2005

Efficiency in Thin-film Liquid System for Hosta Micropropagation

Jeffrey Adelberg
Clemson University, jadlbrg@clemson.edu

EFFICIENCY IN THIN-FILM LIQUID SYSTEM FOR HOSTA MICROPROPAGATION

Jeffrey Adelberg, Dept. of Horticulture, Clemson University, Clemson SC 29634 USA

Email: jadlbrg@clemson.edu

Abstract. Three varieties of Hosta (H. 'Striptease', H. 'Minuteman' and H. 'Stiletto') at four densities (40, 80, 120 and 200 explants / L) were micropropagated on semi-solid agar and a thin-film liquid system with intermittent wetting of plant tissue. The mechanics of wetting by a small wave front required a larger rectangular vessel (11 x 27 = 297 cm^2) compared to the common cylindrical baby food jar (18 cm^2). Plants multiplied more rapidly in the agitated thin-film system than on agar. Lower plant densities increased rates of multiplication in liquid, but had little or no effect on multiplication rate on agar. Increasing plant density lowered the overall multiplication rate, but yielded greater numbers of plants per vessel. Yield, tabulated for utilization of shelf-space in growth room, was greater at all densities in rectangular vessels of liquid than conventional jars of agar media. Increased plant density lowered the sugar residual in media following the culture cycle and liquid media had less residual sugar than agar media. A liquid media with 50g / L sucrose was concentrated enough so that sugar depletion did not limit growth, even at the highest densities. The liquid system allows the technician to skip the step of manually spacing and orienting the freshly cut bud tissue at the transfer station. Harvesting 75 - 100 plants per vessel from the large rectangular vessels resulted in most efficient use of technician time. Plants from liquid and agar acclimatized to greenhouse. Increased multiplication, space utilization, sugar availability

and worker efficiency was demonstrated to be greater in thin-film liquid than more conventional agar-based system.

Additional index words: Mass flow, propagation, liquid culture, bioreactor

Introduction

Hostas are among the most valuable tissue cultured plants in North America (Zimmerman 1996). Virtually all commercial micropropagtion, including Hosta, uses semi-solid agar-based media. Hosta micropropagated on liquid media in shaker flasks had greater dry weights than from agar medium (Adelberg et. al. 2000). The plants from liquid media also acclimatized and grew at a faster rate in the mist bed and outdoor nursery than plants from agar. Acclimatization requires functional changes in leaf anatomy - cuticle, stomata, and mesophyl, and conductive functions in vessel and root systems, before water relations and photosynthetic competence are balanced for growth (Ziv 1995). Carbohydrates accumulated *in vitro,* are necessary for plant metabolism *ex vitro* until photosynthetic growth is established (Williams 1995). It is possible that Hosta from liquid culture have greater carbohydrate reserves, along with greater dry weights.

Labor is the largest cost component in micropropagation (Chu 1995). Liquid culture allows innovative methods in workstation mechanization for the cutting and transfer during subculture. Further savings in media preparation (labor and materials), dishwashing and the manual removal of sugar containing agar from plants prior to planting-out, is realized in liquid systems. Mechanical cutters and large liquid culture vessels allows cost reductions greater than 50% to be predicted (Gross and Levin 1998).

A man-motion study of technician activities showed 'sorting and placing' buds in fresh media required 45% of the time at the transfer station (Alper et al. 1994). A 'cut and dump process' was demonstrated for shoot bud clusters, where labor was reduced by non-oriented cutting and bulk transfer of buds into a vessel of soft agar. With 'cut and dump', yield per vessel was reduced to 41%, but overall system efficiency increased by a factor of 4.8 (Alper et al. 1994). Hosta buds grown in agitated shaker flasks of liquid were obviously never oriented or spaced by the technician and grew, at least as well as, that planted carefully in semi-solid agar (Adelberg et al. 2000). Liquid micropropagation systems may simultaneously reduce inputs and increase quality of the propagated plants, but will only be implemented when a system is deemed easily adapted and cost-effective for an industry.

Mechanized systems for temporary immersion in liquid media have been designed and allow vigorous growth of high quality plants while increasing the efficiency of labor and laboratory shelf space (Etienne and Berthouly 2002). The first such system, a tilting rocker for flasks, was described twenty years ago (Harris and Mason 1983) with no further reports since that time. A simplified, scaled-up rocker was designed, fabricated and tested (Fig. 1; Adelberg and Simpson 2002) to intermittently wet tissue by slowly moving wave fronts in large rectangular vessels (e.g. Nalgene Biosafe, Nalge Nunc Intl., Inc.). High vessel costs, the large number of mechanical parts in each Biosafe vessel, and the failure of this vessel to maintain asepsis during multiple uses, prompted work with Southern Sun BioSystems (Hodges, SC, USA) to devise a less costly vessel with fewer parts for plant micropropagation.

This current study evaluates plant responses during micropropagation in a device developed to facilitate non-oriented bulk transfer process in a simple, scaled-up liquid culture system. Three varieties of Hosta were compared in the thin-film culture and semi-solid agar media at different planting densities. Efficiency was described in terms of (1) multiplication rate, (2) facility utilization, (3) sugar availability, and (4) technician labor. Plant quality was verified in greenhouse-finished transplant liners.

Materials and Methods

Three varieties of Hosta (H. 'Striptease', H. 'Minuteman' and H. 'Stilletto') from commercial stage II cultures were prepared in liquid media modified from Murashige and Skoog (MS) media (Murashige and Skoog, 1962) that included (1) addition of 170 mg/L sodium phosphate, (2) increased $CuSO_4 \cdot 5\ H_20$ concentration to 25 mg/L, and (3) organic constituents of the medium included a vitamin solution 0.5 ml/ L of MS vitamin solution (2.0 g/ L glycine, 100.0 g/ L myo-inositol, 0.50 g/ L nicotinic acid, 0.50 g/ L pyridoxine hydrochloride, 0.10 g/ L thiamine hydrochloride), adenine hemisulfate (0.92 mg/L), sucrose (50 g/L), benzyladenine (1 µM). The pH of the media was adjusted to 5.7 before being dispensed. Liquid culture vessels were maintained on a thin-film rocker system (Adelberg and Simpson 2002) that produced a 1-rpm pitch every 15 minutes. Plant buds were carefully trimmed free of leaves and roots. Crowns were divided into single bud units and agar vessels were planted with vertical and spatial orientations carefully maintained. For each variety; 1, 2, 3 and 5 buds were placed in each of 8 baby food jars (180 ml) containing 25 ml of media solidified with 0.07% agar (PhytoTechnology Plant Tissue Culture Grade Agar A111, Shawnee Mission, KS). Buds

for liquid treatments were cut, trimmed and collected in units of 8, 16, 24, or 40 buds per sterile empty jar, and dumped with one motion without regard for orientation or spacing. For each variety, 4 rocker vessels (Fig. 2; Southern Sun BioSystems, Hodges SC) were prepared at each density, containing 200 ml of liquid media. Densities of 40, 80, 120 and 200 buds / L media were directly comparable between agar and liquid media. The 180 ml cylindrical baby food jar containing 25 ml of media typified a standard practice in agar and was compared with a dissimilar proportioned rectangular vessel of liquid. One rectangular rocker vessel occupies the same shelf space as 8 jars and so a scale-up factor of 8 was applicable to both media volume and shelf space. The relationship for surface area within vessels was approximately 16 : 1 for jar to rocker vessels (18 cm^2 per jar, 297 cm^2 per vessel). Plants were grown for six weeks with 16 h photoperiod at 25 to 35 $\mu mol\ m^{-2}\ s^{-1}$ PPF provided by cool white fluorescent lamps and temperature was maintained at 24 $\pm$ 2°C. After six weeks, shoots were divided into single bud units and returned for a second culture cycle.

Residual sugar concentration was measured from vessels of expended media with a hand refractometer. Sugar was assayed from semi-solid agar by disrupting the matrix with repeated forceful shearing of the medium through a pipette tip. After several pump actions, a bead of liquid slurry was expressed on the lens of the refractometer. The refractometer is an inexpensive tool to rapidly measure sugar with minimal sample preparation. Using the refractometer requires understanding an inherent limitation - the reading of % BRIX in media is a molar summation of sucrose, and its monomers, glucose and fructose. For tissue culture application: (1) a fraction of sucrose hydrolyzes to glucose and fructose in the autoclave, (2) plant cell wall invertase further hydrolyzes

some sucrose to glucose and fructose and returns monomers to the media, and (3) water uptake by the growing plant will increase the molar concentration of the solution by decreasing the volume of diluent. These three factors will cause underestimates of sugar uptake when reading % BRIX in residual medium in comparison to initial sucrose concentrations.

A completely randomized design compared semi-solid agar and liquid media with the three varieties, at four different densities and two subculture cycles, for bud multiplication, plant yield and residual sugar concentrations. Sixteen jars, or eight rocker vessels per treatment factor level combination were pooled from two subculture cycles. Analysis was made on JMP version 3.2.6 (SAS Inst., Cary NC).

Following the second culture cycle, 72 plants from liquid and agar, for each variety were planted in greenhouse mist bed. Two weeks later plants were moved to conventional greenhouse bench and observations of survival were made after a total of four weeks in greenhouse culture.

A data archive from the commercial micropropagation process at the beta-site was assembled. The technicians performing subcultures on 40 varieties of Hosta in Nalgene Biosafe vessels had logged over 6 months in production hood hours and yield data. The technicians were not aware of any experimental design or bias.

Results and Discussion

Plants multiplied equally well during both cycles of subculture and observations were pooled for analysis (data not presented). The three varieties of Hosta had multiplied at similar rates. All three varieties were more prolific in the agitated, thin-films of liquid

media than on agar (Table 1). Better growth in liquid systems has been reported on numerous occasions and may be attributed to the lack of impurities from agar, better water availability, better nutrient availability and larger vessels (Smith and Spomer 1995; Bethouly and Etienne 2002).

Plant density had a great effect on multiplication rate for the liquid system. On agar, plant density did not effect multiplication rate of two varieties, and had a relatively small effect on the third (Table. 1). With a density of 200 explants / L multiplication rates were on average 1.6x and 2.3x, respectively, for agar and liquid media. This equates to 5 buds in a baby food jar with 25 ml media or 40 buds in a rocker box with 200 ml media. When bud density was decreased to 40 / L (1 bud in 25 ml agar or 8 in the liquid culture vessel), multiplication rates increased, to an average of 2.0x on agar or 3.4x in liquid. If efficient micropropagation were simply a comparison of the predicted numbers of plants propagated from a hypothetical bud in an annual crop cycle, and 8, six-week subcultures were performed in that year, than the liquid system with 40 / L would produce 1176 plants ($8^{3.4}$). Similarly, the agar system at the same density would only produce 64 plants ($8^{2.0}$) in the same year. It can be seen that with a valuable new plant where rapid increase was the overriding concern, efficient use of the liquid system could use the large rectangular rocker box at a low density of tissue (8 plants /vessel with 200 ml media).

In practice many considerations besides multiplication of buds impacts efficiency of a micropropagation laboratory. The total number of plants generated by that facility is critical since the bench space available for plant growth puts seasonal limitations on production. When larger numbers of buds were used to initiate a culture, more plants

were harvested six weeks later (Table 1). Reducing plant density increased multiplication rate, but the highest yields were still from highest density planting. The significant linear increase in yield over the ranges of planting density tested implies higher yields are possible with greater explant density. In all cases, yields were higher in liquid than agar per volume media. Eight jars placed in 4 x 2 arrangement create the same 'footprint' on the culture room shelf as one rectangular thin film vessel. Within the parameters established for this experiment, media volume and area shelf space were directly related by the same factor (8 jars : 1 vessel) and comparing plant yield between vessels and jars for shelf utilization in the growth room is identical to yield comparisons by media volume. Fewer, large rectangular vessels create less void space between vessels on a culture room shelf than larger numbers of smaller cylindrical vessels. Eight cylindrical jars of agar ($8 * 18 = 144$ cm^2) has less than half the interior surface for the growth of plants than one rectangular vessel of liquid ($11 \times 27 = 297$ cm^2). The increased yield in the liquid system was partially due to the greater area of growth surface inside the large, rectangular vessels.

When compared to agar, the liquid allowed plants to multiply more vigorously and grow more densely in the same volumes of media. This raised concern as to whether the liquid media contained adequate nutrients to support the increased growth. The residual sugar in semi-solid agar media following six weeks of culture was generally higher than the 5% sucrose used at the onset of cultures (Table 1). With 3% sucrose as an initial concentration in strawberry and apple, the hydrolysis of sucrose proceeded to near completion during the culture cycle (Kozai et al. 1991; Kahru 1997), but with kiwifruit residual sugar remained mostly as sucrose (Monaclean et al. 2003). In our

current work with 5% sucrose as at initiation, finding residual BRIX values greater than 5% at the end of culture did not mean sugar was not used, but indicated hydrolysis and water uptake were relatively large compared to sugar uptake. Sugar residual in agar at the highest plant density had decreased slightly below 5% in two of the three varieties (Table 1). It is clear that the 5% sugar supplied was ample to support plant growth in agar for the density ranges tested.

There was more sugar used during plant growth on liquid media than agar (Table 1). At the higher plant densities in liquid media there was a further reduction in levels of residual sugar. At the highest densities in this experiment, the 2 or 3% sugar residual (BRIX) present made it unlikely that sugar depletion had limited growth. Even if sugars were completely in monomer form, significant concentrations of sugars were present throughout the culture cycles. However sugar may become growth limiting if an optimization plan further increased plant density. Similarly, other nutrients that were not monitored may become growth limiting sooner than sugar, with high-density thin-film cultures of Hosta.

Sugars are known to have regulatory, nutritive and osmotic effects on in vitro plant growth. HPLC analyses of mono and di-saccharides and/or colorimetric procedures measure sugars more specifically than the refractometer. Analyzing endogenous levels of soluble sugars in plant tissue would yield more critical information to optimize plant quality. However, the refractometer assay of media, with zero reagent cost and real-time feedback, is so readily adaptable to practical situations, the author suggests use of this tool be considered preferable to the likely alternative of proceeding without any information on sugar.

Practical alternative micropropagation methods should not increase the amount of man-hours spent in the transfer hood. A bulk dump process eliminated the significant time component when the technician individually oriented and spaced each bud on agar. Spacing of plants in a thin-film rocker is a mechanized-passive process and the buds orient themselves with respect to light during the culture cycle. Hosta grew well under these conditions, and plants were larger with longer petioles than when grown on agar (Fig. 3). Passive spacing and orientation in the large vessels allowed high yields despite the larger size of the plants.

Labor efficiency is of critical importance for commercial application. While designing a vessel for a thin-film bulk dump process in the Nalgene Biosafe, technicians logged time at transfer station and output was quantified as buds cut per hour. Variance in cuts per hour was partitioned for main effects of treatment factors including: the individual technician, plant variety, media formulation, time of day, day of week, weeks in culture, volume of media, number of explants in, and number of plants harvested per vessel (data not presented). The most significant factor that impacted technician efficiency was the number of plants harvested from the vessel (Fig. 4). Observations over six months of production data in Nalgene Biosafe with 22 technicians cutting 40 varieties of Hosta indicated that worker efficiency at the transfer station increased dramatically as numbers of plants harvested reached about 100 per vessel. In this current work, labor efficiency reached the optimal range that when a minimum of 40 buds were dumped into 200 ml of liquid media and plant yield was 77-103 per vessel (95% confidence).

There were two causes for the quantitative relationship between buds harvested and cuts per hour dedicated at the hood station. First, a certain fixed portion of off-task time per vessel included setting up tools, opening the vessel, recording data and sealing the vessel. This remained relatively constant regardless of the number of plants per vessel and obviously constituted a lower proportion of hood time as the worker processed more buds per vessel. Secondly and more subtle, there was a likely period of concentrated effort when cutting proceeded at a brisk pace and was not interrupted as frequently by the off-task functions.

These observations were not from a time-motion study designed for costing this system. Information was logged from a vessel, the Nalgene Biosafe, which was eliminated as being too awkward. The numbers of cuts per hour also involve technicians cutting every plant into single bud divisions and counting each division for quantification. The purpose of the data was to establish factors related to improved worker performance in bulk dump process. The closure system for the rocker vessel, presentation of the rocker vessel from autoclave to the hood, and securing vessel closure in the hood were unfinished at time of this research. Time-motion studies and cost analyses need follow completion of the mechanical vessel system and could not be abstracted from the information presented.

Hundreds of thousands of Hosta plants from the commercial beta-site have been acclimatized to greenhouse and nursery. With retained sub-samples, plants from liquid were compared to plants from agar culture in the greenhouse during acclimatization and plant quality was good (Fig. 5). All plants from liquid and agar survived and grew well in greenhouse. Liquid-cultured Hosta plants tend to grow more quickly than agar-

cultured plants in the greenhouse and nursery (Adelberg et al. 2000) and it can be surmised that Hosta from thin film systems would convey the same advantage.

Conclusion

Liquid culture in a thin-film rocker system presents opportunities for more efficient micropropagation than agar. The magnitude of these efficiencies depended largely on selecting appropriate plant density. Low-density culture results in the most rapid multiplication and has the largest incremental advantage over agar-based systems if high-value plants are in scarce supply. High-density culture results in the most efficient use of hood labor, least cost, and the highest yield from an optimized process (Fig. 6). It was observed that as density increased, so did sugar utilization, and nutrient formulation may warrant further consideration, if system would be further optimized for high-density culture. In other reports on micropropagation in liquid media, dependent on the crop species - sugar, nitrogen salts, phosphate or water may be most limiting to growth (Hale et al. 1992; Desamero et al. 1993; Moncalean et al. 2003; Adelberg et al. 1997).

The thin-film system and rocker vessels offered several improvements in plant growth and system efficiencies over agar-based methods. Plants were enabled to perform functions of spacing and orientation without direct human intervention. Solute transfer from media to plant is greater and allows more rapid growth. Technicians are more focused on the germane tasks of carefully dividing the plant buds. The ergonomic and biological benefits may help to justify up-front inputs required to implement a new system.

References

Adelberg J & Simpson EP (2002) Intermittent Immersion Vessel Apparatus and Process for Plant Propagation. Intl. S/N: PCT/US01/06586.

Adelberg J, Kroggel M, and Toler J (2000) Greenhouse and nursery growth of micropropagated Hostas from liquid culture. HortTech 10: 754-757.

Adelberg J. Desamero N, Hale SA & Young (1997) Long-term nutrient and water use during micropropagation of *Cattleya* orchid on liquid/membrane system. Plant Cell Tiss. Org. Cult. 48:1-7.

Alper Y, Young R, Adelberg J & Rhodes B (1994) Mass handling of watermelon microcuttings. Trans. Amer. Soc. Ag. Eng. 37:1337-1343.

Chu I (1995) Economic analysis of automated micropropagation. p. 19-26. *In:* J Aitken Christie, T Kozai and MAL. Smith (eds.). Automation and environmental control in plant tissue culture. Kluwer Academic, Dordecht, Netherlands.

Desamero N, Adelberg J, Hale SA, Young R & Rhodes B (1993) Nutrient utilization in liquid/membrane system for watermelon micropropagation. Plant Cell Tiss. Org. Cult. 33:265-271.

Etienne H & Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell Tiss. Org. Cult. 69:215-231

Gross,A & Levin R (1988) Design considerations for a mechanized micropropagation laboratory. pp. 637-642. In: Altman A, Ziv M, & Izhar S (eds), Plant Biotechnology and In vitro Biology in 21st Centrury, Kluwer Academic Publishers, Dordecht, Netherlands.

Hale A, Young R, Adelberg J, Keese R & Camper D (1992) Bioreactor development for continual-flow, liquid plant tissue culture. Acta Hort. 319:107-112.

Harris R & E Mason (1983) Two machines for in vitro propagation of plants in liquid media. Can. J. Plant Sci. 63:311-316.

Karhu ST (1997) Sugar use in relation to shoot induction by sorbitol and cytokinin in apple. J. Am. Soc. Hort. Sci. 122: 476-480.

Kozai T, Iwabuchi K, Watanabe K & Watanabe I (1991) Photoautotrophic and photomixotrophic growth of strawberry plants in vitro and changes in nutrient composition of the medium. Plant Cell Tiss. Org. Cult. 25:107-115.

Moncalean P, Jesus Canal M, Fernandez H, Fernandez B & Rodriguez A (2003) Nutritional and gibberellic acid requirements in kiwifruit vitroponic cultures. In Vitro Cell Dev. Biol. - Plant 39:49-55.

Murashige T & Skoog F. (1962) A revised media for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15:473-497.

Smith MAL & Spomer LA (1995) Vessels, gels, liquid media and support systems, pp. 371-404. In: Aitken-Christie J, Kozai T & MAL Smith (eds). Automation and environmental control in plant tissue culture. Kluwer Academic, Dordecht, Netherlands.

Williams RR (1995) The chemical microenvironmnet, pp 405-439. In: Aitken-Christie J, Kozai T & MAL Smith (eds). Automation and environmental control in plant tissue culture. Kluwer Academic, Dordecht, Netherlands.

Zimmerman R (1996) Commercial application of tissue culture to horticultural crops in the United States. J. Kor. Soc. Hort. Sci. 37:486-490.

Ziv M (1995) In vitro acclimatization, pp. 493-516. In: Aitken-Christie J, Kozai T & MAL Smith (eds). Automation and environmental control in plant tissue culture. Kluwer Academic, Dordecht, Netherlands.

This work is contribution no. 4788 of the SC Agriculture Research and Forestry Service (SCAFRS). The author acknowledges the cooperation of Southern Sun BioSystems Inc. Special acknowledgement is given to Jacqueline Naylor-Adelberg - manager at the Norris test site, and her staff, who were most helpful in during this experiment.

Figure 1. Agitated thin-film rocker system is a simple, temporary immersion liquid culture.

Figure 2. Rectangular vessels facilitated a slow moving wave of media to migrate the length of the vessel with the motion of the shelf.

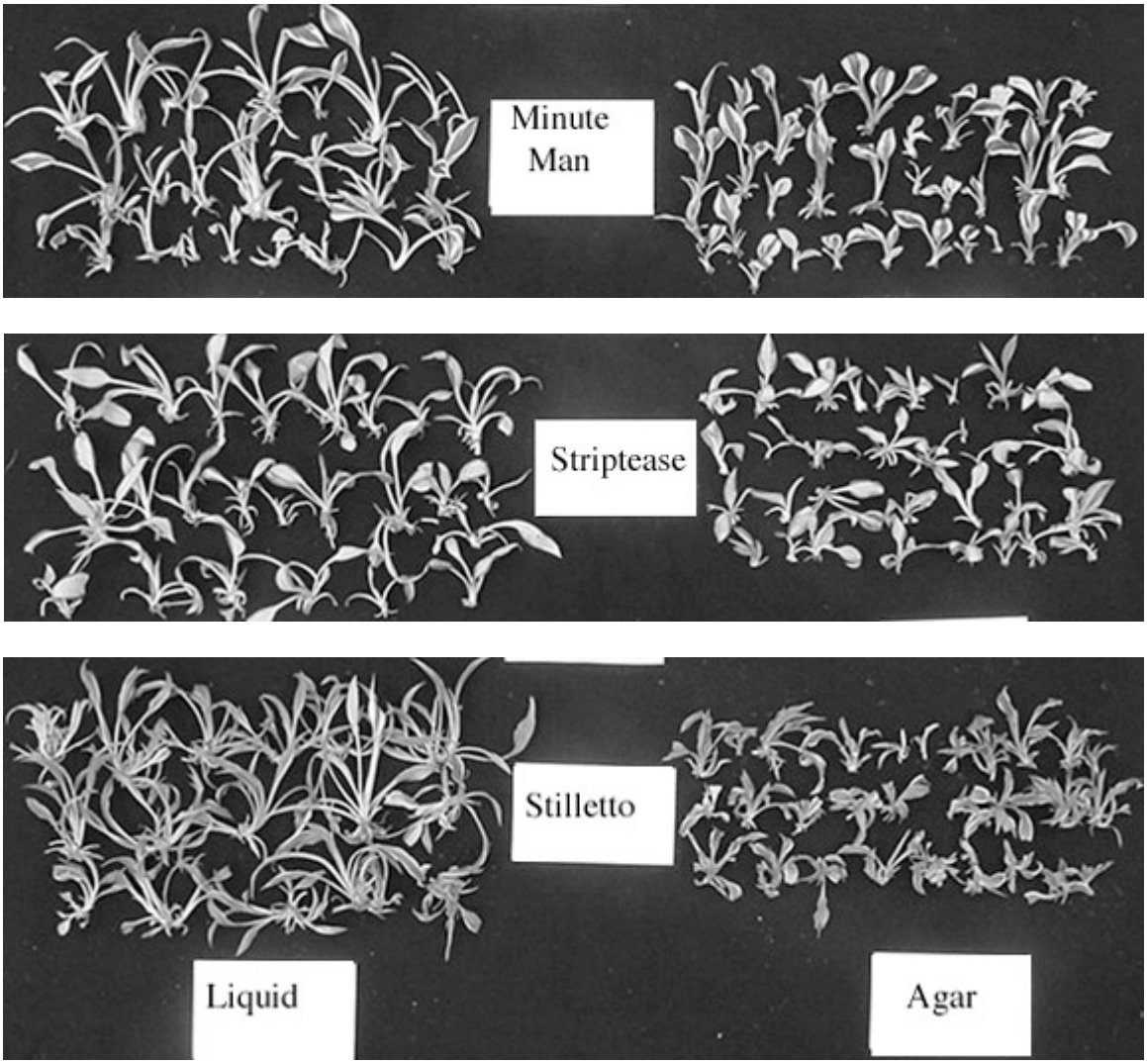


Figure 3. The three hosta varieties after 6 weeks in culture on liquid, thin-film culture (right) and agar media (left), supplemented with 1 µM BA.

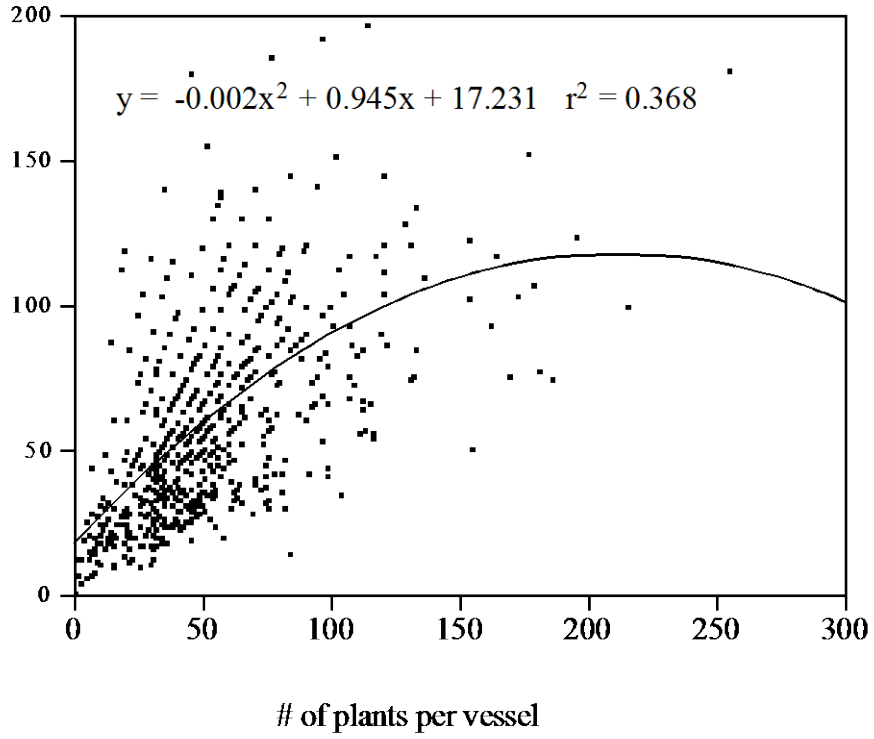
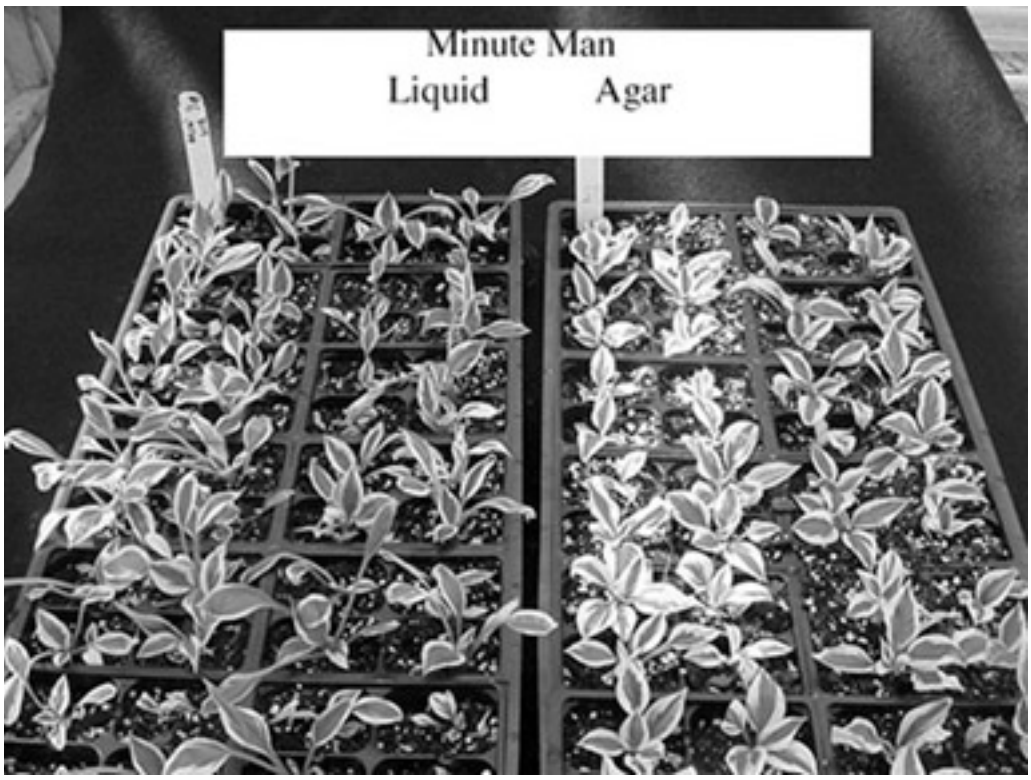
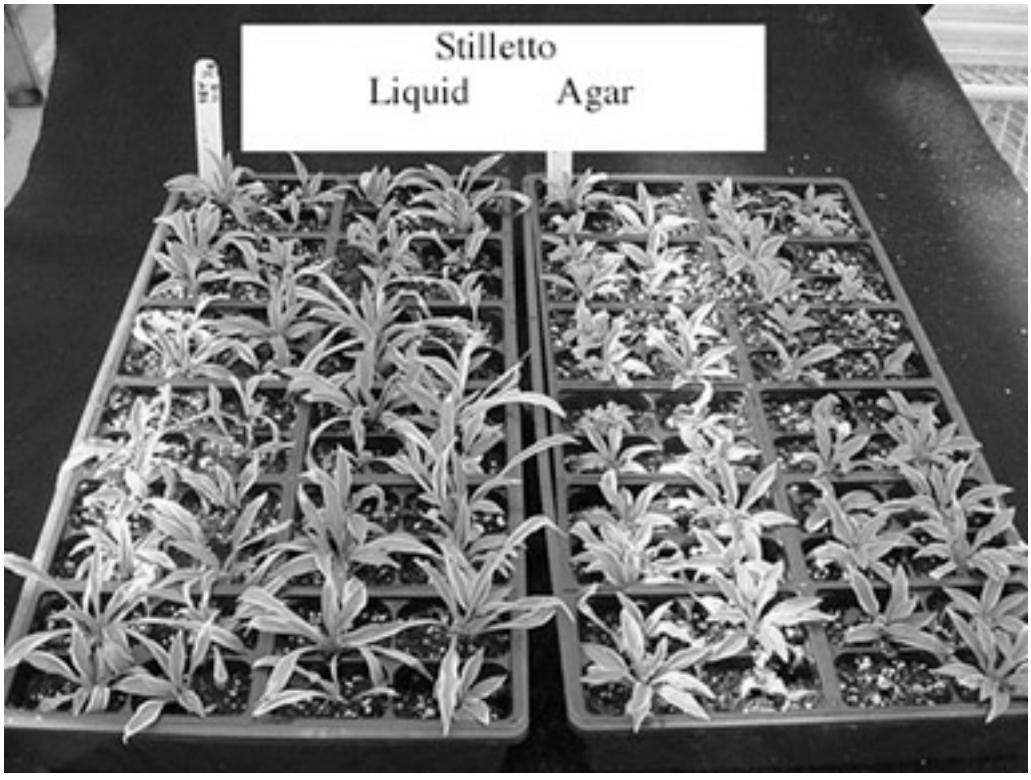


Figure 4. Labor input for twenty-two technicians propagating 40 varieties of Hosta from over six months of observations.


Minute Man
Liquid
Agar


Stilletto
Liquid
Agar

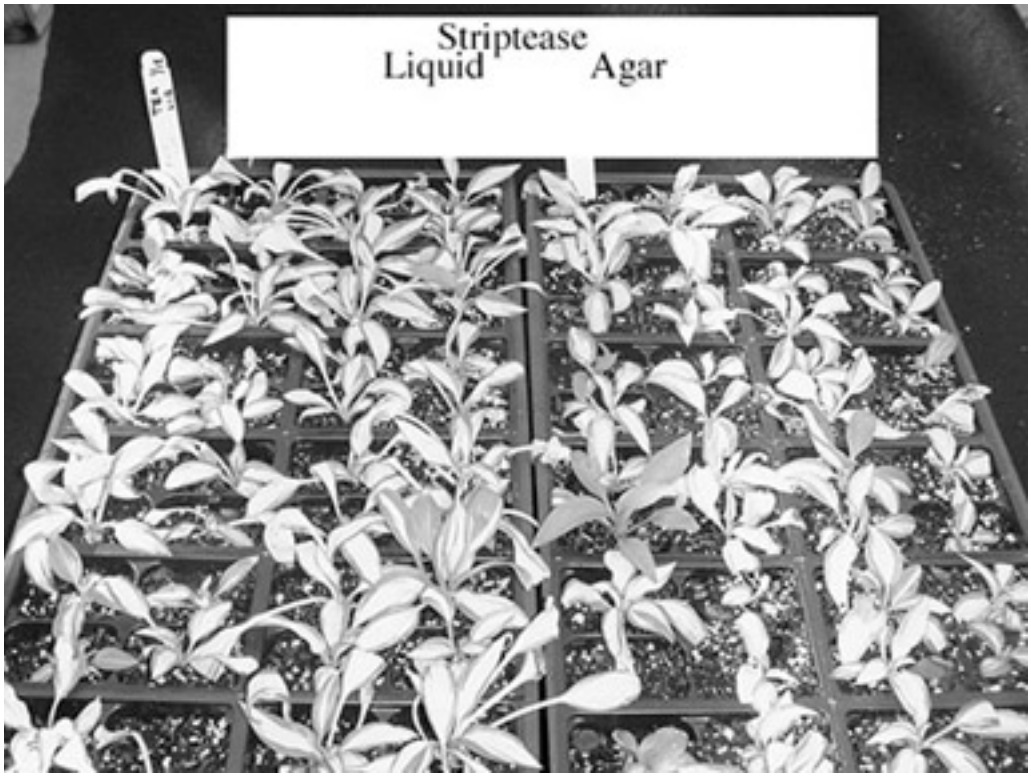


Figure 5. The three hosta varieties from liquid thin-film and agar media, after 4 weeks acclimatization and growth in greenhouse.

Figure 6. Thin film rocker system scaled-up for efficient space utilization in culture room.