Chapter 9

Multiplication of *Chrysanthemum* shoots in bioreactors as affected by culture method and inoculation density of single node stems

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Abstract: Single node cuttings (1 cm in length) of *Chrysanthemum* were cultured on gelled and liquid media to compare shoot multiplication efficiency. Liquid culture resulted in greater fresh weight, dry weight, shoot length and leaf area compared to gelled culture. Shoots from liquid culture grew vigorously without hyperhydricity, showing 100% *ex vitro* survival. To determine optimal inoculation density of single nodes in a bioreactor, different numbers of single nodes (20 or 40 or 60 or 80) were placed into a 10-litre column-type bioreactor. Shoot length was greatest at the 80-node inoculation, with the least number of branches, indicating the best inoculation density tested for shoot multiplication in bioreactors. In the final experiment, single-node cuttings in bioreactors were treated with three different culture systems: ebb and flood, deep flow technique (DFT) culture and immersion. Results indicated that the DFT culture led to the greatest fresh weight, shoot length and leaf area, followed by the ebb and flood culture, while the immersion culture suppressed shoot multiplication due to the lack of oxygen and the high water potential. Our results suggested the possibility of large-scale production of *Chrysanthemum* shoots in bioreactors.

Key words: deep flow technique, ebb and flood culture, immersion culture, gelled culture

Abbreviations: DFT – deep flow technique; DW – dry weight; FW – fresh weight; PPF - photosynthetic photon flux; vvm – volume of gas per volume of liquid per minute

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1. Introduction

Chrysanthemum is one of the most popular cut flowers, cultivated in the largest area in South Korea (Hahn, 1998). Plant multiplication has mostly been *via* cuttings, but this method has a low multiplication rate and often the plants are of low quality. Because cuttings are obtained repeatedly from mother plants, they may be subjected to any virus infection and degeneration, thereby increasing production costs (Hahn et al., 1998; Kim, 2001).

These problems have been solved by applying micropropagation methods, which are routinely applied to the clonal propagation of a variety of horticultural plants including *Chrysanthemum* (Ben-Jaacov and Langhans, 1972; Earle and Langhans, 1974a, 1974b; Pierik, 1988). Once disease-free shoots are obtained through shoot tip cultures, a large number of nodal cuttings can be obtained within a short period, thus showing the advantages of high multiplication rate and plant uniformity compared with conventional vegetative propagation *via* cuttings (Chu, 1992; Debergh and Read, 1991).

Although there have been advances in micropropagation systems through improvement of the microenvironments (Kozai et al., 1992), there are still problems to be solved. In general, micropropagation has been done through conventional gelled culture systems using small-scale culture vessels where sucrose concentrations and relative humidity are extremely high, and CO_2 concentrations and photosynthetic photon fluxes (PPF) are low. As a result, conventional micropropagation requires is costly and has a relatively low multiplication efficiency (Bi et al., 1997).

To increase multiplication efficiency, micropropagation should be scaled-up. Bioreactor systems have been introduced for mass propagation of horticultural plants (Levin and Vasil, 1989; Takahashi et al., 1992) and have proved their potential for large-scale micropropagation. They are highly effective for mass production of valuable plants because so many propagules can be obtained at a time. There are reports of mass propagation of some horticultural plants using bioreactor systems, for example for *Phalaenopsis* (Park et al., 2000), oriental lily (Lian et al., 2003), garlic (Kim et al., 2003) and potato (Piao et al., 2003). However bioreactor systems are still mostly used for cell and root cultures for secondary metabolite production (Paek et al., 2001; Rittershaus et al., 1989).

To establish large-scale micropropagation of *Chrysanthemum* through a bioreactor system, we first compared multiplication rate of *Chrysanthemum* shoots between gelled and liquid cultures. Based on those results, shoots were propagated in bioreactors using variations of culture method and inoculation density to determine the optimal conditions.

2. Materials and methods

2.1 Plant material

Axillary buds of *Chrysanthemum* (*Dendranthema grandiflorum* Kitam 'Cheonsu') were washed with running tap water and surface-sterilized in a 1.0% sodium hypochlorite solution for 30 seconds followed by rinsing 2-3 times with sterilized distilled water. Shoot tips, with two leaf primordia, were excised from the axillary buds and placed in 100 ml Erlenmeyer flasks containing 20 ml MS (Murashige and Skoog, 1962) gelled media supplemented with 0.5 mg l^{-1} benzyladenine and 30 g l^{-1} sucrose to induce adventitious shoots. The pH of the medium was adjusted to 5.8 before autoclaving. After 5 weeks of culture, the adventitious shoots were cut into 1.5 cm-length single nodes and propagated in polypropylene growth vessels (107 × 107 × 97 mm, Osmotek, Israel) containing 50 ml MS basal media supplemented with 30 g l^{-1} sucrose. Cultures were maintained at 25/18°C (day and night) with 70 μmol m^{-2} s^{-1} PPF during the 16-h photoperiod.

2.2 Gelled and liquid cultures

Ten single node explants were placed into a 900 ml square-type glass vessel containing 200 ml gelled or liquid MS medium supplemented with 30 g l^{-1} sucrose. To gel the medium, 2.4 g l^{-1} gelrite was used. In liquid culture, a plastic net was placed inside the culture vessel to support the explants. Gas-permeable microporous filters (Mill-Seal, Millipore, Tokyo; pore size 0.5 μm) were attached on top of the culture vessels and the air exchange rate in the culture vessel was controlled to 0.1 vvm. Cultures were maintained for 5 weeks as above. Fresh weight, dry weight, shoot length, stem diameter, number of leaves, leaf area, water potential and chlorophyll content of leaves were recorded after 5 weeks. Leaf area and chlorophyll content of fully-developed leaves were measured with a leaf area meter (Skye Co, UK) and a chlorophyll meter (SPAD-502, Minolta, Japan). Leaf water potential was recorded for the fourth–youngest, fully-developed leaves using a water potential measuring instrument (Tru Psi, Decagon, USA). After measurements, shoots were cut into 1.5 cm-length single nodes, transplanted to plug trays (2.7 × 2.7 × 4 cm; 128 cells per tray) filled with sand and grown for 4 weeks in greenhouse conditions to investigate the number of plants that survived.

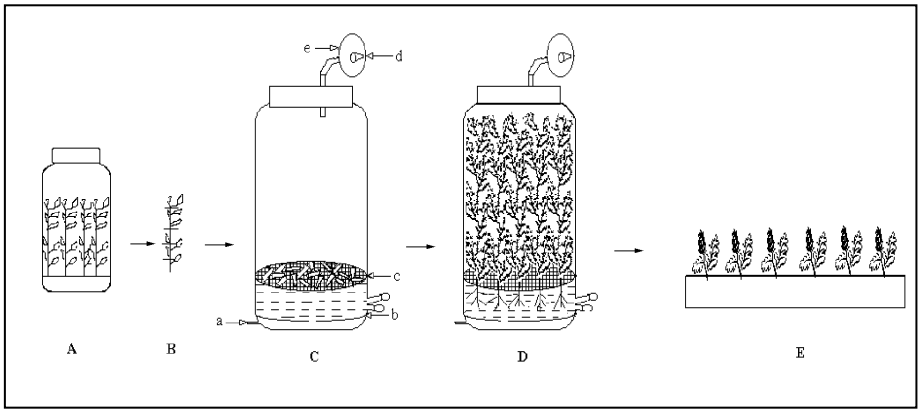

Figure 1: Schematic diagram of multiplication of *Chrysanthemum* shoots in bioreactors: A: Shoots induced from meristem culture; B: Single node stems (1.5 cm in length); C: Bioreactor culture of single node stems (a: Air inlet, b: Sparger, c: Plastic net to support explants, d: Air outlet, e: Membrane filter); D: Shoot multiplication in a bioreactor; E: *Ex vitro* rooting of single node cuttings.

2.3 Bioreactor culture: effect of the number of single nodes inoculated into a bioreactor

Plant material, culture medium, air temperature, PPF, the number of air exchanges and CO_2 concentration inside the vessels were maintained the same as those in liquid culture (above). Groups of single node cuttings (20, 40, 60, or 80) were placed into 10-litre column-type bioreactors containing 4 litres MS liquid medium supplemented with 30 g l^{-1} sucrose. Cultures were maintained for 12 weeks, followed by measurements of fresh weight, plant height, stem diameter, number of branches and leaves. The process of bioreactor culture of *Chrysanthemum* shoots is described in figure 1.

2.4 Effect of culture method

Five-litre column-type bioreactors were used for the experiment. Forty single nodes were placed into a bioreactor and cultured for 10 weeks by three different ways: ebb and flood culture, deep flow technique (DFT) culture and immersion culture. For the ebb and flood culture and the DFT culture, a plastic net was placed into the bioreactor to suspend the explants and the medium was supplied to the underneath of them. In the ebb and flood culture, 1.5 litre MS liquid medium containing 30 g l^{-1} sucrose was supplied for 30 min at 2-hour intervals for the first 2 weeks and at 1-hour intervals from the third week until the end of culture. The medium was sub-

irrigated and circulated using a timer and a solenoid valve (Seki Co., Seoul, Korea). In DFT culture, the medium was continuously supplied to the underneath of the explants. In the immersion culture, the explants were placed on the base of the bioreactor (without a net) and cultured in the medium. CO_2 concentration inside the bioreactor was recorded every two weeks during culture period using: analysis was by gas chromatography (HP 6890, Hewlett Packard, Wilmington, USA). Measurements were made after 10 weeks, of fresh weight, shoot length, leaf number, leaf area, chlorophyll content, and water potential.

3. Results and discussion

3.1 Gelled and liquid culture

There was a significant difference in growth of C*hrysanthemum* plantlets grown on gelled and liquid media. Total fresh weight (2292 mg per plantlet) and total dry weight (185 mg per plantlet) in liquid culture were more than double those in gelled culture (929 mg per plantlet FW and 72 mg per plantlet DW) (Table 1). Liquid culture also resulted in greater shoot length and leaf area compared with gelled culture. Number of leaves, chlorophyll content and stem diameter were also greater in liquid culture. Furthermore, shoots from liquid cultures grew vigorously without hyperhydricity, showing similar leaf water potentials to those in gelled culture. Survival, *ex vitro,* was 100% regardless of whether the culture medium was gelled or liquid (Table 1).

The absorption of nutrients from the medium was accelerated when the nutrients were supplied in liquid medium (Takayama and Akita, 1994); growth was greater compared with that in gelled medium. Avila et al. (1998) also reported leaf areas that were double and shoots with greater length nodes for potato cultures in liquid media, as we found in our results. Hyperhydricity is generally known to occur more frequently in liquid media due to the high water potential of leaves (Paek and Han, 1989). In our results, however, leaf water potential in liquid and gelled media was not different, with slightly lower values of water potential and no hyperhydricity (data not shown) in liquid cultures. This result indicated that the air supply (0.1 vvm) decreased the relative humidity inside the culture vessel (Hahn and Paek, 2001) and the leaf water potential, preventing hyperhydricity. There have been similar results for *Rehmannia glutinosa* and several horticultural crops (Cui et al., 2000; Kubota et al., 1997). These results suggest that bioreactor culture may be possible for the large-scale shoot multiplication of *Chrysanthemum*.

Table 1: Growth of *Chrysanthemum* plantlets after 5 weeks of gelled and liquid culture

		Gelled culture	Liquid culture
Fresh weight (mg per plantlet)[y]	Shoot	844 ± 21	1986 ± 226
	Root	86 ± 14	306 ± 36
Dry weight (mg per plantlet)[y]	Shoot	66 ± 4	160 ± 14
	Root	6 ± 1	26 ± 2
Shoot length (cm)		4.8 ± 0.1	8.3 ± 0.4
No. leaves/plantlet		11.2± 0.2	13.4 ± 0.5
Leaf area (cm^2 per plantlet)		20.4 ± 1.5	49.9 ± 1.3
Chlorophyll content[z]		40.1 ± 1.4	42.3 ± 4.5
Stem diameter (mm)		2.1 ± 0.02	2.5 ± 0.02
Water potential (MPa)		-2.40 ± 0.5	- 2.84 ± 0.7
Ex vitro survival (%)[x]		100	100

[z]SPAD value.
[y]Each value represents mean±standard error of 3 replicate vessels each with 5 plantlets recorded after five weeks of culture.
[x]100 single nodes produced *in vitro* per number of single nodes that grow to 3 cm-length transplants × 100.

Table 2: Effects, after 12 weeks growth in liquid medium, of the number of single nodes inoculated into a 10-litre bioreactor on their fresh weight, shoot length, stem diameter, number of branches and leaves and *ex vitro* survival

Number of single nodes inoculated	Fresh weight (g)[x]	Stem length (cm)	Stem diameter (mm)	No. branches per plantlet	No. leaves per plantlet	*Ex vitro* survival (%)[y]
20	7.84 ± 1.1	23.4 ± 2.3	2.5 ± 0.05	8.33 ± 1.5	59.2 ± 6.8	100
40	9.67 ± 1.7	26.7 ± 1.5	2.4 ± 0.11	6.61 ± 0.8	63.8 ± 5.2	100
60	9.54 ± 2.1	26.9 ± 2.4	2.2 ± 0.09	6.58 ± 1.0	62.7 ± 6.4	100
80	8.44 ± 0.9	28.3 ± 2.0	2.2 ± 0.07	4.52 ± 0.9	54.1 ± 4.1	100

[x]Each value represents mean±standard error of 2 replicate vessels each with 10 plantlets recorded after 12 weeks of culture.
[y]200 single nodes produced *in vitro* per number of single nodes that grow to 3 cm-length transplants × 100.

Table 3: Effects of culture system on fresh weight, stem length, stem diameter, leaf number, leaf area and leaf water potential of *Chrysanthemum* plantlets after 10 weeks of bioreactor culture

Liquid culture system	Fresh weight (g per plantlet)	Stem length (cm)	Stem diameter (mm)	No. leaves per plantlet	Leaf area (cm^2 per plantlet)	Water potential (MPa)
Ebb and flood	1.73 b[z]	12.8 b	1.9 b	17.5 a	36.9 b	- 3.29
DFT	2.63 a	15.8 a	2.2 a	17.0 a	45.3 a	- 2.91
Immersion	1.54 b	11.9 b	1.9 b	14.0 b	34.5 b	- 3.76

[z]Mean separation within columns by Duncan's multiplication range test, 5% level.

3.2 Bioreactor culture: the effect of the number of single node inoculum

Shoot multiplication in early stage was enhanced by high inoculation density (80 single-node cuttings), but became similar among 40, 60, and 80-node inoculations as the cultures proceeded (data not shown). Shoot lengths increased with increasing numbers of single nodes, but stem diameters and the numbers of branches were reduced (Table 2). Shoot length was greatest with 80 nodes per inoculation, but there was least branching (branches are not considered to be shoots and require much longer time for acclimatization, with less plantlet survival due to shoot fragility), indicating that the largest number of single nodes for rooting would be obtained from this treatment. Twenty-node inoculation resulted in least fresh weight and shoot length but with greater numbers of branches, which resulted in smaller number of single nodes for rooting, compared to other treatments (Table 2).

Niu and Kozai (1997) reported the decrease of photosynthesis and shoot growth with higher numbers of explants when potatoes were cultured *in vitro*, in contrast to our results. This could be explained by the difference in the volume of the culture vessels between the experiments. Under ventilation, CO_2 concentration inside a culture vessel gets higher with the increase of the vessel volume. (Paek et al., 2001; Li et al., 2001). Our result suggested that a high inoculation density did not inhibit shoot multiplication if a large volume of culture vessel was used. In this regard, a 80-node inoculation was shown to be effective in obtaining large numbers of single-node cuttings, which survived 100% after transplanting.

3.3 The effect of culture method

DFT culture led to greatest fresh weight, shoot length and leaf area but the differences were minor compared to plant development in the ebb and flood culture. On the other hand, growth was inhibited in immersion culture (Table 3). The negative effect of immersion culture on plantlet growth was mainly due to the lack of oxygen and an high water potential, because the explants were immersed into the medium for the entire culture period (Nguyen and Kozai, 1998; Ziv, 1991). As a result, immersion culture suppressed photosynthetic activity and increased the number of vitrified leaves (data not shown). Difference in plantlet growth among the three culture systems was associated with CO_2 concentrations inside the culture vessels during the light periods. CO_2 concentration in each bioreactor was approximately 1500 μmol mol^{-1} at the initial stage of culture. CO_2 concentration in DFT culture decreased by 400 μmol mol^{-1} after 2 weeks of culture; in ebb and flood culture CO_2 concentration decreased to 750 μmol mol^{-1}, after which remained constant up to week ten. However, CO_2 concentrations >1400 μmol mol^{-1}, was recorded after 2 weeks in immersion culture, then the CO_2 concentration gradually decreased (Figure 2). The results indicated that the explants in DFT and ebb and flood culture systems started to photosynthesize from their initial stages, but photosynthesis in immersion culture was severely suppressed during the early stage of culture. Photosynthesis of plantlets *in vitro* and CO_2 concentration inside the culture vessel are closely related each other, as reported elsewhere (Fujiwara et al., 1987; Kubota et al., 1997; Kim et al., 2003).

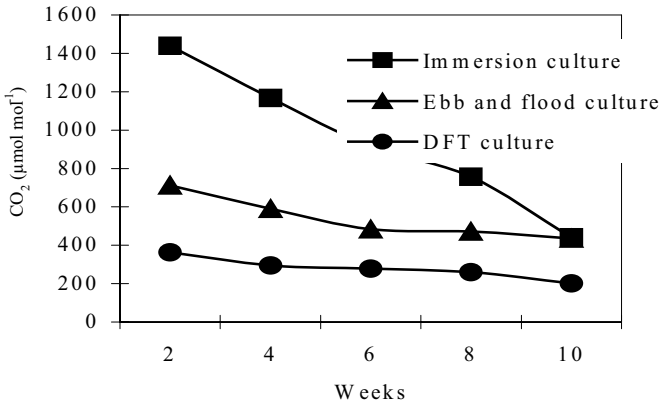

Figure 2: Changes of CO_2 concentration inside 5-litre column-type bioreactors during multiplication of *Chrysanthemum* shoots.

4. Conclusion

Multiplication of horticultural plants through bioreactor systems is, initially, costly, but long-term, it can increase multiplication efficiency remarkably by applying large-scale culture vessels and optimizing the physical and chemical environments. Since plantlets in bioreactors are grown in optimized culture conditions, proliferation rates are much greater than those in conventional gelled cultures. In addition, 100% survival *ex vitro* can be achieved with faster growth after transplantation. Our results suggested the possibility of large-scale production of *Chrysanthemum* shoots through bioreactor systems. Further studies are needed to optimise culture conditions, such as medium composition, rate and frequency of air exchanges and CO_2 enrichment to maximize multiplication efficiency.

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