberry (SB), and *Cnidium officinale* (CO) plants in TIS and semi-solid and liquid cultures. (**A**) Acclimatized chrysanthemum plants after 4 weeks harvested from semi-solid, liquid, and TIS cultures. (**B**) Number of acclimatized plants after acclimatization of semi-solid, liquid, and TIS cultures. NS: no significance. Different letters differ significantly from each other by Duncan's multiple-range test (DMRT; $p \leq 0.05$).

4. Conclusions

The results of current experiments have demonstrated that TIS is an efficient system for mass propagation of plantlets as compared to the conventional in vitro regeneration systems such as semi-solid culture and liquid stationary cultures. Regeneration, growth, and accumulation of biomass by the plantlets were optimal in TIS. Histological observations have revealed that the excellent growth of regenerated plants in TIS was a result of an increase in cell size. In particular, chrysanthemum showed that the development of the secondary xylem from the stem is essential for the survival and growth of plants upon ex vitro transplantation. The results of this study showed that the TIS can be an optimal system for mass production of regeneration of ornamentals, crops, and medicinal plants.

Author Contributions: H.-D.H. and S.-H.K. contributed to data acquisition, experiment, and wrote the manuscript. S.-W.Y. and S.-S.P. participated in data interpretation and H.N.M. participated in editing of the manuscript. S.-Y.P. made substantial contributions to data interpretation, revised the manuscript, and the conception and design of this study. All authors have read and agreed to the published version of the manuscript.

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