Production of potato (*Solanum tuberosum*, L.) microtubers using plastic culture bags

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Abstract  We have developed a system using plastic culture bags with forced aeration system for both liquid medium and gaseous phase to produce microtubers of potato (*Solanum tuberosum* L.). The production of microtubers under sterile conditions is a good way to produce disease-free materials for crop production, and bioreactors have been used for this purpose. However, bioreactors are expensive and difficult to handle. The plastic culture bags are relatively inexpensive and are easy to store and sterilize because they can be flattened. Microtuber production involves two stages: plant proliferation in one medium, followed by microtuber production in a different medium. Both steps are carried out using the same culture bag. Using this system, we produced 100 to 300 microtubers per 8 l culture bag, depending on the potato cultivar. We varied the nutrient concentrations in the media and found that a lower sucrose concentration in the plant proliferation medium and lower nitrogen concentration in the microtuber production medium both increased the total numbers of microtubers per bag. Notably, a higher concentration of potassium phosphate increased the numbers of larger microtubers. This is beneficial because larger microtubers are much more tolerant to field conditions. We produced about 250,000 microtubers per year in a 66 m² tissue culture room using the culture bag system. These microtubers have been planted directly in the field and utilized for seed potato production.

Key words: microtuber, plastic culture bag, *Solanum tuberosum* L., tissue culture.

Introduction

Potato (*Solanum tuberosum* L.) crops are vegetatively propagated using tubers and facing many risks caused by pathogens. It is very important to prevent infection of pathogens during propagation. In vitro plants are free of most pathogens but may still be infected with viruses. Since viral diseases can cause severe yield losses, it is essential to use virus-free plants via meristem culture for propagation. Healthy seed potatoes are produced from virus-free in vitro plants through several generations of propagation. During propagation in the field, however, some of the disease-free plants can become newly infected by pathogens. Therefore, it is important to propagate disease-free plants or tubers as many as possible using in vitro techniques, and improved tissue culture propagation methods are needed for this purpose.

Microtubers are small potato tubers produced by tissue culture (see Coleman et al. 2001 and Donnelly et al. 2003 for reviews). Microtubers are easier to handle than in vitro plants; they can be stored for several months and then planted directly in the field. Therefore, microtubers are useful for seed potato production. Moreover, microtubers can be used to produce transgenic plants (Kumar et al. 1995; Snyder and Belknap 1993) and to conserve germplasm (Estrada et al. 1986; Gopal et al. 2004).

Although microtubers can be produced using either agar-solidified or liquid medium, liquid medium is favorable for scaled-up and automated production with reduced labor costs (McCown and Joyce 1991). During the culture period, the tissue culture medium must be changed to switch from the plant proliferation stage to the microtuber production stage, and this operation is much easier in liquid medium. Bioreactors have been used for the mass production of microtubers (Akita and Takayama 1994; Piao et al. 2003). However, bioreactors are expensive and give an additional cost. The larger bioreactor makes the cost higher and has more risk of microbial contamination.

We have established a system for the mass production of potato microtubers using plastic culture bags. This system requires much lower apparatus costs than those associated with conventional bioreactors. Furthermore, we have optimized the composition of some components of the culture medium for efficient microtuber production in this system.
Materials and methods

Plant materials and plant culture

The potato cultivar 'Sassy' was used as the main plant material. Virus-free plants were maintained and propagated in plastic boxes (60×60×100 mm) with MS medium (Murashige and Skoog 1962) supplemented with 30 g L⁻¹ sucrose and 8 g L⁻¹ agar. The temperature of the culture room was kept at 20°C and the photosynthetic photon flux was 45 μmol m⁻² s⁻¹ with a 16 h photoperiod of fluorescent light.

Media preparation and sterilization

For all media, the pH was adjusted to 5.8. Agar-solidified media were autoclaved at 121°C for 15 min. Liquid media were prepared in a tank and sterilized by pumping through filter units. Two filter units were connected in series; the first was an MCP-JX-E105 Advantec Polypropylene Pleats Compact/ Capsule cartridge filter and the second was an LAGL04TP6 Millipore Optiseal Durapore cartridge filter. Before use, the filters were autoclaved at 121°C for 30 min.

Production of microtubers in culture-bags

Microtubers are produced via two steps: plant proliferation followed by microtuber development, in the same culture bag. These steps are similar to those used for microtuber production in bioreactors (Akita and Takayama 1994; Piao et al. 2003). The major differences between the two steps are in the light conditions (16 h photoperiod for step 1 followed by continuous dark for step 2), the sucrose concentration (lower for plant propagation and higher for microtuber production), and aeration.

Each bag was 300 mm × 490 mm (total volume 8 l, manufactured by Fujimori Kogyo Co., Ltd.) and the film was composed of 3 layers of the materials, cast polypropylene, nylon and polyethylene terephthalate in the order from the inner surface to the outer surface. Aeration of the gaseous phase in the bioreactor is very important in microtuber production (Onishi et al. 1999). Therefore, our propagation system was developed to allow for aeration of both the liquid and gaseous phases within the plastic culture bags. There were three ports (inner diameter 4.4 mm) on the bags: one for aeration of the liquid medium, one for aeration of the gaseous phase above the liquid medium, and the third was an exhaust port. Atmospheric gases were compressed using pumps and delivered via silicone tubes to the ports in the culture bags. In the plant proliferation step, the air was delivered only into the liquid medium, with a flow rate of 300 ml min⁻¹. In the microtuber production step, the air was delivered to both the liquid medium and the gaseous phase above the liquid, with flow rates of 200 ml min⁻¹ and 800 ml min⁻¹, respectively.

Before use, the culture bags were lapped with non-woven fabric and autoclaved at 121°C for 1 h. The volume of the liquid medium in each bag was 2.5 l for the plant proliferation step and 2 l for the microtuber production step. Six in vitro plants with the length about 100 mm were inoculated into each culture bag containing the filter-sterilized liquid medium, then the upper end of the bag was heat sealed. The plants were grown in the plant proliferation medium for 6 weeks with a 16 h photoperiod. The upper end of the culture bag was cut and the old medium was discarded. Then the medium for microtuber production was added to the same culture bag and the upper end was resealed. The plants were cultured for another 6 weeks under continuous dark conditions.

Experiments with varied concentrations of nutrients in the liquid media

For both plant proliferation and microtuber production steps, we used MS medium supplemented with sucrose and benzyladenine (BA). For plant proliferation, the standard sucrose concentration was 10 g L⁻¹ and the medium was supplemented with 0.02 mg L⁻¹ BA. For the microtuber production step, the standard sucrose concentration was 100 g L⁻¹ and the medium was supplemented with 2 mg L⁻¹ BA.

As described in the Results section, we performed a series of experiments to determine the effects on microtuber production of differing concentrations of phosphate, sucrose, calcium, and nitrogen. Three to 10 culture bags were used for each treatment in each experiment. After growth for 6 weeks in the plant proliferation medium and 6 weeks in the microtuber production medium, the microtubers were harvested, washed in water to remove residual sugars, and dried for about two weeks in the culture room (20°C, 16 h photoperiod). Because the microtubers smaller than 0.3 g easily shrunk during cold storage, only those larger than 0.3 g were collected, classified into 4 groups according to their weights and counted. The weight classes were 0.3–0.5 g, 0.5–1 g, 1–3 g, and larger than 3 g.

Relationship between plant proliferation and number of microtubers per culture bag

To determine the relationship between plant proliferation and number of microtubers per culture bag, we used four potato cultivars: 'Sassy', 'Cynthia', 'Alowa', and 'Jagakids Purple'. Nine to 14 culture bags were used for each cultivar. At the end of the plant proliferation step, the medium was discarded and the culture bags containing the proliferated plants were weighed. After harvesting the microtubers, the empty culture bags were weighed. The weights of the plants at the end of the plant proliferation step were calculated by subtracting the weights of the empty culture bags from the weights of the culture bags containing the proliferated plants. Coefficients of correlation between the numbers of microtubers and the plant weights were analyzed.

Results

The system for producing microtubers in plastic culture bags

The culture bags used in this study were kept in sterile condition throughout the culture period. As shown in Figure 1A, the inoculated plants grew up to the tops of...
the culture bags after 6 weeks in the plant proliferation medium. After changing to the medium for microtuber production, many stolons elongated first, then the microtubers formed on the stolons. The microtubers were distributed from the surface of the liquid medium up to the tops of the culture bags (Figure 1B). We were able to place 12 culture bags in each shelf space with dimensions of 120 cm width, 60 cm depth, and 65 cm height. The condition of the culture room was fixed for 16 h photoperiod or continuous dark. In this way, we produced about 250,000 microtubers per year in a 66 m² tissue culture room (Figure 2). The rate of microbial contamination was about 5% of the bags at the time the microtubers were harvested. These microtubers have been planted directly in the field and utilized for seed potato production.

**Effects of variations in the phosphate, sucrose, calcium, and nitrogen concentrations on microtuber production**

We performed a series of experiments to determine the effects on microtuber production of differing concentrations of phosphate, sucrose, calcium, and nitrogen.

The standard concentration of monopotassium phosphate in MS medium is 170 mg l⁻¹. To test the effect of increased phosphate in the medium, we compared media containing the standard concentration with those containing three times the standard concentration (510 mg l⁻¹). These concentrations were used in both the shoot proliferation and the microtuber production media. Other components were used at the standard concentrations described above. The results are shown in Figure 3. The growth of plants was similar between the standard concentration and the higher concentration. The higher concentration of monopotassium phosphate significantly increased the number of microtubers in the size class larger than 3 g compared to the standard concentration. On the other hand, there were no significant differences in the microtuber production of the other size classes between these two concentrations. Because larger microtubers were advantageous in practical use, we used 510 mg l⁻¹ concentration in the following experiments.

We next tested various sucrose concentrations (10, 20, and 30 g l⁻¹) in the shoot proliferation medium. The sucrose concentration in the microtuber production medium was kept at 100 g l⁻¹, and the monopotassium phosphate concentration in both media was 510 mg l⁻¹. Other components were used in their standard concentrations as described above. We found that the lowest concentration of sucrose, 10 g l⁻¹, produced significantly more microtubers in all size classes smaller...
than 3 g. There were no significant differences between the 20 g l\(^{-1}\) and 30 g l\(^{-1}\) concentrations in microtubers of any size classes (Figure 4).

The standard concentration of calcium chloride dihydrate in MS medium is 440 mg l\(^{-1}\). To test the effects of increased calcium, we compared media containing the standard concentration with media containing four times the standard concentration (1760 mg l\(^{-1}\)). These concentrations were used in both the shoot proliferation and the microtuber production media. Other ingredients were used in their standard concentrations described above, except the monopotassium phosphate, which was used at a concentration of 510 mg l\(^{-1}\). The results of this experiment are shown in Figure 5. The numbers of microtubers in all size classes were higher in the high calcium medium, although the differences were not statistically significant.

We also tested the effect of varied concentration of nitrogen in the microtuber production medium. For this experiment, we compared a medium containing the standard concentrations of ammonium nitrate and potassium nitrate (1,650 mg l\(^{-1}\) and 1,900 mg l\(^{-1}\), respectively) with a medium containing half the standard concentrations (825 mg l\(^{-1}\) and 950 mg l\(^{-1}\), respectively). The plant proliferation medium contained the standard concentrations of these nitrate components. All other components were used in their standard concentrations in both media except the calcium chloride dihydrate (1,760 mg l\(^{-1}\)) and the monopotassium phosphate (510 mg l\(^{-1}\)). We found that the medium containing the half strength nitrogen produced significantly higher total numbers of microtubers, although the differences within each size class were not significant (Figure 6).

**Relationship between plant proliferation rate and number of microtubers per culture bag**

Since different potato cultivars have different growth and productivity, we used four cultivars (‘Sassy,’ ‘Cynthia,’ ‘Alowa,’ and ‘Jagakids Purple’) to assess the relationships between plant proliferation rates and microtuber yields. As expected, we found that the numbers of microtubers per culture bag varied among cultivars. In productivity order from the highest to the lowest, the average numbers of microtubers per culture bag were 276.4 for ‘Alowa,’ 198.7 for ‘Sassy,’ 189.8 for ‘Cynthia,’ and 91.5 for
'Jagakids Purple.' The average weights of plants at the end of the plant proliferation step for each of these cultivars were 1,101.8 g, 871.8 g, 944.6 g, and 613.6 g, respectively. A scatter plot of the data is shown in Figure 7. There was a highly significant positive correlation ($r = 0.834$) between the weights of plants and the numbers of microtubers per culture bag when the correlation coefficient was calculated with all cultivars included. When the correlation coefficients were calculated for each cultivar, there were significant positive correlations for Cynthia and Jagakids Purple ($r = 0.861$ and $r = 0.716$) and there were not significant correlations for Sassy and Alowa.

Discussion

We have developed a system for large scale microtuber production using plastic culture bags in the present study, and successfully produced 100 to 300 microtubers per culture bag, depending on the cultivar. There are several advantages in using the culture bags rather than bioreactors. The first is that culture bags are much cheaper than bioreactors, and the bags can be used several times if cleaned and autoclaved between uses. Secondly, the culture bags are much easier to sterilize than bioreactors because they can be flattened. Only two sets of bioreactor with total volume of 8 l can be sterilized in an autoclave with the inner volume of about 50 l (for example, TOMY ES-315). On the other hand, 8–10 pieces of the culture bag can be sterilized in the same autoclave. The space needed to store unused culture bags is also much smaller than the space needed for bioreactors. The third advantage is that bioreactors made of glass are very fragile, whereas culture bags are easily and safely handled. Because of these advantages, culture bags are ideal for large-scale microtuber production. These microtubers were planted in the field and successfully utilized for seed potato production.

In the previous studies, microtubers were mostly formed at the surface of the medium, although tuberization was observed in all parts of plants grown in jar fermentors with semi-continuous medium surface level control (Akita and Takayama 1993, 1994) and in a temporary immersion system (Jiménez et al. 1999). In contrast, microtubers were formed from the surface of the medium up to the tops of the culture bags in the present study. Since the liquid and gaseous phases inside the culture bags were aerated with flow rate of 200 ml min$^{-1}$ and 800 ml min$^{-1}$, it is likely that the aeration of the gaseous phase in the culture bags had a similar effect to the semi-continuous control of the medium surface level in the system used by Akita and Takayama (1993, 1994). However, the aeration of the gaseous phase is much simpler, and suitable for a low-cost production system. Zobayed et al. (2001) found that the growth and quality of plantlets, and sizes of the microtubers were greatly enhanced by introducing a forced ventilation system, although they used an agar-solidified medium.

In the present study, we succeeded to increase the number of microtubers per culture bag by changing the concentrations of some medium components. It is notable that the higher concentration of phosphate increased the numbers of large microtubers. Haverkort et al. (1991) found that when planted in the field, larger microtubers grew into plants with more stems and more tubers per plant, greater tuber yields per plant, and higher harvest indices. Lommen and Struik (1994) reported that heavier minitubers gave more consistent emergence, faster ground cover soon after emergence, higher dry-matter yields, and higher fresh tuber yields. Large microtubers are more tolerant to field conditions than smaller microtubers (Ranalli 1997). Therefore, it is very important to produce large microtubers in practical
The concentration of sucrose in the plant proliferation medium affected the numbers of microtubers per culture bag. In a preliminary experiment, we found that sucrose concentrations lower than 10 g l\(^{-1}\) gave poor plant growth (unpublished results). Therefore, we tested sucrose concentrations of 10, 20, and 30 g l\(^{-1}\) in the shoot proliferation medium. When the concentration was 30 g l\(^{-1}\), some shoots became thickened and the numbers of shoots decreased. The lower sucrose concentration (10 g l\(^{-1}\)) led to the development of higher numbers of shoots and microtubers. We also studied the concentration of sucrose (10 g l\(^{-1}\) and 15 g l\(^{-1}\)) in the medium for microtuber production step, and there was no difference in microtuber production between these two concentrations (data not shown). This result was similar with the results in a review by Donnelly et al. (2003).

Arvin et al. (2005) studied the calcium concentration in media for microtuber production, and obtained highest yields with 10 mM Ca (1470 mg l\(^{-1}\) calcium chloride dihydrate). In our study, higher calcium concentrations resulted in higher total yields, but the data showed no significant differences.

Plant growth is very important in the production of microtubers, and in general, plants with more shoots produce more microtubers. The ‘Alowa’ plants grew well and there was no room for more plant proliferation in the culture bags we used. Therefore, the volume of the culture bag may be a limiting factor in microtuber production with ‘Alowa.’ The growth of ‘Jagakids Purple’ was poor comparing to the other cultivars, and improvements in plant proliferation rates might lead to higher yields with this cultivar.

Potato microtubers have dormancy periods that vary depending on the cultivar. Microtubers of cultivars with longer dormancy periods can be produced earlier in the year, and those with shorter dormancy periods can be produced later. Therefore, we can produce microtubers about 3 times in a year using the same culture rooms. In this way, large numbers of microtubers can be produced and planted directly into the field for seed potato production.

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