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BARD

FINAL REPORT

Project No. US-1678-89

Alternative Techniques for Plant Micropropagation

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BARD Project No. US-1678-89 Alternative Techniques for Plant Micropropagation

ABSTRACT

During the three years of BARD Project No. US-1678-89, alternative techniques for plant micropropagation have evolved around the concept of liquid/membrane culture. Research occurred in the following phases: (1) identification of an alternative support matrix for conventional agar, (2) development and automation of continual-flow, liquid/membrane bioreactors, (3) studies of nutrient depletion with a liquid/membrane culture, (4) growth assessment of several species in liquid/membrane cultures and (5) development and testing of mechanical devices for separation and transfer of plant tissue.

A microporous, polypropylene membrane (Celgard[®], Hoechst Celanese Separation Products Division, Charlotte, NC) emerged as an alternative support matrix on liquid media that produced superior or at least equal growth of several species to that on conventional agar. The liquid/membrane concept had new potentials for mechanization and was also a microbial barrier against contamination. The microporous polypropylene membrane was commercialized by Sigma Chemical Co. as membrane rafts with rigid polypropylene sidewalls and a membrane bottom (Models M 3929 and M 4380). Scale-up liquid bioreactors were developed utilizing the Nalgene "Bio-Safe" polycarbonate box. These bioreactors accommodated 10 Sigma membrane rafts and, as modified, enabled automatic sensing and computer control of sucrose and dextrose medium composition. Growth of tobacco disc initiation cultures was visibly increased over agar in a 28-day liquid/membrane culture cycle with automated control of medium concentration.

Nutrient depletion studies of watermelon tissues in proliferating and elongating liquid medium revealed that Ca^{++} and K^{+} reduced minimally over a 35-day growth period while NO_3^{-} and NH_4^{+} depleted significantly. Sugars remained high at the end of the experiments with 72.3% and 42.8% of initial levels for proliferation and elongation, respectively. Doubling or greater of fresh weight gains have been observed with *Rex begonia* cv. Peacock, *Nicotiana tabacum* cv. Burley 21 and *Citrullus lanatus* cv. 'Charlee'. Higher relative dry matter content of watermelon, however, in agar-solidified than in liquid medium indicated a unique vitrification tendency. Similar enhancements in growth rates were also observed for three species of orchids in liquid/membrane culture. Other species observed to have greater growth on liquid/membrane culture include rhododendron, cherry, tomato, hosta, fern, banana and sweet potato.

Wire grid cutting devices have increased the cutting output over scalpel and forceps by a factor of 14. When utilized in a cut-and-dump protocol, this device became 4.8 times more productive for the total transfer process with both time and yield of viable bud clusters considered than scalpel and forcep procedures.

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Introduction

Conventional plant tissue culture technology involves growing micropropagules in small vessels on nutrient medium contained in semisolid agar or, less frequently, in liquid suspension which is constantly agitated to enhance aeration. From the initiation of these cultures, both methods are characterized by medium in which one or more components progressively deplete. Furthermore, both methods involve frequent, tedious, highly labor-intensive subcultures, or transfers, onto fresh medium after 4-8 weeks. Competitiveness of micropropagated plants with those produced via traditional propagation is currently limited to crops with high profit margins such as ornamentals or plants of superior properties. This research was directed first at a mechanism, namely liquid, for continually replenishing medium while providing aeration of the tissue comparable to an agar culture to assure optimal growth. A flowing liquid medium with an artificial support material replaced agar. The liquid/membrane culture technique was scaled-up into a larger polycarbonate bioreactor that accommodated 10 membrane raft units and could be automatically sampled and computer controlled to maintain a setpoint level of sugar concentration. Depletions of several nutrient components in liquid medium were monitored and quantified using ion selective electrodes.

Secondly, a priority of this project was defining the hand labor components in Stage II culture and exploring the potentials of simple mechanical devices as alternatives to scalpel and forceps for explant separation and transfer. The concept of unitized, non-selective mass cutting with a wire cutter device was tested.

Objectives

Initially, there were two primary objectives for this research:

- * To investigate and evaluate a continual-flow, nutrient-controlled liquid plant tissue culture methodology compatible with commercial production;
- * To develop and evaluate mechanical techniques and devices for mass separation, singulation and transfer of micropropagules.

The various research activities conducted under this project have adhered very closely to these two original objectives. Perhaps one extended activity was the nutrient depletion work done with watermelon. Such research has never been possible with agar since melting it to obtain measurable samples would likely alter composition. The liquid/membrane

culture technique affords a very important research tool that can now be used extensively by plant tissue culturist to achieve optimal medium compositions for specific growth and morphology objectives. Moreover, this capability to determine *in situ* levels of media components permits automated adjustments that can optimize the outcome of a given culture.

Methods and Materials

Alternative Support Matrices

The most apparent deficiency of liquid suspension culture compared to agar culture is its inability to support the tissue so that it receives sufficient aeration for non-vitrified, or non-hyperhydrated, growth. Consequently, as we initiated our work with continual-flow liquid culturing, we surveyed various materials as alternatives to agar for physical support of tissue. Based on criteria such as autoclavability, strength, absorbancy, inertness to plant tissue and reusability, we screened approximately 30 material options including paper, plastics and wire screening. Four materials were selected for testing: non-woven polypropylene fiber, glass beads, microporous polypropylene membrane and polypropylene netting.

Each of the materials were used as growth support matrices in both static vessels and in dynamic flow vessels. The static liquid medium tests were conducted in Magenta GA-7 containers, square plastic vessels designed specifically for the plant tissue culture industry. Dynamic liquid flow tests were conducted in larger bioreactor vessels constructed initially from modified glass beakers and later from a Nalgene "Bio-Safe" polycarbonate box.

Continual Flow Bioreactor Development

The development of a scale-up whole plant bioreactor involved a progression through three basic designs. The first attempt coupled a group of Magenta vessels together with tubes feeding into the bottoms of each vessel. Media were pumped into and from all vessels simultaneously through a common, "autoclavable" diaphragm pump in alternate wetting and drying cycles. Media were reused from a single supply reservoir.

A second-generation continual-flow apparatus used modified, 7-inch diameter, heavy duty glass beakers with media supplied by siphoning or a peristaltic pump from a supply reservoir, Figure 1. The beakers were closed by flat glass tops seated on flat ground beaker rims. Liquid media entered each beaker through a port in the sidewall approximately 50 mm above the bottom. This port was sufficiently high above the bottom of the beaker to assure a physical discontinuity of liquid between the beaker and the supply lines to restrict contaminant transport. Spent media were drained at desired intervals by gravity flow through the bottoms of the beakers into a common waste reservoir. Media were not reused.

Based on these two previous experiences, the following design criteria were established for a third bioreactor:

- All components had to be autoclavable;
- The growth chamber must be transparent for adequate light transmission and visibility;
- Materials must be more breakage resistant than glass;
- Components must be easily disassembled for thorough cleaning;
- Number of openings and component connections must be minimized to reduce possible contamination entry.

A commercially-available polycarbonate Nalgene "Bio-Safe" box was adapted as the growth chamber of the bioreactor system. It accommodated 10 microporous polypropylene Sigma membrane rafts scaled for the conventional Magenta GA-7 vessel (55 cm x 55 cm). The growth chamber was connected by flexible silicone tubing through separate media inlet and outlet ports to two polypropylene jars (1.9 l and 3.8 l) which functioned as media supply and waste reservoirs, respectively (Figure 2). Connections were made with polypropylene luer lock tubing fittings and silicone O-rings. Each jar lid had a filtered gas exchange vent. The lid of the supply media jar contained a third filtered tube which facilitated adding fresh, non-sterile media to the bioreactor even after it had been inoculated with plant tissue. The end of the polypropylene tubing for the media inlet inside the chamber was positioned high enough to provide a physical break between itself and the liquid nutrient pool. Removal of nutrient from the chamber was facilitated by using the Bio-Safe box in an inverted orientation so that a recessed annulus in its upper half provided a lowest-level drainage line to the open end of an outlet port. A non-invasive nutrient sampling port was constructed in the lower end of the growth chamber by placing a septum and silicon O-ring combination in the end of a

Figure 1. Continual flow beaker bioreactor.

Figure 2. Nalgene "Bio-Safe" box reactor.

polypropylene pipe adapter compression fitting designed for rigid and semi-rigid tubing. The septum created a sterile seal which was easily pierced by a syringe for periodic nutrient sampling and resealed. Chamber gas exchange was facilitated by three venting ports in its upper half. An entry port was connected through a hydrophobic, microbial ($0.2\ \mu\text{m}$) filter inside the chamber to a rigid 3.2 mm diameter polypropylene distribution tube with holes drilled at 12.7 mm intervals over the length of the chamber. Similar filter elements were interior-mounted over the two gas exit ports in the opposite end of the chamber.

Figure 3. Automated nutrient-controlled bioreactor.

intervals and analyzed for sucrose and dextrose concentration. These data were sent to a computer which used specially developed software to compare with a setpoint reference level for sucrose. If the measured levels deviated from setpoint the computer activated control valves and a supply control pump to mix appropriate portions of concentrated sucrose with concentrated mineral salts and hormones and distilled deionized water to correct automatically the concentration and volume of medium in the bioreactor.

Nutrient Depletion Studies with Watermelon

Proliferating shoot meristems of watermelon (*Citrullus lanatus*) from established shoot cultures were inoculated on modified Murashige and Skoog (MS) medium. The MS medium was supplemented with $10\ \mu\text{M}$ 6-benzyladenine (BA) for shoot proliferation and with $1\ \mu\text{M}$ BA and $10\ \mu\text{M}$ gibberellic acid (GA_3) for shoot elongation. Both agar-solidified medium and microporous polypropylene membrane rafts in liquid medium were used to support the tissue. Growth comparisons were made for proliferating and elongating tissues in liquid/membrane and agar culture for equal culture times. In the liquid/membrane cultures, nutrient depletion was periodically quantified with ion selective electrodes.

The "Bio-Safe" bioreactor was subsequently altered to the configuration shown in Figure 3 to develop an automatically controlled level of sugars in the medium. A YSI 2700 sugar analyzer was placed inline with a media circulation pump which constantly kept the medium uniformly mixed by creating a recycling loop with the bioreactor. Small aliquots of medium were extracted from the bioreactor at set time

Selected Growth Studies

Three plant varieties were selected for performance testing of the bioreactor system: *Rex begonia* cv. Peacock (begonia), *Nicotiana tabacum* L. cv. Burley 21 (tobacco) and *Citrullus lanatus* cv. 'Charlee' (watermelon). A three-way comparison was made of tissue growth (1) in the continual-flow bioreactor, (2) on membrane rafts in static, non-replenished liquid in Magenta GA-7 vessels and (3) on agar in Magenta GA-7 vessels. Explants for the begonia and watermelon were shoot clusters from previous agar cultures. Leaf discs constituted the explants for the tobacco initiation cultures. After 28-day culture cycles with each species, final fresh weight, total fresh weight gain and final dry weight were recorded and percentage dry matter was calculated.

Growth studies were also made with several varieties of *Cattleya* orchids to compare number and size of plantlets cultures by the liquid/membrane and agar methods. Explants were mericlone plantlets provided by a local tissue culture laboratory. Tissues of comparable size and weight were selected for culture inoculations. A modified MS medium with 30 g sucrose, 100 mg i-inositol, 30 mg adanine sulfate, 2 mg glycine, 0.5 mg thiamine HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine and 0.3 mg NAA per liter at pH 5.7 was used. All cultures were made in Magenta GA-7 vessels with either 40 ml of liquid medium or 0.7% agar medium. At conclusion of the culture cycle, tissues were divided into single apex rooted plantlets. Shoots without roots or less than 0.1 gram were not scored as harvestable. Harvested plantlets were graded by sizes as more than 1gm, 0.5 - 1 gm and 0.1- 0.5 gm. Transplants were rinsed with tap water and soaked in an antibiotic, natriphene (100 µg/ml). These were placed in flats containing a fir bark, perlite, peat moss mix and placed in the greenhouse under mist (eight 15-second pulses daily). Successful acclimation was defined as fully turgid plants after six weeks in greenhouse culture.

Mechanized Separation and Transfer

Several devices for separation and transfer of tissue have been conceived and developed as alternatives to the conventional scalpel and forceps method. They included the following: (1) a trifold configuration of 8 x 8 mesh (i.e., 8 grids/in in a square pattern) polypropylene netting, (2) a sandwich type membrane raft with a microporous membrane film bottom and a polypropylene top, (3) a stainless steel wire cutter and (4) a growth-orienting mini-tray.

Explants were placed between the bottom and middle folds of the trifold netting. This device inherently served as a physical support for the explant as well as method to train the growth of plantlets through the grid spacings. The sandwich raft in Figure 4 was an extension of the concept of training plantlets to grow through a grid whereby an electric knife could be registered on the top of the grid to harvest plantlets while the less differentiated tissue could continue to propagate on liquid medium.

Figure 4. Sandwich type membrane-netting device.

Figure 5. Unitizing wire cutter.

The unitizing cutter, Figure 5, consisted of a stainless steel wire (0.025 mm diameter) mounted in a square grid arrangement on a pivotable, aluminum handle. Cutting occurred by pressing the wire grid through the plant tissue into slots made on the upper face of the cutting block. The wire grid dimensions and slot dimensions were precisely matched. Tissue was transferred by forceps to the upper face of the cutting block while the pivotal handle containing the cutting wire grid was in the open position. After being cut, tissue segments remained on the top of the cutting block ready for transfer to new vessels. Figure 6 shows an alternative configuration of the cutting block that positioned the tissue within cylindrical cells and oriented cuts with respect to the tissue clusters.

Operator functions during subculture time studies were partitioned into three components: (1) time to remove tissue from the original vessel, (2) time to cut tissue and (3) time to place divided tissue into a new vessel. These parameters were compared for scalpel and forceps (hand) manipulations versus the unitizing cutter. Three Magenta GA-7 vessels containing 16 bud clusters each were analyzed for both methods of cutting through three culture cycles. Analyses were made of tissue yields in terms of numbers of clusters containing buds and tissue fresh weights for hand cutting and unitizing cutters over three culture cycles. Subculture growth periods of 14, 18 and 22 days were compared.

Grid spacings of the wire cutters were compared by constructing three unitizing cutters of the first configuration in Figure 5 and a fourth of the configuration in Figure 6. The three grid spacings for the flat top cutting block were 4.3, 4.9 and 5.7 mm. The cylindrical cell cutting block had four equally spaced cells of 12.8 mm diameter and 9.5 mm depth. The cylindrical cells allowed insertion of the basal portion of the shoot bud cluster. Each of these cells was slit to permit the cutter wire to divide plant material in the cell into four sections. The three non-selective flat top and the one cellular unitizing cutters were compared with hand cuts through five culture cycles of 20-22 days each.

In Figure 7, the unitizing wire cutter was inverted and mounted to an aluminum support base to allow direct cutting and dumping into an open Magenta vessel(s) placed under the square grid. Clusters of plant tissue were placed on the upper surface of the wire grid and then pressed through the wire grid by the slotted cutting block. Dissected tissue segments dropped directly into the open Magenta vessel below.

An experiment was conducted to compare time efficiency and shoot bud growth among three cutting and handling, or transfer, treatments:

- (A) Hand cutting and transferring using conventional scalpel and forceps manipulations;
- (B) Cut-and-dump using the unitized, non-selective wire cutter; and
- (C) Cut-sort-and-dump using the same wire cutter but collecting the dissected tissue in a sterile petri dish and sorting out only pieces containing visibly viable buds and subsequently dumping the buds by hand into new vessels.

Another experiment was conducted to study growth and oriented cuts of watermelon shoot buds growing in a restricted cell space. Polycarbonate blocks (50 x 50 x 13 mm; 2 x 2 x ½ in) were drilled with five holes each of three treatment cell diameters: 9.5, 12.7 and

Figure 6. Oriented-cell wire cutter.

Figure 7. Unitized cutter mounted on base for cut-and-dump operation.

15.9 mm ($\frac{3}{8} \times \frac{1}{2} \times \frac{5}{8}$ in). These blocks were placed inside the rigid frames of polypropylene Sigma^{*} membrane raft squares (Sigma Chemical Company, St. Louis, MO) with the membranes removed, Figure 8. The combination will be designated as a "mini-tray." Cell diameters were selected after previous experience with cells smaller than 9.5 mm diameter showed restrictions to growth of watermelon buds. Magenta GA-7 vessels were filled with 40 ml of medium at an agar concentration of 7 g/l. The mini-trays were placed in the Magenta vessel while the agar was molten. Six vessels served as replications for each

Figure 8. Mini-tray with the restricted growing cells.

cell size treatment and a control treatment where the buds grew conventionally on agar without any structure. The three cell size (mini-tray) treatments coupled with oriented cell cutting were compared with conventional agar vessels and hand cutting with scalpel and forceps. The experiment was run through three culture cycles of 20-22 days each. Three of the six treatments (vessels) with the plug trays were cut by hand and used for subculture and data collection. The other three vessels were cut by the oriented cutter, Figure 6, and counted for data collection. Half of the vessels grown in the normal way were cut by hand with a scalpel and used for subculture and data collection, and the other half were dissected by the 5.7 mm square grid wire cutter, Figure 5, and counted for data collection. Tissue fresh weight and the number of dissected segments with visible buds were recorded at each subculture; sizes of dissected segments were measured after the third (final) subculture.

Results and Discussion

Support Matrices

The microporous polypropylene membrane (Celgard^R membrane by Hoechst Celanese, Separation Products Division, Charlotte, NC) was a very intriguing support material which had compatibility with mechanization. It provided physical separation of the

*Use of specific company names is for identification purposes only and does not indicate endorsement by Clemson University over other brands of similar functional equipment.

Figure 9. Sigma membrane raft.

ears" to curl the sides up. Another configuration had the membrane glued onto a square rim of closed cell polypropylene foam to enhance buoyancy. Hoechst Celanese also provided a limited number of membrane "boats". These boats had a square solid polypropylene sidewall heat-sealed to the membrane which served as a bottom. A similar configuration was later commercialized by Sigma Chemical Company, St Louis, MO as shown in Figure 9.

Figure 10 compares mean fresh weight gains for initiating cultures of tomato cotyledons and transfers of cultures of begonia in static medium in Magenta GA-7 vessels on four support materials: agar, Celgard^R microporous membrane, nonwoven polypropylene fiber and glass beads. Four or five replications of each support matrix were made in Magenta vessels containing 4-6 tomato cotyledons of equivalent initial age, and combined fresh weights were recorded. Cotyledons on microporous membranes had the highest mean fresh

tissue from the liquid nutrient yet allowed transfer of nutrient to the plant tissue through the micropores (0.075 micron). Moreover, the membrane pore size was sufficiently small to create a microbial barrier to the transmission of bacteria or fungi. Its buoyancy enabled it to float a small biomass, even as a flat sheet lying on the liquid surface. It also provided essentially 100% surface contact with the liquid media, a feature which improved uniformity of tissue growth. However, additional buoyancy and/or edge walls were needed to prevent flooding as the tissue mass increased. Several configurations of devices utilizing this membrane were tried to assure floating as the plant tissue expanded. One of the earliest configurations had the flat membrane sheet pinched and glued at the corners into "dog-

Figure 10. Comparison of mean tissue fresh weight gains on four alternative support matrices.

weight gain of 0.77 g, significantly greater ($\alpha=0.05$) than tissue grown on traditional agar (0.47 g). In fact, growth on microporous membrane was also significantly greater than growth on glass beads (0.26 g) and nonwoven polypropylene fiber (0.37 g). Fresh weight gain on glass beads (0.26 g) was significantly less than gain on agar.

Figure 10 also shows mean fresh weight gains measured for transferred cultures of callus clusters of begonia tissue. Three replications of each of the four support matrices were used in Magenta vessels with static medium. Five to six clusters of begonia tissue divided from established *in vitro* agar cultures were transferred to each Magenta vessel. Combined weights of clusters in each vessel were recorded. Mean fresh weight gains of 7.60 g on microporous membrane and 7.64 g on nonwoven polypropylene fiber were essentially equivalent, and the highest for the four support matrices. A mean fresh weight gain of 4.08 g on agar was the lowest of the matrices tested. Glass beads yielded a weight gain of 6.46 g, which was not significantly different ($\alpha=0.05$) from microporous membrane and nonwoven fiber. All three alternative matrices - microporous membrane, nonwoven fiber and glass beads - yielded significantly higher mean fresh weight gains of transferred begonia tissue than agar. Closer relative growth performance of begonia than tomato on glass beads, nonwoven polypropylene fiber and microporous membrane may have resulted because of greater care to maintain critical liquid levels, knowledge gained from earlier tomato cotyledon studies. Moreover, the begonia tissue transferred from established *in vitro* (agar) cultures appeared to be less susceptible to contamination and perhaps more resilient to environmental shock than the initiation cultures of tomato cotyledons.

Bioreactors

The initial bioreactor connecting several Magenta vessels through their bottoms to a single nutrient reservoir and repeatedly pumping and draining liquid nutrient through a diaphragm pump was seriously flawed. Contamination at any one location rapidly spread through the entire system. The diaphragm pump could not withstand repeated autoclavings because of varying coefficients of expansion of its metal and plastic parts.

The large glass beaker vessels were significantly superior to the Magenta system. The larger volume of the individual vessel reduced the number of connections (potential leaks). Positioning of the supply port 50 mm above the bottom of the beaker created a physical barrier to rapid migration of contamination back to the supply medium. Either a siphon or a peristaltic pump eliminated the sterilization difficulties associated with an in-line, diaphragm pump and was simpler. Separate inflow and outflow media lines eliminated crossover contamination. Even while working under a laminar flow hood, however, one had to exercise extreme caution to open the large top of the beaker only partially when inserting plant tissue. Contaminants could still very readily enter the top opening of this beaker reactor.

The Nalgene "Bio-Safe" box was very effective as modified for a scaled-up bioreactor. Explant tissue could be aseptically exposed to a continual-flow of liquid nutrients and to

exchanges of atmospheric gases. The bioreactor and plastic carboys were highly fracture resistant and could withstand repeated autoclavings. Carboys, the bioreactor and connecting tubing could be preassembled and then autoclaved as a unit so that asepsis was easier to maintain. Only the top of the Bio-Safe box needed to be tilted slightly to permit quick inoculation with plant tissue after sterilization of the assembled system. This top provided an opening sufficiently large to enable easy access, yet its tilted posture did not leave the bioreactor "top-exposed" so that microbial contaminants could readily enter when it was being inoculated. The liquid sampling port functioned effectively for removal of small aliquots of liquid medium using steril syringes without disturbing the plant tissue or introducing contaminants. The polycarbonate allowed high light transmission. For thorough cleaning between culture cycles, quick disconnect luer lock fittings allowed easy dismantling and effective cleaning with soapy water and clean water rinses. Filter sterilization of non-sterile media into the system enabled additions at any point in a culture cycle without threatening asepsis. Incorporation of the YSI Analyzer and computer control software, along with appropriate valving, independent supply carboys for concentrated sugar, other nutrient components and sterile distilled water, and calibrated peristaltic pumps proved to be very functional as a closed loop, automatic control system for nutrient monitoring and control. A pleasant and unanticipated result of increased growth of tobacco leaf discs by a factor of approximately 3-4 was repeatable with the closed loop nutrient control system. This automated liquid/membrane bioreactor will be a very valuable tool for the tissue culturists in future studies of tissue physiology. Already we have a PhD plant physiology graduate student using the system for studying carbohydrate utilization in plant micropropagation.

Nutrient Depletion - Watermelon

In both proliferation and shoot elongation media, fresh weights of watermelon tissue were greater in liquid than in agar-solidified medium. Relative dry matter content, however, was greater in agar-solidified than in liquid medium. More shoots elongated in agar-solidified than in liquid medium. The numbers of buds or unelongated shoot meristems, however, were comparable for both the liquid and agar-solidified medium. Proliferating and elongating tissues in liquid medium used Ca^{++} and K^+ minimally. NO_3^- was utilized but not depleted by proliferating tissues. NH_4^+ , however, was depleted. Most of the NH_4^+ was utilized by the proliferating tissues within 21 days of culture when growth rate was greatest. At 35 days, residual Ca^{++} , K^+ , NO_3^- and NH_4^+ in proliferation medium were 81.0%, 67.8%, 55.7% and 1.2% of initial levels, respectively. NO_3^- and NH_4^+ in shoot elongation medium were depleted. The greatest NO_3^- and NH_4^+ utilization was observed during the first 14 days of culture when the largest growth rate was obtained. The residual Ca^{++} , K^+ , NO_3^- and NH_4^+ in shoot elongation medium at 38 days were 63.5%, 37.9%, 21.2% and 24.3% of initial concentrations, respectively. At the end of the experiment, 72.3% and 42.8% of initial sugars were still remaining in the shoot proliferation and elongation media, respectively.

Tissue Growth

Liquid begonia cultures (static and bioreactor) had tissue fresh weight gains of about double that of agar cultures (Table 1). Dry weights were not significantly different. Percentage of dry matter, however, was found to be statistically higher for agar than liquid cultures. Perhaps this phenomenon occurred because water was more tightly bound chemically and less available for plant uptake in the agar-based nutrient. Although several membrane units were found to be contaminated with the non-pathogenic bacteria *Bacillus polymyxa*, media in the bioreactors remained uncontaminated because of the microporous membrane barriers of the rafts.

Tobacco initiation cultures had fresh weight gains in the bioreactor 76% greater than on agar and 3.6 times greater than on the static liquid cultures (Table 2). Liquid level was noted to influence growth of tobacco significantly. Diminished levels in the non-replenished, static liquid vessels very probably accounted for their low fresh weight gains. No significant difference was observed in final dry weight or percentage dry matter of tobacco between the bioreactor and agar-solidified cultures.

Watermelon on both shoot proliferation and elongation media had significantly higher fresh weight gains on liquid cultures of 2.25 and 2.8 times greater, respectively, than on agar (Table 3). Final dry weights were significantly higher in the bioreactor than on agar, yet the percentage dry matter was significantly greater for agar cultures. Vitrification of watermelon was a very common observation for either liquid culture technique. Continuing studies with relative humidity and the gas exchange capability of the bioreactor will hopefully reveal protocols which can control vitrification.

Varying qualities of tissue growth among these three tissue types suggest that different operational protocols for nutrient and gas exchange with the bioreactor will be appropriate for optimizing cultures. Nevertheless, fresh weight gains of nearly double or greater have been consistently realized with the continual-flow, liquid bioreactor.

Both the size and number of plantlets of the *Cattleya* orchids were increased on the liquid/membrane cultures versus on agar. Agar cultures attained most of their growth in the first 40 to 90 days of culture while tissue on liquid/membrane demonstrated greater growth rates during initial stages and continued to grow over extended periods in culture. Eight-month cultures on membrane without subculture displayed 100% acclimation to the greenhouse, but perhaps more stressed plantlets from 16-month cultures without subculture were not quite as successful through greenhouse transfer. A particularly interesting observation was that lengthy liquid cultures supplemented with nutrients revealed less growth enhancement than cultures supplemented with distilled water only. This response might suggest that the Murashige and Skoog formulation is too concentrated for optimum growth of orchids on membranes where nutrient availability is likely enhanced by liquid medium. It is also possible that as plantlets get larger they have a greater demand for

Table 1. *Rex begonia* cv. Peacock growth response on agar medium, static liquid medium and in bioreactors with 7-day medium replenishment.

Contamination Status	Treatment	Fresh Weight Measurements			Dry Weight Measurements		
		Number of Observations	Final Weight (g)	Total Weight Gain (g)	Number of Observations	Final Weight (g)	Percentage Dry Matter
Uncontaminated	Agar	7	5.79 ± 0.35	5.44 ± 0.35	3	0.31 ± 0.02 ^e	4.82 ± 0.02 ^g
	Static	5	11.53 ± 0.56 ^a	11.24 ± 0.58 ^e	2	0.48 ± 0.01 ^e	3.74 ± 0.04 ^{gh}
	Bioreactor	5	12.09 ± 1.16 ^a	11.77 ± 1.19 ^e	5	0.39 ± 0.07 ^e	3.32 ± 0.61 ^h
Contaminated	Agar	2	4.45 ± 1.05	4.18 ± 1.05	2	0.22 ± 0.04 ^f	5.01 ± 0.28 ⁱ
	Static	5	9.83 ± 0.72 ^b	9.50 ± 0.72 ^d	0	n/a	n/a
	Bioreactor	15	8.36 ± 0.72 ^b	8.06 ± 0.72 ^d	15	0.35 ± 0.02 ^f	4.27 ± 0.13 ⁱ

Table 2. *Nicotiana tabacum* L. cv. Burley 21 leaf disc initiation culture growth.

Treatment	Fresh Weight Measurements			Dry Weight Measurements		
	Observations	Final Weight (g)	Total Weight Gain (g)	Observations	Final Weight (g)	Percentage Dry Matter
Agar	7	6.51 ± 0.89	6.47 ± 0.89	7	0.27 ± 0.02	4.29 ± 0.25
Static	44	3.20 ± 0.97	3.15 ± 0.97	44	0.18 ± 0.02	3.79 ± 0.07
Bioreactor	6	11.42 ± 0.90	11.37 ± 0.89	4	0.41 ± 0.04	3.42 ± 0.16

Table 3. *Citrullus lanatus* cv. 'Charlee' proliferation and elongation growth responses.

Medium Type	Treatment	Fresh Weight Measurements			Dry Weight Measurements		
		Number of Observations	Final Weight (g)	Total Weight Gain (g)	Number of Observations	Final Weight (g)	Percentage Dry Matter
Proliferation	Agar	5	3.43 ± 0.15	3.09 ± 0.14	4	0.24 ± 0.02	7.11 ± 0.78 ^d
	Static	10	6.01 ± 0.23 ^a	5.67 ± 0.21 ^b	9	0.33 ± 0.01 ^c	5.48 ± 0.28
	Bioreactor	5	7.23 ± 0.49 ^a	6.95 ± 0.48 ^b	4	0.38 ± 0.03 ^c	5.45 ± 0.52
Elongation	Agar	5	6.38 ± 0.82 ^g	6.04 ± 0.83 ^j	5	0.38 ± 0.03	7.47 ± 0.40 ^m
	Static	10	8.31 ± 0.65 ^f	8.02 ± 0.64 ⁱ	10	0.38 ± 0.03	5.51 ± 0.25
	Bioreactor	5	17.29 ± 1.04 ^e	16.98 ± 1.04 ^b	5	0.91 ± 0.06 ^k	5.55 ± 0.16

NOTE: Common superscripts indicate values found not to be significantly different. Valid comparisons are possible for only measurements within a single contamination status grouping. Not all plant material included in fresh weight observations was included in dry weight measurements.

water, i.e., more dilute media. Enhanced growth in the early stage of tissue culture of orchids on the liquid/membrane media continued in the greenhouse media and resulted in some observations of earlier flowering than orchids originating on agar cultures.

Cutting and Separating Devices

The trifold polypropylene netting enabled examination of two methods of mechanically separating the plantlets from the calli and poorly differentiated tissue. When the upper layer of netting was lifted vertically, or peeled away from the middle layer, plantlets did not separate cleanly from the calli, but tended to strip through the grids of the netting and to be damaged. On the other hand, separation of the plantlet from the calli was easily achieved by shearing the upper netting layer laterally past the middle layer. The use of a modified electric knife with the "sandwich" raft was able to apply the shear principle in a more positive manner to harvest older plantlets while leaving less differentiated tissue for further Stage II multiplication. It did not, however, lend itself to subdivision of proliferating tissue which might further increase the multiplication potential of plant micropropagation. It also had some limitations set by the inherent morphology of varying species of plantlets. Consequently, our research evolved toward the unitizing wire cutter devices.

The greatest impact of using the unitizing, non-selective wire cutter instead of hand-cutting with a scalpel and forceps occurred for the actual cutting time **per bud cluster**. This time was reduced from 4.2 seconds to 0.7 seconds, from approximately 47% of the total process time to 5%). The other two job functions in the transfer process - (1) removing and staging source tissue from the initial vessel for cutting and (2) sorting freshly cut bud clusters to fill a new culture vessel - were both performed faster with hand cutting than with the unitizing cutter. The removing and staging function increased from 0.7 to 1.4 seconds, or from about 8% to 20 % of the total time. The filling time increased from 3.9 to 5.1 seconds, or from about 45% to 75% of the total process time. The unitizing cutter required 6.8 seconds total process time per bud cluster compared to 8.8 seconds per bud cluster with the hand method. The reduction in total processing time with the new device was, therefore, about 29% per acceptable bud cluster.

Table 4 summarizes the time studies comparing appropriate job functions involved in conventional hand (scalpel and forceps), cut-and-dump and cut-sort-and-dump transfer procedures per **four bud clusters** used for establishing each new vessel. The mean *total transfer* time spent per four bud clusters were 160.0, 21.2 and 60.8 seconds, respectively, for the hand, cut-and-dump and cut-sort-and-dump processes. Consequently, the cut-and-dump procedure consumed 13.3% as much time as hand transfer, and the cut-sort-and-dump procedure 38.0% as much. In other words, cut-and-dump reduced the total processing time by a factor of 7.5, and cut-sort-and-dump reduced it by a factor of 2.6.

Table 4. Time studies using groups of four bud clusters to compare job functions for three Stage II watermelon tissue culture transfer techniques: hand cut, cut-and-dump and cut-sort-and-dump.

Job Function	Hand Cut		Cut-and-Dump		Cut-Sort-and-Dump	
	(sec) $\pm$ S.E.	(%)	(sec) $\pm$ S.E.	(%)	(sec) $\pm$ S.E.	(%)
(1) Taking Out	11.3 $\pm$ 0.55	7.15	15.4 $\pm$ 0.64	72.6	12.1 $\pm$ 0.61	19.9
(2) Cutting	76.2 $\pm$ 4.91	47.6	--	--	5.2 $\pm$ 0.28	8.6
(3) Sorting & positioning	72.5 $\pm$ 3.36	45.3	--	--	--	--
(2,3) Cut & Dump	--	--	5.8 $\pm$ 0.32	27.4	--	--
(2) Sorting	--	--	--	--	34.3 $\pm$ 1.73	56.4
(3) Dumping	--	--	--	--	9.2 $\pm$ 0.53	15.1
TOTAL TRANSFER	160.0 $\pm$ 7.37	100.0	21.2 $\pm$ 0.70	100.0	60.8 $\pm$ 1.91	100.0
Unitized/Hand (%)	--	--	13.3	--	38.0	--

The times per four bud clusters spent taking the tissue from the original vessel and placing it for dissection (*removing*) were similar for all three techniques. *Cutting* times were reduced from 76.2 seconds by hand to 5.2 seconds by cut-sort-and-dump and to 5.8 seconds by cut-and-dump, factors of reduction of 14.7 and 13.1, respectively. These reductions of times for the cutting function are very comparable to the 14:1 reduction factor observed in our earlier experiments.

The mean number (with standard errors) of dissected segments with visible buds for the time studies in Table 4 were 17.3 ± 0.80 for hand cutting and 11.3 ± 0.58 for cut-and-dump and cut-sort-and-dump. Since dissected segments from the cut-and-dump technique were placed directly into the new vessel, there was no opportunity to count them. Therefore, they were assumed to equal the number of segments actually counted for the cut-sort-and-dump technique. Production rates (segments/second) could be calculated by dividing the number of segments by the total transfer times in Table 4 for the corresponding transfer techniques. Therefore, the productions rates were 0.11, 0.53 and 0.19 segments/second for hand cut, cut-and-dump and cut-sort-and-dump, respectively. From the standpoint of relative productive ratios, the cut-and-dump technique was 4.8 times more productive than the hand cut technique, and the cut-sort-and-dump technique was 1.7 times more productive than hand cut. The cut-and-dump technique was also 2.8 times more productive than the cut-sort-and-dump technique.

It was observed from Table 4 that the largest job function with the cut-and-dump technique was still removing tissue from the initial vessel, about 73% of the total process time. We envisioned that reducing the number of bud clusters to be lifted by the forceps

might further improve productive efficiency. This eventuality could be achieved by the use of the mini-trays.

Table 5 indicates that fresh weights per dissected segment cut from the 15.7-mm-cell mini-trays with the corresponding 15.9 mm oriented wire cutter cell were significantly greater at the 10% level than other tissues cut in correspondingly sized oriented cutter cells or with the 5.7 mm unitized cutter for the tissue from the conventional vessels. All tissue segments in the oriented cutter column of Table 5 were significantly heavier than segments

Table 5. Fresh weights per dissected segment with visible buds immediately after cutting tissue from the third (final) culture cycles by hand and with the appropriate oriented cutter cell. ($P \leq 0.10$)

Cell Diameter Treatments	Final Fresh Wt. per Segment (mg) $\pm$ S.E.	
	Hand Cut	Oriented Cutter
9.5 mm	53.5 $\pm$ 4.2 ^a	104.3 $\pm$ 22.5 ^c
12.7 mm	37.5 $\pm$ 2.5 ^b	126.1 $\pm$ 20.7 ^c
15.9 mm	61.3 $\pm$ 8.2 ^a	177.6 $\pm$ 17.6 ^d
Conventional	52.3 $\pm$ 5.5 ^a *	95.4 $\pm$ 12.1 ^c

* Cut with the 5.7 mm unitized wire cutter

in the hand cut column at the 1% level. Standard errors for tissues cut by the oriented cutter were also noticeably large, indicating wide variability in sizes of tissue clusters divided into four quadrants. Perhaps some variability was induced by more tissue growing toward one quadrant space than another or uneven division of more vertically grown tissue by the downward movement of the cutting wires. The oriented cutter yielded between 71% to 85% as many dissected segments as hand cutting among the various diameter cell sizes. On the other hand, the unitized, non-oriented 5.7 mm cutter yielded only 60% as many dissected segments as hand cutting. Consequently, orientations of growth and cutting of tissue increased the percentage of segments yielded. This response should positively influence the productive efficiency of the unitized cutting technique. The mini-tray concept reduced the number of tissue clusters to be removed from a single vessel; therefore, it should intuitively reduce time required to remove tissue from the culture vessel. This circumstance should further enhance the productive efficiency of the unitized cutting concept over hand cutting. Time studies have not been conducted at this time. Further research on these aspects needs to be conducted.

Summary

The liquid/membrane culture technique has demonstrated equal or superior growth of several plant species in micropropagation over conventional agar-based cultures. Scaled-up bioreactors have demonstrated the capacity to replenish liquid medium automatically by computer control and to rescue tissue from contamination. Liquid media also enable research with plant tissue physiology and media optimization as well as potential commercial alternatives more compatible than agar to mechanized handling.

By applying the principle of unitization, wire grid cutting devices increased the productivity of the cutting function in plant micropropagation by a factor of 14. Configuring the wire cutter into an inverted posture over the receiving vessel(s) enabled a cut-and-dump technique that became 4.8 times more productive for the total transfer process than the conventional scalpel and forceps technique when both time and yield of dissected segments with visible buds were considered. The mini-tray concept has the potential to further enhance productive efficiency by reducing the tissue removal job function, which is the largest fraction job function in the cut-and-dump technique. Because of their simplicity, both the unitized, non-selective wire cutter and the mini-trays can be incorporated into typical manual operations as operator aids and potentially be more effective than sophisticated robotic or automated concepts. Yet they may become effective components with the end effectors of automated robotic systems for handling plant micropropagation transfer processes.

COOPERATION

Cooperation between US and Israeli investigators on this project has been extremely good throughout the life of the project. Dr. Young, the principal US investigator, was in Israel under other sponsorship in May 1990 and was able to participate in direct person-to-person planning with both the Volcani engineers and the Rosh Hanikra tissue culturists in anticipation of potential funding of the project. These in-person exchanges of investigators in both countries greatly enhanced familiarity and communications so important for productive working relationships. In addition, visits of Drs. Peiper and Alper and Prof. Wolf to Clemson in May 1991 allowed us to exchange ideas and accomplishments of our respective first-year efforts directly and to plan for progression into the second year. Active electronic mail (BITNET) communication afforded us rapid, almost daily contact at various times throughout the project. The US's second generation, polycarbonate bioreactor and membrane rafts were shipped to Bet Dagan, and Israel prepared a modified electric knife for the US project.

Cooperation between US and Israeli investigators during the second project year continued to be extremely good. Beginning in November 1991, Dr. Alper spent a year in Clemson and conducted a very commendable research effort there. His presence stimulated more active communications with the Volcani researchers through frequent E-mail, FAX, telephone and mail messages. Prof. Levav visited Clemson in May 1992 and shared his activities with Rosh Hanikra and offered beneficial inputs to our efforts. During the third year we worked closely together to complete our algorithms for control of sugars in the Nalgene liquid media bioreactor and to refine our cutting and separation devices and techniques for various types of plant morphology. This project was truly a cooperative effort between Israel and the US.

EVALUATION OF RESEARCH ACHIEVEMENTS

The project has been successful in achieving its original anticipated results as stated:

1. Basic knowledge of plant tissue culture interactions with media, environment and manipulation in a continual-flow, nutrient-controlled liquid culture system and
2. Evaluation of technical concepts which will reduce the tedium and intensity of manual labor and enhance the productivity of plant tissue culture facilities.

The liquid/membrane technique has produced very successful cultures for several plant species. It has several inherent advantages over agar-based cultures to include (1) increased availability of nutrient constituents through the liquid format in 100% contact with the membrane surface, (2) easier handling of tissue in a membrane raft, (3) protection from contamination by virtue of the microbial barrier property of the microporous membrane, (4) research potential of liquid medium to determine nutrient compositions which will optimize

growth and physiological responses of plant tissue and (5) flexibility to utilize various configurations for scaled-up bioreactors and materials handling devices to automate the process and to optimize labor productivity.

One commercial product has resulted from the project in the form of the Sigma membrane raft. Proprietary disclosures have been made relative to the automated bioreactor and to a sealed vessel that was a spinoff of the project. Two concepts have followed the project and are currently under further development: (1) a wheel bioreactor to utilize volume space on growth benches and (2) a microhydroponic system to interface between the laboratory and the greenhouse. High voltage electric discharge (ozone) sterilization for devices and vessels used in plant tissue culture is another concept being explored subsequent to the conduct of this plant micropropagation mechanization project.

PROJECT RELATED PUBLICATIONS

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Submitted:

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Alper, Y., R. E. Young, J. W. Adelberg, and B. B. Rhodes. 1993. Mass handling of watermelon microcuttings. Submitted to Transactions of the ASAE.