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MULTIPLE SHOOT CULTURE OF *DIANTHUS CARYOPHYLLUS* USING MIST CULTURE SYSTEM

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SUMMARY

A mist bioreactor system for the plant tissue cultures was developed. Using this system, the growth of *Dianthus caryophyllus* multiple shoots was directly measured. Tissue growth in mist bioreactor system was far better than that on agar medium and almost comparable to that in liquid medium. The mass increase (final dry weight/initial dry weight) in the mist culture was 2.85 while 3.28 in the liquid flask culture. Shoots were seriously vitrified in flask culture but these vitrifications could be considerably cured by using the mist culture system.

INTRODUCTION

Mass propagation of plants is a labor intensive process because of slow growth of plants and difficulty in scale-up. In recent years, there has been much research carried out to solve these problems(He *et al.*, 1992; Simonton *et al.*, 1991; Kruger *et al.*, 1991; Smith *et al.*, 1989; Weathers and Giles, 1988) and the mist culture system was developed by Weathers *et al.* (1988) as one of possible systems.

Limited nutrient diffusion in conventional agar culture can be overcome by using liquid media. However, the problem of adquate gas diffusion still remains. Weathers and Giles (1988) reported that various plants were successfully regenerated and efficiently increased in number by using mist culture and Kurata *et al.* (1991) also obtained good results using an improved mist culture system.

These efforts were, however, focused on the qualitative comparison of shoot growth with agar culture and therefore it is obvious that quantitave engineering data should be deficient. Because of large size and shear sensitivity, it has been reported difficult to cultivate organized tissues in a liquid medium (Park *et al.*, 1989). Furthermore, since serious vitrification of shoots takes place in liquid midium, acclimation step of the shoots may be time-consuming or even unsuccessful (Debergh *et al*, 1992). In this report, aspects of engineering for multiple shoot culture in a mist bioreactor system are compared with the liquid flask culture system.

MATERIALS AND METHODS

Estabilishment of multiple shoot culture

In vitro plantlets of *D. caryophyllus* used in this work were kindly supplied by Dr. H.G. Nam of Life Science Department, POSTECH and multiple shoot culture was induced in our laboratory. Effects of growth regulators on shoot growth were examined with various combinations of benzyladenine (BA) and 1-naphthalene-acetic acid (NAA) concentrations (BA 1, 2, 5 mg/*l* and NAA 0.02, 0.1, 1 mg/*l*) in agar medium. The combination of 5 mg/*l* of BA and 1 mg/*l* of NAA was found to be the best condition for the growth of multiple shoots. The multiple shoot culture was cultivated in MS medium containing 5 mg/*l* BA, 1 mg/*l* NAA, and 30 g/*l* sucrose for all experiments described thereafter (All chemicals were purchased from the Sigma Chem. Co., U.S.A.). The medium was prepared in double distillated water and the pH was adjusted to 5.8 with 0.1N NaOH before autoclaving (15psi, 121°C for 20min.).

Development of mist culture system

Nutrient mist, produced by an ultrasonic transducer(TDK, TU-24X) fixed to the mistifier, was delivered by air flow into the bioreactor containing the multiple shoot culture (Fig.1). Excess mist was condensed and collected in a reservoir and this medium was recycled into the mistifier. The bioreactor was double jacketed (Pyrex, 150mm ID x 180mm H, 3.2*l*) and covered with a lid (Teflon, 272mm D), which had eight Swagelok stainless steel fittings to connect with mist flow tubing and various probes. The bottom of the bioreactor was slanted to allow the excess medium to flow spontaneously and a support matrix consisting of stainless steel screen (1mm mesh) was placed on the bottom to hold the shoot culture. The mistifier was also double jacketed (Pyrex, 150mm ID x 150mm H, 2.65*l*) and covered with a lid (Teflon, 272mm D), which had seven ports for various connections. The ultrasonic transducer was fixed to the silicon plug in a hole at the bottom of mistifier. The operations of air blower, mist generator, and fluorescent lamps were controlled by a timer. Temperatures of both bioreactor and mistifier were controlled by circulating water from a water bath. The total weight of bioreactor containing inoculum was obtained by a load cell (Mettler PJ12) and the changes in weight during the culture period were recorded. These changes were a reflection of shoot growth.

Flask culture of multiple shoots

Shoot cultures grown on agar medium were transferred into 250 ml Erlenmeyer flasks containing 25 ml medium and incubated on a New Brunswick rotary shaker (100rpm) under cool-light fluorescent illumination of 1,500 Lux for 18 hr per day at 25℃. These cultures were subcultured every 2 weeks by transferring into fresh medium. Multiple shoots grown in flasks were used as the inoculum for the bioreactor of the mist culture system.

Mist reactor culture of multiple shoots

Before starting the culture, the bioreactor, the mistifier (including transducer), the mist collecting reservoir, and all the tubings were sterilized by autoclaving. The bioreactor was inoculated by placing 80 g multiple shoots (grown in flasks for 2 weeks) directly onto the support. Initial volume of the liquid medium in mistifier was 1000 ml. Temperature and illumination condition were kept the same as in the liquid flask culture. Air flow of 1000 ml/min and nutrient mist were supplied periodically (15 min on / 15 min off). Fresh weight of shoots and remaining liquid volume were measured throughout the culture and 5ml of liquid medium was sampled everyday for sugar analysis.

Measurement of shoot growth and sugar analysis

The fresh weight of shoot in liquid flask culture was measured after suction on Whatman no. 1 filter paper for 30 sec. to remove excess water. The dry weight was determined by drying the shoots in an oven at 80℃ for 24 hr. The weight of the empty bioreactor without shoots was weighed by load cell and the fresh weight of shoots in the bioreactor was determined from the weight difference with the empty bioreactor. Sugar concentrations (sucrose, glucose, fructose) in the culture medium were measured using a

Carbopac PA1 column (250x4 mm, Dionex) connected to a Dionex IC system with an integrated amperometry detector. 160mM NaOH was eluted at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Development of mist culture system

In the early stage of system development, transducing ability was seriously reduced after autoclaving. This problem was later found due to water entering through the leak between the plastic case surrounding the ultrasonic transducer. It could be solved by mechanical tightening and the use of epoxy adhesive. As the air flow rate increased, the mist flow rate into the bioreactor also proportionally increased (data not shown) but the optimal air flow rate with minimal dessication of plant tissues existed at about 0.6 vvm. However, this optimal air flow rate could be changed by changing the volume of reactor and mistifier as well as transducing capacity. Mist was not coalesced completely or was lost considerably by evaporation into the air outlet at a too high air flow rate. In this experiment, the air flow rate was maintained at 2 l/min (0.6 vvm) during the mist culture. About 70 ml of liquid midium per day was delivered into the reactor by mist at this air flow rate.

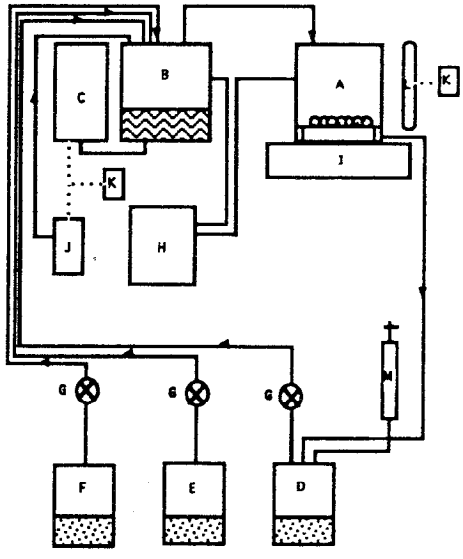

Fig. 1. The schematic diagram of the mist culture system
A: Bioreactor, B: Mistifier, C: Mist generator, D: Mist collector, E: Concentrated medium, F: Pure water, G: Peristaltic pump, H: Water bath, I: Load cell, J: Air blower, K: Timer, L: Light, M: Mist coalescer.

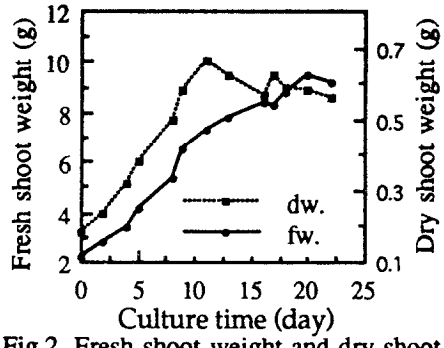

Fig.2. Fresh shoot weight and dry shoot weight changes over the culture period in flask cultures.

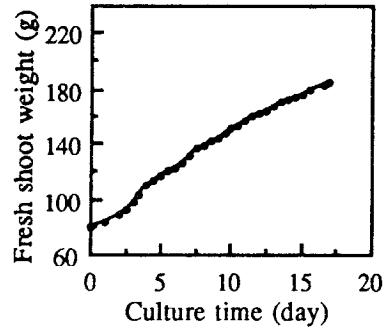

Fig.3. Fresh shoot weight increase during the culture period in the mist culture.

Shoot growth in liquid flask culture

As can be seen in Fig. 2, the multiple shoots in liquid flask culture grew linearly without a lag phase. The fresh weight of shoot culture increased from 2.2 g to 8.8 g per flask in 18 days. On the other hand, the dry weight of shoots increased 0.18 g to about 0.7 g per flask for 11 days and decreased thereafter. This implied that water content in fresh shoots increased due to serious vitrification in the late culture period (Debergh *et al.*, 1992). Although shoots of flask cultures were only partially submerged in the liquid they were still vitrified and if shoots were cultivated under completely submerged conditions, using a conventional reactor vessel, their morphology and growth was badly affected (Levin *et al.*, 1988).

Shoot growth in mist reactor culture

In large scale shoot culture using conventional reactors, the mass of shoots and culture fluid could not be treated as a continuum, and therefore consistent and representative samples from the reactor vessel could not be easily obtained (Park *et al.*, 1989). However, in the mist culture, the fresh weight of shoots could be directly measured by weighing the whole bioreactor as described above since there is no disturbing factor such as air sparging in the air-lift reactor which affects the measurement of the total weight of reactor. In our experiments, the fluctuation of the whole reactor weight could be kept in the range of 2.5 g for more than 8 days blank operation (supplying the mist into the empty reactor vessel weighing 5 kg) once the mist flow reached a steady state (in about 12 hr). Fig. 3 shows that shoots in the mist culture grew linearly without a lag phase similarly to those in liquid flask culture. The fresh weight of shoots increased from 80 g to 185.5 g per reactor in 17 days. On a fresh weight basis, the increment of shoot mass in mist culture was lower than that in flask culture (Table 1). These results indicate the difference in water content between mist cultured and flask cultured shoots. It was also observed that the morphology of shoots in mist culture was different from that in flask culture. The multiple shoots grown in flasks were severely vitrified as described before while the shoots grown in mist bioreactor were normal and healthy as those of agar culture. The size of the shoot lump in mist cultrue was about 1.5 times larger than that in flask culture, and leaves of shoots were not brittle but soft and not translucent but dark green (picture not shown). In the mist culture, nutrients could be supplied under mild environmental conditions compared to the flask or conventional air-lift reactor culture which gave mechanical stresses to shoots by shaking or mixing. Moreover, the air(oxygen) could be supplied in a gas phase while in the liquid culture the supply of oxygen was in a dissolved form, which helped the growth of shoots with a normal morphology.

Sugar consumption during shoot culture

Since the water content in fresh shoots was over 90% during the cultivation, the

amount of water in the liquid medium was significantly reduced according to the shoot growth due to the incorporation of water into the shoot mass. Therefore, the total content of sugar and not its concentration in the liquid medium should be considered during the culture (Fig. 4, 5). As seen in Fig. 4, some portion of initial sucrose was hydrolyzed into glucose and fructose by autoclaving and the amount of remaining sugars declined adversely with increments of shoot mass. The levels of monosaccharides (glucose and fructose) in the medium remained unchanged for about 7 days and declined thereafter along with sucrose. From this trend, it was supposed that the sucrose was preferably consumed by the shoot culture or the sucrose uptake was mediated by both hydrolytic and non-hydrolytic processes. All sugars were completely consumed by the 11th day and the growth of shoots reached the stationary phase at the same time which implied that sugars were taken up and consumed in a short time without storage in the cells.

Table 1. Comparisons of results of liquid flask culture with those of mist reactor culture

	Liquid	Mist reactor
FWi, Initial fresh weight (g)	2.2	80.0
FWf, Final fresh weight (g)	8.8	185.5
Growth index (FWf/FWi)	4.0	2.3
DWi, Initial dry weight (g)	0.18	4.88
DWf, Final dry weight (g)	0.59	13.91
Growth index (DWf/DWi)	3.28	2.85
Initial water content (%)	91.8	93.9
Final water content (%)	93.3	92.5
Initial sucrose content (g)	0.53	27.5
Final sucrose content (g)	0	6.89

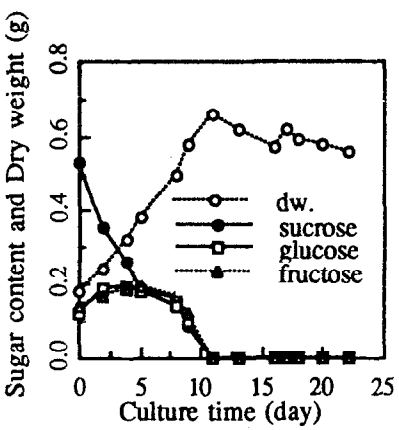

Fig. 4. Sucrose, glucose, fructose content and dry weight changes in the medium for the flask culture

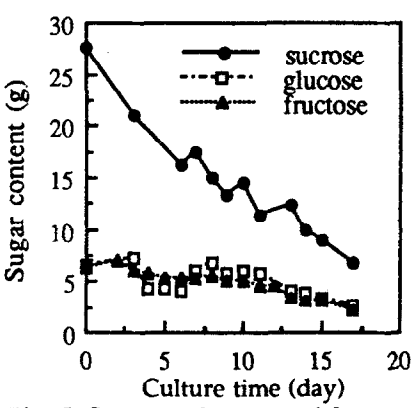

Fig. 5. Sucrose, glucose, and fructose content in the medium for the mist culture.

As can be seen in Fig. 4 and Fig. 5, the trend of sugar consumption in the mist culture was similar to that in flask culture. However, the glucose and fructose were

consumed very slowly since sucrose remained at higher level than those sugars. From Fig. 4 and Fig. 5, it could be concluded that sucrose uptake was carried out only by a non-hydrolytic process and that the monosaccharides were initially produced only by autoclaving. Mist culture was terminated at the 17th day because the medium was exhausted by evaporation of mist into the air outlet in addition to the incorporation into shoots. The amount of evaporated medium could be evaluated by a simple mass balance equation and was estimated to 25.5 ml per day. It is believed that a supply of prehumidified air and additional fresh medium would prolong the stable mist culture.

CONCLUSIONS AND FUTURE WORK

In order to develop a large scale plant tissue culture system which is commercially feasible, quantitative analyses of the system are required in addition to qualitative data describing the tissue growth. In this research, engineering approaches based on quantitative analyses of growth and sugar consumption were carried out to examine the growth kinetics of *D. caryophyllus* multiple shoot culture in the mist bioreactor. It was found that the morphology of shoots in mist culture was normal without vitrification and the growth of shoots was almost comparable to that in liquid flask culture. Further research on other aspects of the mist culture system and mist flow dynamics is currently under way with the ultimate goal of commercial application of the mist culture system. It is also our plan to cultivate other types of plant tissues such as root, hairy root or even callus in our mist culture system.

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