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Microponic and hydroponic techniques in disease-free chrysanthemum (*Chrysanthemum* sp.) production

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Abstract

In this paper, microponic and hydroponic systems of chrysanthemum (*Chrysanthemum* sp.) in which cuttings can grow more rapidly and vigorously were described. Cuttings (3.0 cm in length) of chrysanthemum excised from shoot clusters multipled *in vitro* were pre-treated with NAA (α-naphthaleneacetic acid) and grown for 28 days in microponic culture system. The NAA concentration at 500 ppm for 20 min were proven to be the optimal (compared to 100, 1000 ppm, and pre-treatment time periods of 10, 30 min). Fresh weight, shoot length, root length and survival rate were 0.31 g, 5.99 cm, 3.08 cm and 100%, respectively. Cuttings were planted in hydroponic culture system to avoid massive contamination in soil. In this system, cuttings (10 cm in length) of chrysanthemum excised from shoots multipled *in vitro* were pre-treated with 500 ppm NAA for 20 min and grown for 28 days. Different nutrient solutions were examined to determine the most appropriate solution for hydroponic system. Results obtained in the system using half-strength Murashige and Skoog (MS) solution (shoot length, number of leaves, leaf length, leaf diameter, number of roots, root length, fresh weight were 14.85 cm, 7.0, 6.46 cm, 4.82 cm, 28.6, 2.47 cm and 2.24 g, respectively) was considered as the optimal treatment. In half-strength MS solution, the density of 20 cuttings per box (about 60 cm^2) gave survival rate of 100% during 14 days of culture. This is the appropriate density for non-circulating hydroponic system.

Key words: *Chrysanthemum, Dendranthema grandifolium*, auxins, NAA, microponics, hydroponics.

Introduction

Chrysanthemum (*Chrysanthemum* sp.) has been propagated successfully using several methods including conventional propagation methods, such as cutting culture, *in vitro* apical meristem culture (Huong and Nhut, 2004), callus culture and plantlet regeneration, protoplast culture (Rout and Das, 1997). Several kinds of commonly used medium are originated from Murashige and Skoog's formulation (MS) (1962) and Gamborg *et al.* (1968). Researchers have also developed gene transfer systems using *Agrobacterium* (Rout and Das, 1997) and many studies in cell biotechnology and molecular biotechnology have been carried out in order to meet the demand.

Conventional *in vitro* culture methods have successfully produced disease-free chrysanthemum for the last few decades but some limitations still remain. Meristem culture is a very useful approach giving high quality plantlets with high virus-free rate. Shoots originated from these methods, however, have to undergo rooting periods on solid media (containing agar) and are subsequently transferred into soil. This may lead to disease contamination and quality reduction, especially product "clean" level, in the next generations. Thus, there is always a requirement for plantlets derived from *in vitro* culture. In addition, culture in tight vessels leads to shoot malformation, stomata malfunction, limitation of micropropagation product diversity, negative effects of plant growth regulators, etc. due to low carbon dioxide (CO_2) concentration, extremely high relative humidity (over 95%) within culture vessles (Debergh, 1987; Gaspar *et al.*, 1987; Jackson *et al.*, 1991; Ziv, 1991). Moreover, conventional methods require complicated procedures. The sterile culture conditions have great impact on the growth and development of plantlets after transferring to nursery garden (acclimatization). All of these limitations make the product very expensive.

Alternative techniques for propagation have been studied, including autotrophic tissue culture systems (Fujirawa *et al.*, 1988) and microponics. In autotrophic culture systems, plantlets are grown in large vessels whose atmosphere contains both carbon dioxide and oxygen with appropriate concentration; relative humidity and nutrient components are also controlled. However, internal environment in these systems also need to be sterile and controlling the culture conditions increases the production cost. Whereas, several preferable features are advantages of microponic systems which is the combination of micropropagation and hydroponics, making a novel technique in plant tissue culture. In microponic system, nutrients are supplied in solution in small-scale.

Hahn *et al.* (1998, 2000) reported that chrysanthemum plantlets in microponic culture system grew more rapidly and vigorously compared to those in conventional *in vitro* system. The requirement of complicated culture steps were not necessary. In addition, labour and time could be saved because primary culture, multiplication, shoot formation and rooting steps are carried out continuously. Microponic system does not require completely sterile conditions. However, plantlets (or cuttings) should be placed under normally "clean" conditions. This facilitates several kinds of opening for propagation system, promoting automation in plant tissue culture and decrease in production cost.

The large-scale microponics/hydroponics, is a technique in which plantlets (or cuttings) are grown in nutrient solution supplying all necessary nutrient components for the optimal growth conditions. Inert substrata may be used in these systems. Hydroponics was practiced centuries ago in Amazon region, Babylon, Egypt, China and India. Later, plant physiologists started to grow plants in liquid medium with specific nutrients for experimental purposes, they called it "*nutriculture*". As early as in 1929, at the University of California, Dr William succeeded in growing tomato vines of 7.5 m height, in nutrient solutions and this new production system was named as "*hydroponics*".

In hydroponics, disease sources, nematodes and insects from soil can be controlled. This was crucial in tropical areas, where these pests grow continuously over a year. Hydroponics offers a clean working environment and thus hiring labour is easy. Hydroponic systems are divided into opening systems (nutrient solution is not recycled) and closing systems (nutrient solution is recovered, refilled and recycled). They could also be called non-circulating and circulating system, respectively. In this study, non-circulating system was applied.

The objective of this investigation was to study the effect of different factors involved in disease-free chrysanthemum multiplication under hydroponic and microponic systems.

Materials and methods

Cuttings were excised from *Chrysanthemum* shoot obtained from *in vitro* meristem culture. Shoot multiplication medium was MS basal medium (Murashige and Skoog, 1962) added with 0.5 mg l^{-1} 6-benzyl adenine (BA), 0.2 mg l^{-1} NAA, 30 g l^{-1} sugar (sucrose) and 8 g.l^{-1} agar (agar *Hai Phong*, Vietnam).

Transparent plastic cylindrical boxes (*Dai Dong Tien*, Ho Chi Minh City, Vietnam), 8.5 cm in height with top and bottom surface, 12 and 9 cm in diameter, respectively were used for experimant (Fig. 1).

Microponic culture system preparation: Rectangular pieces of transparent polypropylene, curved into small tubes, were arranged vertically in boxes with the densities of 18-20 tubes per box (Fig. 1). Microponic nutrient solution was half-strength MS medium. Hydroponic nutrient solutions were tap-water, MS, 1/2 MS, 1/5 MS, 1/10 MS, *Song Gianh* solution (Song Gianh Co., ltd., Ha Noi, Vietnam). The pH values of these solutions were adjusted to 5.8, except tap-water and *Song Gianh* solution.

Hydroponic culture system preparation: The plastic rectangle trays whose upper surface was 51 x 33 cm and lower surface was 49 x 28 cm, 10 cm in height, were used. Wire nets were shaped to make frames for putting chrysanthemum cuttings. Wire net was placed into the plastic rectangle trays about 5 cm from the bottom. Nutrient solution was poured into these trays with 3 litres per tray, and the solution level was 3 cm. Auxin used in the experiments was NAA at concentrations of 100, 500, and 1,000 ppm. Hydroponic culture system was carried out in *ex vitro* conditions, under natural light in net-house where 50-70% of sun light was shaded. The air temperature in net-house was 20 ± 2°C and relative humidity was 75-80%.

Culture conditions: *In vitro* culture and microponic culture system were placed in growth chamber where environmental conditions were adjusted to 25 ± 2°C air temperature, 75-80% relative humidity, under photosynthetic photon density flux (PPDF) of 55- 65 μmol $s^{-1}m^{-2}$ with photoperiod of 16 hours per day.

Experimental design: After 30 days of meristem culture, vigorous plantlets were obtained with 12 cm in length and used as explants. In microponics, before planting in the system (Fig. 2), shoots derived from *in vitro* culture were excised into cuttings each of which contained two nodes (2.5-3 cm). Leaves in basal parts of cuttings were removed. In hydroponics, before planting in the system, shoots of plantlets derived from *in vitro* were cut into cuttings, 10 cm in length and leaves in basal parts were removed.

Effect of concentration and time period of NAA pre-treatment on the growth and development of cuttings in microponic system: The basal part of the cuttings was dipped into NAA solution at concentrations of 100, 500 and 1000 ppm. At each concentration, cuttings were pre-treated for different time period: 10, 20, and 30 min. Cuttings not pre-treated with NAA were as control. Some were kept on culturing on MS medium containing agar within fertile vessels in order to make comparision with microponic method. Results were recorded after 28 days of cultureon morphology and growth of roots, fresh weight, shoot length and root length.

Effect of nutrient solution on the growth and development of cuttings in hydroponic system: Non-circulating hydroponic system was established. Shoot cuttings, 10 cm in length, were pre-treated with NAA 500 ppm for 20 min, planted in plastic trays containing nets. Different kinds of solution were poured into these plastic trays correlated with 6 treatments: MS, 1/2 MS, 1/5 MS, 1/10 MS, tap-water and *Song Gianh* solution. Observations were recorded after 28 days of culture on morphology, shoot length, number of leaves, leaf length, leaf diameter, number of roots, fresh weight.

Effect of cutting density on their growth and development in hydroponic system: Shoot cuttings, 10 cm in length, were pre-treated with NAA 500 ppm for 20 min, planted in plastic boxes containing tiny polypropylene tubes. Four different shoot densities examined were: 5, 10, 15, and 20 cuttings per box. Data were recorded after 7 and 14 days of culture on morphology and development of roots, number of leaves, shoot length and pH values. Root length recorded only on 14th day. After 14 days of culture in hydroponic system, plantlets were transferred to soil in order to make comparison with those derived from cuttings rooted in soil.

Statistical analysis: Each experiment was repeated three times. Data were analysed by Duncan's multiple range test (Duncan, 1995).

Results and discussion

Effect of concentration and time period of NAA pre-treatment on the growth and development of cuttings in microponic system: Except the cuttings which were not pre-treated with NAA, the survival rate of cuttings was 100%. Those which were pre-treated with NAA possessed high and straight shoots. Shoot length, root length, fresh weight were highest at 500 ppm NAA (Table 1). Cuttings pre-treated with 500 ppm NAA had more and larger leaves than those pre-treated at 1000 ppm.

Auxins enhanced adventitious root formation, promoted rooting of hard-rooting plants, shortened rooting time, increased number of roots and root length (Davis *et al.*, 1988; Al-Salem and Karam, 2001). However, long duration auxin treatment may inhibit further root development. This lead to the fact that cuttings pre-treated for 20 min have better development than those pre-treated for 30 min. However, ten min was too short for auxin to have an effect on cuttings, therefore, 20 min was the optimal duration in this experiment.

Auxins have positive effect on rooting at low concentrations but give negative effect at high concentrations (De Klerk *et al.*, 1997; De Klerk, 2002). Cuttings pre-treated with NAA at high concentration (1000 ppm) gave lower fresh weight than those pre-treated at 100 and 500 ppm. This result showed that 500 ppm was the optimal concentration for enhancing rooting. The concentration of 100 ppm was too low to promote rooting well. Those pre-treated at 100 ppm had shortest roots. Similarly, the concentration of 1000 ppm was very high, hence inhibiting rooting. The NAA pre-treatment at the concentration of 500 ppm for 20 min was proven to be the optimal for rooting. Cuttings which had vigorous roots were transferred to greenhouse.

Auxins play an important role in rooting, they are essential for rooting in many species (Al-Saqri and Alderson, 1996; Carpenter and Cornell, 1992; Erwin *et al.*, 1997). The results, however, may vary among different species (Hinesley *et al.*, 1994; Maynard and Bassuk, 1990). Auxins are only required in the initial steps of rooting and somatic embryogenesis; in late periods, they suppress development, organogenesis and new embryogenesis (Davies, 1995).

Effect of nutrient solution on the growth and development of cuttings in hydroponic system: The survival rate was 100% in all treatments (except tap-water treatment as the control). Of the treatments, 1/2 MS gave the best results. Cuttings planted in 1/2 MS solution had higher shoot length, number of leaves, leaf length, leaf diameter, number of roots, root length and fresh weight than those planted in the other solutions. Cuttings in 1/2 MS grew better than in *Song Gianh* solution which is commercially available. The results indicated that 1/2 MS was the optimal solution to be used for hydroponic system with cuttings pre-treated with 500 ppm NAA for 20 min. Cuttings grew poorly in tap-water for they were not provided macro- and micro-elements, nutrients, vitamins enough to grow and develop.

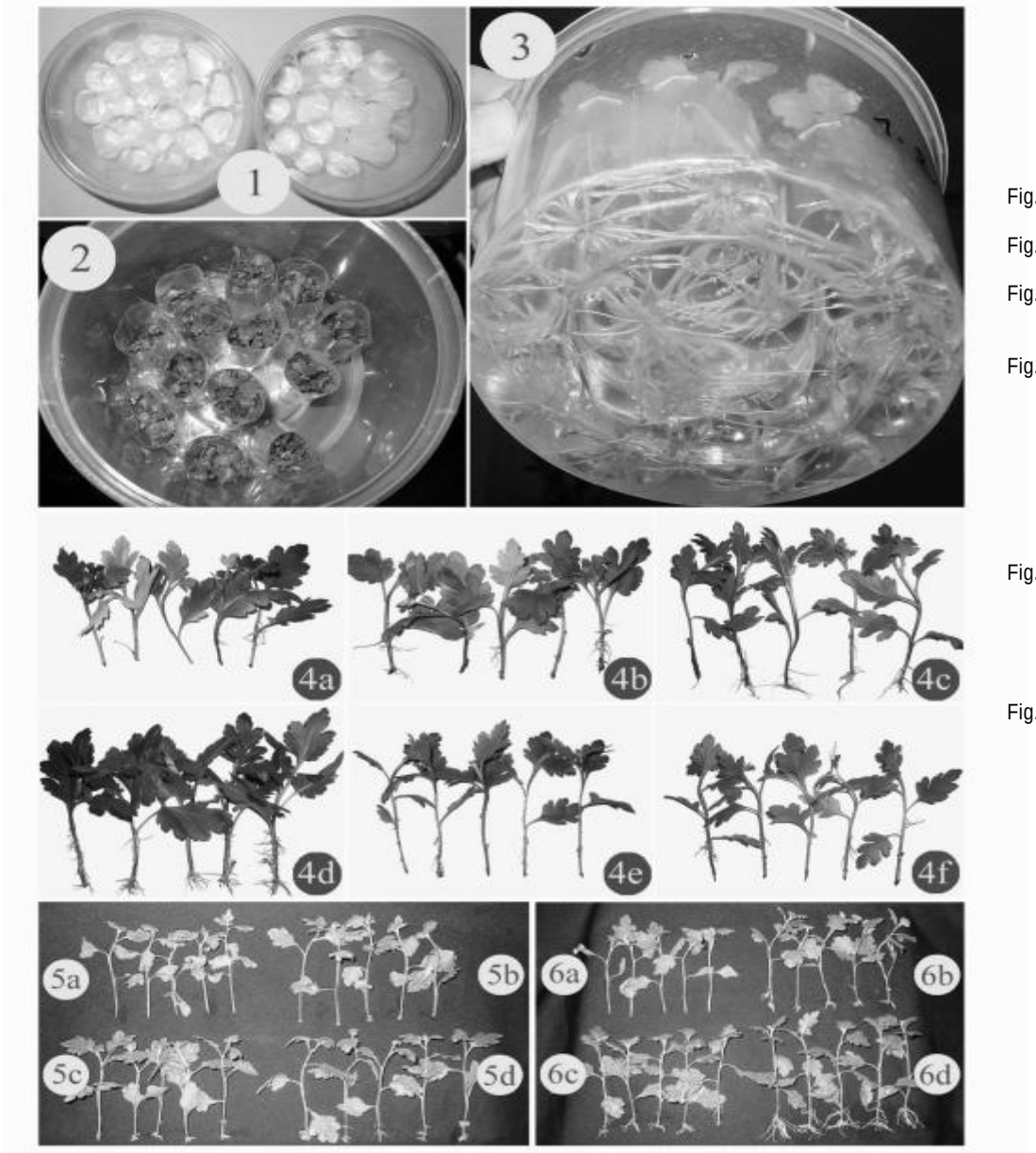

Fig. 1. The polypropylene tubes are placed in plastic boxes.

Fig. 2. Cuttings were planted into microponic system.

Fig. 3. Rooting of cuttings pre-treated with NAA at 500 ppm after 28 days of culture.

Fig. 4. Rooted cuttings after 28 days of culture in hydroponic system with different nutrient solutions:
a.Tap-water
b. *Song Gianh* solution
c. MS medium
d. 1/2 MS medium
e. 1/5 MS medium
f. 1/10 MS medium

Fig. 5. Rooted cuttings after 7 days of culture in hydroponic system:
a. 5 cuttings/box
b. 10 cuttings/box
c. 15 cuttings/box
d. 20 cuttings/box

Fig. 6. Rooted cuttings after 14 days of culture in hydroponic system:
a. 5 cuttings/box
b. 10 cuttings/box
c. 15 cuttings/box
d. 20 cuttings/box

Table 1. Effect of concentrations and time lengths of NAA pre-treatment on the growth and development of cuttings after 28 days of culture in microponic system

NAA (ppm)	Time period (min)	Fresh weight (mg)	Shoot length (cm)	Root length (cm)	Survival rate (%)
0 ppm	0	90f[w]	5.18f	0.90g	30
100 ppm	10	240c	4.68g	1.44f	100
	20	260c	5.70c	2.56d	100
	30	240c	5.52d	2.50d	100
500 ppm	10	220d	5.51d	3.41b	100
	20	310a	5.99a	3.08c	100
	30	250c	5.46d	3.98a	100
1000 ppm	10	200e	5.34e	2.19e	100
	20	290b	5.89b	2.24e	100
	30	200e	5.66c	2.51d	100

[w] Different letters within a column indicate significant difference at $P = 0.05$ (Duncan's multiple range test).

Table 2. Effect of nutrient solutions on growth and development of cuttings after 28 days of culture in hydroponic system

Nutrient solution	Shoot length (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)	Number of roots	Root length (cm)	Fresh weight (mg)
Tap-water	11.41e[x]	6.60b	5.25c	4.19c	5.20f	0.81f	1730b
Song Gianh	12.92d	6.50b	5.91b	4.61b	13.80c	2.14c	1600c
MS	14.01b	6.90a	4.69d	3.46d	17.90b	2.33b	1280d
1/2 MS	14.85a	7.00a	6.46a	4.82a	28.60a	2.47a	2240a
1/5 MS	13.56c	6.10c	4.39e	3.35d	8.00d	1.38d	940e
1/10 MS	12.93d	6.50b	4.20f	3.05e	5.80e	1.10e	960e

[x] Different letters within a column indicate significant difference at $P = 0.05$ (Duncan's multiple range test).

Table 3. Effect of cutting density on rooting, shoot growth and survival in hydroponic system after 7 days of culture

Treatment	Number of roots	Shoot length(cm)	Number of leaves	pH	Survival rate (%)
5 cuttings/box	0.30d[y]	9.70d	4.10c	7.7	60
10 cuttings/box	3.35c	10.33b	5.30b	5.1	80
15 cuttings/box	6.27b	10.07c	5.27b	5.8	100
20 cuttings/box	11.38a	10.68a	6.13a	5.6	100

[y] Different letters within a column indicate significant difference at $P = 0.05$ (Duncan's multiple range test).

Table 4. Effect of cutting density on rooting, shoot growth and survival in hydroponic system after 14 days of culture

Treatment	Number of roots	Root length (cm)	Shoot length (cm)	Number of leaves	pH	Survival rate (%)
5 cuttings/box	3.90d	0.20d	9.70d	5.60d	7.9	30
10 cuttings/box	12.30c	0.90c	10.68c	7.00b	6.2	50
15 cuttings/box	13.73b	1.29b	10.87b	6.80c	6.8	90
20 cuttings/box	18.60a	2.05a	11.33a	7.20a	6.3	100

[z] Different letters within a column indicate significant difference at $P = 0.05$ (Duncan's multiple range test).

The experiment also showed that high concentration of minerals was not suitable for the growth and development of chrysanthemum cuttings. To some plants, high concentration of minerals in MS basal medium seems to be too high and may be harmful for cultured tissues. Therefore, half-strength MS medium was appropriate for the growth and development of plants (Fig. 4).

In 1/5 MS and 1/10 MS solutions, the growth and development of cuttings was weakly correlated with low concentration of minerals and nutrients because the amount of nutrients was too small for cuttings to grow normally (Table 2).

Effect of cutting density on their growth and development in hydroponic system: After 7 days of culture, primordial roots emerged from each cutting (less than 1 cm in length). Significant differences among treatments were recorded (Table 3). The rooting was positively correlated to the density of cuttings. With higher density of cuttings, better growth and development was observed. The same result was also obtained with the survival rate.

After 14 days of culture, roots were long enough to be observed and compared visibly. Similarly, there were obvious differences among the four treatments. In general, the survival rate of cuttings after 14 days of culture was lower than those recorded after 7 days. However, the survival rate remained 100% in the boxes which contained 20 cuttings (Fig. 5).

In this experiment, cuttings were more vigorous in higher density box. The high concentration of nutrients accompanied with the presence of algae and mold in hydroponic solution caused weak vitality of plants. In lower density boxes, there were more spaces for bacteria, fungi and algae to get in touch with and grow rapidly in nutrient solution. Available nutrients in the solution could be utilized by these micro-organism; this may cause the release of harmful substances, changing pH value and nutrient composition. Although no disease was recorded, the presence of micro-organism are supposed to cause poor growth of cuttings and plantlets.

The optimal pH value was about 5.8 – 6.5. Plants show their best growth at pH 6.0 in microponic system (Hahn *et al.*, 1998). However, at the density of 5 cuttings / box, the pH value of solution was high (7.7 on 7th day and 7.9 on 14th day), hence their survival rate was very low (Table 4). High pH values (greater than 7.5) were not appropriate for the growth and development of cuttings in hydroponic culture system. At the density of 10 cuttings / box, pH value was lower than at 5 cuttings / box.

The pH values of hydroponic solutions increased in lower cutting density. At the density of 15 cuttings / box, pH value was 5.8 on 7th day and 6.8 after 14 days of culture. These values were suitable for cuttings to grow. Yet the density of 20 cuttings / box is the optimal condition for cuttings planted in hydroponic system, the results recorded of this treatment were evidently better than those of the others. It was proven that high density of cuttings can prevent the presence of disease-causing organisms from surrounding environment. The pH values recorded after 7 and 14 days of culture increased in all treatments (about 0.14 / day). During the culture, cuttings took up nutrients from hydroponic solution and released certain compounds, this resulted in the change of pH values of nutrient solutions. At the density of 5 cuttings / box, the pH value increased more rapidly than the others due to the presence of fungi and algae in the solution.

Microponic culture system was an effective system for rooting of *in vitro* cuttings of *Chrysanthemum* sp. without *in vitro* rooting stage. Basal part of shoot cuttings dipped with 500 ppm NAA for 20 min was the optimal pre-treatment.

The optimal nutrient solution for non-circulating hydroponic system is half-strength MS solution in short time (less than 1 month). Cuttings gave the optimal growth and development at the density of 20 cuttings per box (bottom surface area is 64 cm^2 and top is 120 cm^2).

Rooting procedures in these two systems could be conducted without soil, hence reducing disease contamination in cuttings and ease for transportation. This can be applied for large-scale production of *Chrysanthemum* sp.

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