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Growth Responses and Construction Costs of Various Tissue Culture Systems

Brent Tisserat¹

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Summary. The influence of the culture chamber size and medium volume on the growth rates of shoot tips of peas, lettuce, kidney beans, and spearmint were determined after 8 weeks of incubation. Cultures were grown in a variety of culture chambers including culture tubes, baby food jars, Magenta GA-7 containers, 1-pint Mason jars, 1-quart Mason jars used with and without an automated plant culture system (APCS), 0.5-gal Mason jars with and without an APCS, Bio-safe chambers with an

APCS, and polycarbonate culture chambers with an APCS having culture chamber volumes of 55, 143, 365, 462, 925, 1850, 6000, and 16,400 ml, respectively. Plans are presented for the construction of various culture chambers used in an APCS. The APCS consisted of a peristaltic pump, media reservoir containing 1 liter of liquid nutrient medium, and a culture chamber. Cultures grown with an APCS consistently produced higher fresh weights than cultures using any of the agar culture systems tested. Growth rates varied considerably depending on the plant species and culture system tested. Peas, lettuce, and spearmint exhibited flowering only when grown in the APCS. A cost comparison using the APCS versus various conventional tissue culture systems is presented.

C current plant tissue culture technology is based on establishing cultures in culture tubes and other small-capacity vials and jars. Periodic reculturing (usually every 8 weeks) is necessary to replenish medium and combat toxins released by growing cultures (Bajaj, 1991). During the reculturing process, roots and shoots are often divided and trimmed to reduce physically the size of the culture in order to facilitate its transfer into new vessels. Division also may promote culture proliferation (Bajaj, 1991).

Recently, automation of plant tissue culture has become a realistic possibility (Bajaj, 1991). The goal of automation is to reduce the cost of producing cultures in vitro. Plant tissue culture automation systems using robotics, bioreactors, and sterile hydroponics systems have been suggested (Bajaj 1991). Robotics mimic the la-

bor-intensive activities currently involved in the reculturing process, but these are prohibitively expensive for the typical researcher. Similarly, bioreactors are expensive (\$5000 to \$25,000) and are also beyond the economic means of most researchers. Bioreactors aerate, stir, and maintain the chemical composition of the liquid medium and have been used mainly to grow plant suspension cultures (Lipsky, 1992; Teng et al., 1993). Bioreactors usually are limited to propagule initiation and somatic embryos <3 cm in length and are not amenable to larger plantlet development.

In contrast, a sterile hydroponic or automated plant culture system (APCS) is considerably less expensive to construct and test than other automation systems. In the APCS, a plant culture is established and maintained in a single chamber while medium is introduced and removed daily. These systems are particularly suitable to large plantlet growth unobtainable in other culture systems. In such systems, the necessity of conducting culture division to promote proliferation is eliminated. Several such novel automated systems have been developed (Aitken-Christie and Davies, 1988; Simonton et al., 1991; Tisserat, 1991; Tisserat and Vandercook, 1985; Weathers et al., 1988). In those reports, a growth comparison between the automated system and the traditional agar-gel culture system was conducted, which demonstrated the superiority of the new technology relative to the traditional medium.

For any plant tissue culture automation system to be used widely, an appraisal must be conducted to determine whether it is financially worthwhile to invest in alternative technologies to culture plants versus retaining the standard culture systems. The first purpose of this study was to construct a low-cost APCS that is assembled easily. With this APCS, accelerated plant growth over that obtained using existing traditional agar systems was achieved. Many automation systems, which tend to be experimental, often use expensive and sophisticated culture chambers and equipment (Bajaj 1991). The costs involved in constructing and operating automated systems usually have been ignored. Secondary purposes for this study was to compare 1) growth rates with various plants over an 8-week period and 2) the costs

¹Research chemist/microbiologist, U.S. Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Fermentation and Biochemistry Research Unit, Peoria, IL 61604.

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for construction and use of a number of tissue culture systems. Through such comparisons, the practicality of using novel automated systems can be determined.

Materials and methods

Cultures and media. Shoot tips of peas (*Pisum sativum* L. 'Super Snappy'), lettuce (*Lactuca sativa* L. 'Great Lakes #118'), kidney beans (*Phaseolus vulgaris* L.), and spearmint (*Mentha spicata* L.) were obtained from seeds germinated under sterile conditions. Nutrient basal medium (BM) contained Murashige and Skoog salts and the following in mg·liter⁻¹: thiamine·HCl, 0.5; i-inositol, 120; sucrose, 30,000; and agar (A 4550) (Sigma Chemical Co., St. Louis), 8000. The pH was adjusted to 5.7 ± 0.1 with 0.1 N HCl or NaOH before adding agar. Liquid medium pH value was adjusted to 5.0. BM was dispensed in 25-ml aliquots into culture tubes [150 mm height × 25 mm diameter; 55 ml capacity (cap)] and baby food containers (76 mm height × 60 mm diameter; 143 ml cap) (Sigma Chemical Co.); 50-ml aliquots were dispensed into Magenta (GA 7) polycarbonate containers (70 mm long × 70 mm wide × 100 mm high; 365 ml cap) (Sigma Chemical Co.), 1-pint Mason jars (130 mm height × 78 mm diameter; 462 ml cap), and 1-quart Mason jars (180 mm height × 91 mm diameter; 925 ml cap); and 100-ml aliquots were dispensed into 0.5-gal Mason jars (250 mm height × 107 mm diameter; 1850 ml cap). Culture chambers were capped with polypropylene lids. Mason jar closures were fitted with cotton plugs. The APCS used a 0.5-gal Mason jar containing 1 liter of BM. All media were sterilized for 15 min at 1.05 kg·cm⁻² and 121°C. Shoot tips of peas, lettuce, and bean were cultured in culture tubes, baby food jars, Magenta containers, 1-pint Mason jars, 1-quart Mason jars, and 1-quart Mason jars coupled to an APCS. In later experiments, spearmint shoot tips were cultured in culture tubes, baby food jars, Magenta containers, 1-pint Mason jars, 1-quart Mason jars, 0.5-gal Mason jars, 0.5-gal Mason jars with APCS, 6000-ml Bio-Safe container with APCS, and 16,400-ml polycarbonate chambers with APCS. Three 2-week-old, 1-cm-long shoot tips were cultured in each system. Ten replicates per treatment were planted originally,

and experiments were repeated three times. After 8 weeks of incubation, data on culture fresh weight, leaf length, plant height, and number of flowers were recorded and analyzed with Student-Newman-Keuls multiple range test when appropriate. Simple correlation coefficients were calculated to compare culture fresh weight, leaf length, or plant height to culture chamber capacity, medium volume used, or culture chamber height. Cultures were incubated at a constant 26 ± 1°C under 16 h daily exposure to 20 μmol·m⁻²·s⁻¹ coolwhite fluorescent lamps.

Construction of the automated systems. A list of items to construct the 16,400-ml capacity mega-APCS is presented in Table 1. The culture chamber was constructed of two transparent, interlocking, polycarbonate food pans (325 mm long × 265 mm wide × 300 mm high; 8200-ml cap) (Cambro, Huntington Beach, Calif.). The cover pan was modified by mounting a silicone tape gasket (112 cm long × 6.3 mm wide × 3.2 mm thick) (Furon, New Haven, Conn.) (Fig. 1A). The

bottom chamber was modified by mounting a silicone gasket (described above), three bacterial hydrophobic air vent filters (Gelman Sciences, Ann Arbor, Mich.), and a polypropylene spigot to allow for the inflow and evacuation of nutrient medium (Fig. 1A). The filters were attached to the pan with silicone tubing to polypropylene 1.6-mm I.D. female barbed fittings (Ark-Plas Products, Flippin, Ark.) and then attached to 1.6-mm I.D. male barbed fittings, which were threaded into 1/8-27 threaded holes. The spigot consisted of a 90° 1.6-mm I.D. female barbed fitting attached to a male barbed fitting mounted to a 1/8-27 NPT tapped hole in the bottom chamber. Polypropylene washers (1.6 cm O.D. × 1.0 cm I.D. × 0.2 mm thick) and silicone O-rings were used to secure the fittings. The culture chamber was attached to the medium reservoir with silicone tubing (145 cm long × 6.5 mm O.D. × 3mm I.D.) (Fig. 1A; Table 1). A layer of glass gravel, 10 mm deep, was applied to the bottom of the chamber. After culture planting

Table 1. Materials required for constructing an APCS.

Item description	Model no.	Quantity	Cost (\$)	Source ^a
Culture chamber				
Cam-Wear food pan	CW22	2	7.00	1
Binding clip	---	6	0.25	2
Silicone rubber tape	---	2	3.52	3
Silicone sealant	---	100 ml	1.50	4
Filter, air vent	4210	3	3.33	5
Silicone tubing	L-06411-66	152.5 cm	0.03/cm	6
Barbed female fitting	081227KF18P	1	0.16	7
Barbed female fitting	071227TF12P	3	0.19	7
Barbed male fitting	011227FA12P	4	0.07	7
Washers, polypropylene	---	3	0.13	8
Bag, polypropylene	2782	1	0.54	9
Clamp, ratchet	L-06833 -00	1	0.50	6
Gravel, glass	---	2.2 kg	3.08	4
Cable ties, nylon	2782	4	0.02	6
O-rings, silicon	---	4	0.11	10
Glass media reservoir				
Mason jar, 0.5-gal	70610-00505	1	0.80	11
Filter, air vent	4210	1	3.33	5
Silicone tube	L-06411-66	5 cm	0.03/cm	6
Polypropylene tubing	L-06400-31	20.3 cm	0.01/cm	6
90° barbed male fitting	081227KM12P	3	0.11	7
Barbed female fitting	071227TF12P	1	0.19	7
Washers, polypropylene	---	2	0.13	8
Cable ties, nylon	2782	2	0.02	6
O-rings, silicon	---	4	0.11	10
Cost			50.38	

^aSource of items: 1 = Cambro Co., Huntington Beach, Calif.; 2 = Local Stationary Store; 3 = Furon, New Haven, Conn.; 4 = Local Pet Store; 5 = Gelman Sciences, Ann Arbor, Mich.; 6 = Cole-Parmer Instrument Co., Chicago, Ill.; 7 = Ark-Plas Products, Flippin, Ark.; 8 = Boker's Inc., Minneapolis, Minn.; 9 = Dynalab Corp., Rochester, N.Y.; 10 = MSC, Plainsview, N.Y.; 11 = Local Supermarket.

Fig. 1. Examples of various culture systems used. (A) Diagrammatic representation of the mega-APCS. Note that a silicone gasket is fitted between the cover and bottom pieces of the culture vessel to provide a barrier to the outside environment. Growth responses of spear mint shoot tips in various culture systems after 8 weeks. (B) Spear mint tips in traditional agar culture systems. From left to right: baby food jar, culture tube, Magenta container, 1-pint jar, 1-quart jar, and 0.5-gl jar. (C) Comparison between spear mint tips grown in culture tube, baby food jar, and APCS using Bio-Safe container. Arrows denote the occurrence of flowers. (D) Growth of spear mint tips in mega-APCS. Bar = 10 cm.

on the surface of the glass gravel, the culture chamber was filled with medium up to the top of the glass gravel, and then the medium was evacuated. Six stationary binding clips (50 mm long) were used to seal the two pans together (Fig. 1D). In some cases, a polycarbonate Bio-Safe container (379 mm long $\times$ 170 mm wide $\times$ 178 mm high; 6000 ml cap) (Nalgene Co., Rochester, N.Y.), a 0.5-gal Mason jar, or a 1-quart Mason jar was used as the culture chamber in the APCS. In cases in which Mason jars were used as cul-

ture chambers, three holes were drilled into the polypropylene closure. Two filters were attached with silicone tubing to 90° 1.6-mm I.D. male barbed fittings that were threaded with 1/8-27 threaded holes. The third hole was used as the medium spigot that consisted of a 90° 1.6-mm I.D. male barbed fitting attached to a 1.6-mm I.D. female barbed fitting. A polypropylene tube attached to the female fitting extended to the bottom of the culture chamber. The Bio-Safe container was modified in the same manner as the mega-APCS, except the air filters were mounted in the top container (Fig. 1C). Nylon cable ties (50 mm long) were used to secure silicone tubing to connectors, and all chambers were autoclave in a clear polypropylene bag (45.7 $\times$ 58.4 cm). Cultures were soaked once daily for 5 min, and then the medium was evacuated back to the reservoir. Media pumping was timed using digital program timers (Intermatic, Spring Grove, Ill.).

Results and discussion

Influence of culture systems on growth. Culture of pea, lettuce, and

bean shoot tips on various culture systems showed that higher fresh weights were obtained consistently using an APCS, i.e., 1-quart Mason jar serving as the culture chamber, compared to using the non-APCSs (Fig. 2). Pea, lettuce, and bean exhibited 1.5-, 4.3-, and 5.3-fold increases in fresh weight, respectively, when grown in APCSs compared to plants grown in culture tubes (Table 2). Bean tips usually produced higher flesh weights when cultured in larger containers, regardless of whether an APCS was co-used. In contrast, pea and lettuce tips did not exhibit any significant difference in fresh weights when cultured on any of the non-APCS vessels (Table 2). It should be noted that the APCSs used greater medium volumes (i.e., 1-liter BM) than the non-APCSs (i.e., 25- or 50-ml BM). The additional BM contributed to superior growth in the APCSs. Significant ($P = 0.05$) positive correlation coefficients occurred between fresh weight and medium volume used. Fresh weights of pea, lettuce, and bean exhibited correlation coefficients, of 0.99, 0.96, and 0.84, respectively, compared to volume of

Fig. 2. Growth of vegetable seedlings in various types of culture systems. Three seeds were cultured per container for each vegetable type. Data were averaged for 10 replications/treatment. Experiments were repeated three times and single representation is presented. Mean separation by Student-Newman-Keuls multiple range test ($P < 0.1$). Columns with the same letter on top were not significantly different.

medium used. In contrast, no significant correlation was found relating culture chamber height or culture chamber area with fresh weights of these plants. Plant height and leaf size were not significantly different among any of the plants tested, regardless of culture system used. Pea and lettuce tips exhibited flowering in the APCS only, but not when cultured in the other systems; beans did not exhibit any flowering in any system tested (data not shown).

Based on these experiments, larger chambers coupled to the APCS were used to test the growth of spearmint shoot tips (Fig. 1 B–D). Using the largest culture chamber tested, the mega-APCS, spearmint cultures exhibited a 10.8-fold increase in fresh weight compared to cultures grown on agar medium in culture tubes (Table 2). Lesser increases occurred using the smaller culture chambers with the APCS, but all APCSs produced substantially more fresh weight than the agar culture tubes (Fig. 1 B–D). Unlike in the previous experiments, highly positive correlations between culture weight, leaf length, or plant height and the culture chamber capacity, media volume, or culture chamber height occurred for spearmint cultures (Fig. 3).

Also associated with growing spearmint in larger culture chambers is the transformation of the vegetative morphology to the formation of reproductive structures (i.e., flowers) (Fig. 1C). Spearmint tips only flowered in the chambers using the APCS. Generally, as the culture chamber size increased using an APCS, a corresponding increase in the number of flowers was exhibited, as was an increase in fresh weight (data not shown). As many as 50 to 60 flowers occurred in the mega-APCS; fewer occurred in the smaller APCS vessels. This result paralleled what occurred when pea and lettuce tips were cultured on APCSs and non-APCSs. Since all the cultures were the same age, culture vessel size

Table 2. Relationship between different culture systems and cost/g fresh weight and fresh weight increases with various plants. Growth increases compared to cultures grown in agar tubes are shown in parenthesis.

Culture system	Costs (\$)/fresh wt (g): (fold x) ^a			
	Spearmint	Peas	Lettuce	Bean
Tube	0.13 (---)	0.26 (---)	0.70 (---)	0.08 (---)
Baby food	0.11 (1.3)	0.27 (1.0)	0.85 (0.9)	0.07 (1.5)
Magenta	0.84 (0.6)	1.28 (0.8)	1.89 (1.4)	0.16 (1.8)
1-Pint jar	0.20 (1.2)	0.62 (0.8)	1.29 (0.1)	0.08 (1.6)
1-Quart jar	0.21 (1.7)	0.60 (1.1)	1.73 (1)	0.07 (2.7)
1-Quart-APCS	--- (---)	4.13 (1.5)	3.74 (4.3)	0.34 (5.3)
0.5-Gal jar	0.19 (2.3)			
0.5-Gal-APCS	0.45 (7.1)			
Bio Safe-APCS	2.75 (4.7)			
Mega-APCS	0.74 (10.8)			

^aCost/g is calculated by adding container costs/g and media cost/g.

Culture Systems

as well as medium volume were important factors for flowering to occur in vitro for pea, lettuce and spearmint.

Notes on cost factors. Comparisons of the costs involved in using several culture systems are presented in Table 3. For example, a 25 × 150-

mm culture tube, 55-ml cap, costs \$0.81 each to construct, whereas the mega-APCS, 16,400 ml cap, costs \$50.38 each to construct. The cost difference between these two systems is great. However, in terms of cost (\$) per sterile volume or capacity, the test

Fig. 3. Relationship between culture weight, leaf length, and plant height of spearmint shoot tips to various culture systems. Correlation coefficients (r) between culture weight, leaf length, and plant height and culture chamber capacity, nutrient medium volume used, or culture chamber height are given. All correlations were significant at $P = 0.05$.

Table 3. Comparative costs of culture systems based on culture volume.

Item	Capacity (ml)	Source ^a	System costs (\$) ^b	Cost (\$)/ml
Culture tube	55	1	0.81	0.015
Magenta container	365	1	3.16	0.009
Baby food jar	143	1	0.85	0.006
1-Pint Mason jar	462	2, 3	1.43	0.003
1-Quart Mason jar	925	2, 3	2.11	0.002
1-Quart jar-APCS	925	2, 3	19.23	0.021
0.5-Gal Mason jar	1850	2, 3	2.51	0.001
0.5-Gal jar-APCS	1850	2	19.66	0.011
Bio-Safe-APCS	6000	2, 4	80.74	0.013
Mega-APCS	16400	2	50.38	0.003

^aSource of items: 1 = Sigma Chemical Co., St. Louis, Mo.; 2 = See suppliers in Table 1; 3 = Consolidated Plastics Co., Twinsburg, Ohio; 4 = Daigger and Co., Wheeling, Ill.

^bCost reflects chamber, closures, and partial use of trays to use culture systems

tube costs about \$0.015/ml and the mega-APCS cost is \$0.003/ml (Table 3). In this perspective, the mega-APCS is more economically priced than the other systems. BM was estimated to cost about \$0.01/25 ml. Another factor to consider is the cost to obtain a fixed amount of culture tissue (i.e., cost (\$)/g fresh weight). This is derived by combining the costs of media and culture vessel and dividing by fresh weight (g) of tissue produced by the system (Table 2). As shown in Table 2, although enhanced growth rates can be achieved using a variety of APCSs (e.g., the mega-APCS produced a 10.8× increase in spearmint growth over growth in culture tubes), it costs five times more (e.g., \$0.13/g in culture tubes versus \$0.74/g in mega-APCS). Appraisals such as these should be included in the discussions of future, novel, automated culture systems to determine their economic merits.

Remarks

Selection of pea, lettuce, bean, and spearmint was arbitrary. Theoretically, it should not matter what plant materials are tested in the various culture systems to determine a culture system's attributes (i.e., growth rates and costs). Nevertheless, plant source was related to the growth rates obtained in the various culture systems and greatly influenced the cost/g of tissue produced. Depending on the plant and culture system used, growth rates were not uniform but were decidedly different. For example, spearmint grew about 10.8× faster in the mega-APCS than in the culture tube containing agar. Pea, lettuce, and bean grew 1.5-, 4.3-, and 5.3-fold faster,

respectively, in the 1-quart Mason jar-APCS than in the culture tube containing agar. These results demonstrate that, although rapid rates of growth can be achieved in a short period (8 weeks) when an APCS is used, growth rates are plant-specific. In other long-term studies, e.g., 9 months, stationary cultures in the APCS clearly grew considerably faster than recultured cultures used in the traditional agar tissue (Tisserat and Vandercook, 1985). However, traditionally plants are recultured every 8 weeks, therefore a comparison over this time-frame was conducted in this study.

One of the major expenses in culturing plants in vitro using an APCS is the cost of the multiple-channel peristaltic pump (Tisserat and Galletta, 1993). An APCS can use any type of peristaltic pump. Using inexpensive, reliable peristaltic pumps is critical to reduce costs of an APCS and make the system cost-competitive with other tissue culture systems (Tisserat and Galletta, 1993). Another way to reduce costs is to use inexpensive digital timers instead of more sophisticated computerized systems. Future APCSs could be procured either commercially at low prices or constructed by researchers with only minimal expertise and time requirements. It is unlikely in the foreseeable future that expensive, patent-protected, highly sophisticated automation systems or robotics will be used widely due to their procurement and operation costs. More likely, the automation systems that will be used widely in the future will be those that are simple to construct, flexible (i.e., can be adapted easily to various conditions as required),

and inexpensive (both to operate and to construct).

In this study, a comparison of growth rates related to culture chamber size, and costs was emphasized. The APCS chambers used in this study had internal capacities of 925, 1850, 6000, and 16,400 ml. Any of these chambers can be used with the peristaltic pump and culture medium reservoir and give enhanced growth compared to existing tissue culture technology. Best growth, however, occurred in the largest chamber tested, i.e., the mega-APCS. Other investigators have used large culture chambers in their automated plant tissue culture studies and similarly obtained superior results to the APCSs presented in this study when compared to traditional agar culture method (Aitken-Christie and Davies, 1988; Simonton et al., 1991; Weathers et al., 1988). Simonton et al. (1991) used a 7000-ml polycarbonate culture chamber and was able to report doubling of the fresh weights of *Spathiphyllum* shoot cultures compared to growing cultures in agar medium in jars after 35 days in culture. Similarly, in this study, a 16,400-ml mega-APCS gave impressive results with spearmint tips. Large, sterile chambers can be obtained inexpensively from various commercial sources. The simplified technology offered in this study should allow any researcher in the plant tissue culture field to experiment with automated systems. Using the larger culture chambers also will allow for the study of the reproductive events in the in vitro environment, which often is not possible using the traditional tissue culture vessels (i.e., test tubes). It also should be noted that additional costs in labor are necessary to prepare and plant additional agar cultures to equal the growth rates obtained from a single APCS. This study revealed mixed results: 1) growth in APCSs exceeds growth obtained in traditional agar culture systems, but 2) APCSs are more expensive than agar culture systems in terms of cost/g fresh weight obtained. Clearly, further work is necessary to reduce the costs of automation with APCSs. Justification for using an APCS could be to obtain enhanced growth due to time constraints or to procure reproductive organs or unique plant developmental patterns only possible in an APCS environment, which traditional agar culture systems cannot provide.

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