COMMERCIAL PROPAGATION OF HERBACEOUS PERENNIALS BY TISSUE CULTURE

MARK ZILIS, DOUGLAS ZWAGERMAN, DAVID LAMBERTS, and LAWRENCE KURTZ

Walters Gardens, Inc.
Zeeland, Michigan 49464

A wide range of herbaceous perennial species can be efficiently propagated on the commercial level using plant tissue culture techniques. Maximum productivity is achieved by finding the optimal physical and chemical conditions for the multiplication and rooting of propagules *in vitro* and in the transition to the soil environment. Utilization of these methods at Walters Gardens, a large wholesale producer of herbaceous perennials, has enabled rapid introduction of new cultivars, propagation of plant species difficult to produce by cuttings or divisions, and production of disease-free plants. The end result has been an increase in the quantity and quality of healthy, vigorous plants.

LABORATORY FACILITIES AT WALTERS GARDENS

The tissue culture laboratory at Walters Gardens covers over 1200 square feet and contains two culturing rooms, a preparation room, a transfer room, and a high humidity room for transplants. Within the culturing rooms, 576 square feet of lighted shelf space is available for the growth of plant tissues. The preparation room contains the equipment used to mix media including an autoclave, refrigerator, sink, a water filtration system, and glassware. The transfer room holds 2 laminar flow hoods, each 6 feet in length. In the high humidity room, 192 square feet of lighted shelf space is available for hardening new transplants.

Four full-time and two part-time people are employed in the laboratory. The main jobs involved in running the facilities are media preparation, transfer work, transplanting, cleanup, and supervision.

HERBACEOUS PERENNIALS IN CULTURE

As a group, herbaceous perennials are easy to propagate *in vitro*. The wide diversity is shown in Table 1 which lists 50 herbaceous perennial species in 18 botanical families produced at Walters Gardens by tissue culture. Tissues from all of these species have been cultured, multiplied, and rooted *in vitro*.

Reasons for propagating a species by tissue culture vary but many of the plants represent introductions of species or cultivars new to the Walters Gardens line of products. For exam-

Table 1. Examples of herbaceous perennial species which can be propagated by tissue culture at Walters Gardens.

Family	Species	Explant	Regeneration/ Multiplication System
Apocynaceae	*Amsonia tabernaemontana*	Shoot tip (ST)	Axillary shoot proliferation
Asclepiadaceae	*Asclepias tuberosa*	ST	Embryoids within callus
Boraginaceae	*Brunnera macropylla*	ST, flower bud, leaf, roots	Adventitious shoot formation from callus
Campanulaceae	*Campanula elatines* var. *garganica*	ST	Axillary shoot proliferation
Campanulaceae	*C. glomerata* 'Superba'	ST	Axillary shoot proliferation
Campanulaceae	*C. rotundifolia*	ST	Axillary shoot proliferation
Campanulaceae	*C. linifolia* (Syn.: *C. scheuchzeri*)	ST	Axillary shoot proliferation
Campanulaceae	Lobelia cardinalis	ST	Axillary shoot proliferation
Caryophyllaceae	*Dianthus gratianopolitanus*	ST	Axillary shoot proliferation
Caryophyllaceae	*Gypsophila paniculata*	ST, flower bud	Axillary shoot proliferation
Caryophyllaceae	*Herniaria glabra*	ST	Axillary shoot proliferation
Caryophyllaceae	*Silene vulgaris* (Syn.: *S. cucubalus*)	ST	Axillary shoot proliferation
Caryophyllaceae	*Petrorhagia Saxifraga* (Syn.: *Tunica saxifraga*)	ST	Axillary shoot proliferation
Compositae	*Aster novi-belgii*	ST	Axillary shoot proliferation
Compositae	*Chrysanthemum maximum*	ST	Axillary shoot proliferation
Compositae	*C. morifolium*	ST	Axillary shoot proliferation
Compositae	*Coreopsis* sp.	ST, flower bud	Axillary shoot proliferation
Compositae	*Inula ensifolia*	ST, leaf	Axillary shoot proliferation and direct shoot formation from leaf base.
Compositae	*Liatris aspera*	ST	Axillary shoot proliferation
Compositae	*Ratibida pinnata*	ST	Axillary shoot proliferation
Compositae	*Rudbeckia fulgida* var. *speciosa*	ST	Axillary shoot proliferation
Fumariaceae	*Dicentra spectabilis*	ST	Axillary shoot proliferation
Hyperiaceae	*Hypericum calycinum*	ST	Axillary shoot proliferation
Labiatae	*Ajuga reptans*	ST	Axillary shoot proliferation
Labiatae	*Lamiastrum Galeobdolon* (Syn.: *Lamium Galeobdolon*)	ST	Axillary shoot proliferation
Labiatae	*Nepeta cataria*	ST	Axillary shoot proliferation

Leguminosae	*Thermopsis caroliniana*	ST	Axillary shoot breaks
Liliaceae	*Hemerocallis* sp.	Flower bud	Shoots developed from callus; callus can be multiplied
Liliaceae	*Hosta* fortunei 'Aoki'	Flower scape sections	Shoot formation directly from scape; multiply by axillary shoot proliferation
Liliaceae	*H. fortunei* 'Hyacinthina'	Dormant veg. bud	Axillary and adventitious shoot formations
Liliaceae	*H. lancifolia*	Flower scape sections	Shoot formation directly from scape; multiply by axillary shoot proliferation
Liliaceae	*H. plantaginea*	Flower scape sections	Shoot formation directly from scape; multiply by axillary shoot proliferation
Liliaceae	*H. undulata*	Dormant veg. bud	Axillary shoot proliferation
Liliaceae	*Lilium* cvs.	Flower bud	Bulblet at multiplication from fl. bud
Liliaceae	*Maianthemum canadense*	ST	Axillary shoot proliferation
Polemoniaceae	*Phlox glaberrima*	ST	Axillary shoot proliferation
Polemoniaceae	*P. paniculata*	ST	Axillary shoot proliferation
Polemoniaceae	*P. sublata*	ST	Axillary shoot proliferation
Primulaceae	*Dodecatheon meadia*	ST	Axillary shoot proliferation
Primulaceae	*D. jeffreyi*	ST	Axillary shoot proliferation
Ranunculaceae	*Aquilegia caerulea*	ST	Axillary shoot proliferation
Ranunculaceae	*Thalictrum aquilegifolium*	ST	Axillary shoot proliferation
Rosaceae	*Geum triflorum*	ST	Axillary shoot proliferation
Rutaceae	*Dictamus albus* 'Rubra'	ST	Axillary shoot proliferation
Saxifragicaceae	*Astilbe* × *arendsii*	ST	Axillary shoot proliferation
Saxifragicaceae	*Bergenia cordifolia*	ST	Axillary shoot proliferation
Saxifragicaceae	*B. ciliata* (Syn.: *B. ligulata*)	ST	Axillary shoot proliferation
Saxifragicaceae	*B. stracheyi*	ST	Axillary shoot proliferation
Scrophulariaceae	*Erinus alpinus*	ST	Axillary shoot proliferation
Scrophulariaceae	*Mimlus rigens*	ST	Axillary shoot proliferation

ple, *Petrorhagia saxifraga* (Syn.: *Tunica saxifraga*), *Campanula rotundifolia, Bergenia cordifolia, Gypsophila paniculata* cultivars, and *Chrysanthemum morifolium* selections have been produced for those reasons.

Tissue culture propagation becomes essential when conventional methods of production are inefficient. For example, field-grown *Hosta* plants yield from 3 to 6 divisions after two years of growth, making rapid buildup of large numbers of plants impossible. By culturing dormant vegetative buds or sections of the flower scape, the rate of *Hosta* multiplication can be accelerated several thousand times. At the present time, over 25 *Hosta* species and cultivars are in culture at Walters Gardens. Other plants produced by tissue culture for similar reasons include *Bergenia cordifolia* cultivars, *Dictamus albus* 'Rubra', and *Lilium* cultivars.

Other advantages of *in vitro* propagation include the ability to produce disease-free material, excellent growth characteristics of cultured plants, the means to develop new cultivars, a speedup of breeding work, and capabilities to propagate large numbers of plants from a small amount of explant material. Also, many species which have been neglected in the horticulture trade, but exhibit excellent ornamental value, can be rapidly introduced. For example, several native American perennials possess colorful and unusual flowering characteristics that rival the best of the cultivated herbaceous perennials, yet they are rarely produced by nurseries. Rapid propagation by tissue culture will enable species such as *Dodecatheon meadia* (common shooting star), *Lobelia cardinalis* (cardinal flower), *Geum triflorum* (prairie smoke), and *Amsonia tabernaemontana* (willow amsonia) to become part of the commercial trade in the near future.

TISSUE CULTURE SYSTEMS FOR HERBACEOUS PERENNIALS

Axillary Shoot Proliferation System. Many types of explants can be used to propagate herbaceous perennials *in vitro* including shoot tips, flower buds, flower scapes, leaves, dormant buds, embryos, and roots (Table 1). For all types, however, the initial goal is the same — to produce shoots. Regeneration in 47 of the 50 species listed in Table 1 involves obtaining shoots directly from the explant in response to specific growth regulator combinations within the medium. Once shoots develop, they can be subcultured for axillary shoot proliferation or for rooting. All explants are surface sterilized in 10% Clorox for 17 minutes followed by 3 rinses in sterile water before culturing.

Shoots can be obtained directly from all types of explant tissues. The easiest route to shoot production utilizes cultured shoot tips from which axillary shoots can be proliferated. This can be done with 90% of the Table 1 species. Flower bud explants, however, can also directly regenerate shoots if taken at an immature stage of development. This has been observed in cultures of *Aster novi-belgii*, *Coreopsis* spp., *Rudbeckia fulgida* var. *speciosa*, *Hosta* 'Nakaimo', and *Gypsophila paniculata*. Cultured flower scape sections are also useful in directly regenerating *Hosta* shoots (8). Shoots can also arise from cultured leaves or leaf sections in many species including *Inula ensifolia*, *Hosta* 'Aoki', *Phlox paniculata*, *Rudbeckia fulgida* var. *speciosa*, and *Bergenia cordifolia*. Although roots readily develop callus, direct shoot formation is rare in culture. Sterile roots (ones developed adventitiously in culture) of *Phlox sublata* have regenerated adventitious shoots.

Once uncontaminated shoot cultures are obtained, they can be subcultured for further proliferation of axillary shoots. Growth cycles from 2 to 8 weeks or more should be established. For many species, the cycle length is critical. Although greater numbers of shoots will develop over extended culturing time, shoot quality usually deteriorates past an optimal time. This can subsequently lower rooting percentages and transplanting survival.

Maintenance of the proper physical environment is essential during shoot multiplication. Generally, temperatures between 20° and 30°C (68° and 86°F) and 300 to 400 footcandles of light from cool-white fluorescent lamps are optimal for culturing herbaceous perennial shoots. Lower light intensities (down to 100 f.c.) tend to slow multiplication and produce poorer quality shoots in such species as *Bergenia cordifolia*, *Gypsophila paniculata*, and *Dicentra spectabilis*. Other species, though, including those in the family Compositae, can be cultured under a broader range of growing conditions.

Shoots produced *in vitro* should be rooted *in vitro*. Although some research (2) has suggested rooting tissue-cultured shoots directly into soil, this technique is not efficient for the production of herbaceous perennials. Rooting cultured shoots can be easily accomplished *in vitro* for most herbaceous perennial species (Table 2).

The proper physical environment within the rooting container leads to success in the rooting and transplanting processes. High light conditions (800 to 1200 f.c. from cool-white fluorescent lamps) and temperatures between 20° and 30°C (68° and 86°F) are optimal for most species. The agar concentration of the rooting medium is another critical factor. Levels between

Table 2. Rooting and transplanting responses for 22 major herbaceous perennial species produced by tissue culture at Walters Gardens.

Species	Rooting Percentage	Transplant Survival[1]
Ajuga reptans	+++	+++
Aster novi-belgii	+++	+++
Bergenia cordifolia	+++	+++
Campanula Elatines var. *garganica*	+++	+++
C. glomerata 'Superba'	+++	+++
C. rotundifolia	+++	+++
C. linifolia (Syn.: *C. scheuchzeri*)	+++	+++
Chrysanthemum maximum	+++	+++
C. morifolium	+++	+++
Dicentra spectabilis	++	+++
Dictamus albus 'Rubra'	+	+
Dodecatheon meadia	+++	+
Gypsophila paniculata	+++	+++
Hosta fortunei 'Aoki'	+++	+++
H. fortunei 'Hyacinthina'	+++	+++
H. lancifolia	+++	+++
H. plantaginea	+++	+++
H. undulata	+++	+++
Lobelia cardinalis	+++	+++
Phlox paniculata	++	+++
Rudbeckia fulgida var. *speciosa*	+++	+++
Petrorhagia Saxifraga (Syn.: *Tunica saxifraga*)	+++	+++

[1] +++ = 80% or greater; ++ = 50 to 80%; + = less than 50%.

10 and 14 grams per liter create dry conditions that enable leaves produced *in vitro* to withstand dessication following transplanting. Length of culture time during rooting also seems to be an important factor. Periods from 2 to 4 weeks are typical for most crops while longer periods *in vitro* reduce survival after transplanting.

The transplanting operation is relatively simple. The rooted plants are removed from the mason jars, transplanted into soil, and placed in a growing room where the relative humidity is maintained at 50%. Light levels are kept at 300 to 400 f.c. for 2 to 3 days after which they can be increased to 1200 f.c. Most crops remain under high humidity for 3 to 10 days and, then, are transferred to the greenhouse. Response to transplanting has been excellent for most major species (Table 2).

Thus, the basic system for the culture of most herbaceous perennial species utilizes axillary shoot proliferation. The system involves regeneration of shoots directly from explant tissues and the proliferation of axillary shoots. Subcultured shoots are rooted under conditions of high light in mason jars containing media with an agar concentration of 10 to 14 g/l. After rooting, the plants are transplanted into soil and moved to conditions of high humidity.

Other Systems of Propagating Herbaceous Perennials *In Vitro.* Although most herbaceous perennials produced by tissue culture at Walters Gardens utilize axillary shoot proliferation, three other regeneration systems have been used: an embryoid germination system; a system in which shoots develop from callus; and the formation of bulblets. Generally, though, these systems have been employed only when attempts to proliferate axillary buds have failed.

Propagation of *Asclepia tuberosa* via asexual embryogenesis represents a relatively unique system. Callus tissues obtained from shoot tip explants develop embryoids in response to growth regulator supplements in the medium. Individual embryoids or callus can be continually subcultured to yield more embryoid-containing callus. To induce embryoid germination, whole cultures are smeared onto the surface of a growth regulator-free medium in quart mason jars. After shoots and roots develop from the germinating embryoids, they can be transplanted into soil. Light levels during the germination period should be maintained at 600 to 800 f.c. from cool-white fluorescent lamps.

An intermediary callus step is necessary in the propagation of *Brunnera macrophylla* and *Hemerocallis* (although other species can be propagated this way in addition to axillary shoot tip proliferation). Shoot tips, flower buds, leaves, and sterile roots of *Brunnera macrophylla* develop callus when cultured in *darkness in response to the proper growth regulator additions* in the medium. Shoots develop in the callus and subsequently the cultures are transferred to light. Little axillary shoot proliferation is noted. Such shoots can be rooted and transplanted in the same manner as axillary shoots.

Daylily flower buds also develop callus in darkness on the proper medium. When callus tissues are subcultured and grown in light, shoots arise which then initiate adventitious roots. These can be transplanted into soil. Similar useful systems for iris (3) and tetraploid daylily cultivars (4) have been developed.

The third alternate system of herbaceous perennial propagation involves the production of adventitious bulblets. Tissues from two members of the lily family, *Lilium* cultivars and *Eremurus* × *isabellinus* (Syn.: *E.* × *shelfordii*), the Shelford desert candle, develop bulblets when cultured *in vitro.* Callus developed from *Eremurus* × *isabellinus* embryos regenerate bulblets in light or darkness in response to specific auxin and cytokinin combinations. Bulblets and callus tissues can be subcultured for the continuous production of bulblets. Rooting techniques are being finalized.

Bulblets can develop directly from *Lilium* flower buds cul-

tured in darkness. It is also possible to initiate bulblet formation directly from the tips of inverted sterile roots. Anderson (1) described a system for the production of lily bulblets from cultured bulb scale sections and for root production from the bulblets. Regardless of explant source, subcultured lily tissues will continuously produce bulblets in light or darkness in response to several growth regulator combinations in the medium. After rooting, the bulblets can be transplanted into soil.

CULTURE MEDIA CONSTITUENTS

The basic medium used for culturing herbaceous perennials *in vitro* consists of inorganic salts, sucrose, vitamins, agar, and water. The inorganic salt formula is identical to the Murashige and Skoog formula (5) except for the KH_2PO_4 concentration which is increased to 300 mg/l. In cultures of a few species, however, the concentrations of the major salts must be reduced to 25 or 50% of full strength during rooting and, occasionally for multiplication, to achieve the optimal response. Sucrose is included at 30 g/l while thiamine·HCl, nicotinic acid, and pyridoxine·HCl are used at 0.5 mg/l each. During multiplication 8 to 10 g/l of agar are incorporated but the level is increased to 10 to 14 g/l during rooting.

Certain combinations of growth regulators contained in the culture medium stimulate the regenerative responses. Most herbaceous perennials proliferate multiple shoots in response to 1.0 to 10.0 mg/l benzyladenine (BA), with or without naphthaleneacetic acid (NAA), in the concentration range 0.02 to 1.0 mg/l. Forty of the 47 species listed in Table 1, multiplying by axillary shoot proliferation, respond better to BA than to other cytokinins while 27 of the 40 utilize a combination of BA and NAA. Six species, however, formed multiple shoots best when kinetin or kinetin plus NAA was included in the medium. Gibberellic acid (GA_3) also has been useful in combination with cytokinins and/or auxins for shoot multiplication. Eight species listed in Table 1 require GA_3 for maximum axillary shoot proliferation. Other growth regulators, such as the auxins, indoleacetic acid (IAA), indolebutyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin, 2-isopentenyladenine, have not proven effective in the shoot multiplication process. Typical growth regulator combinations used in the proliferation of shoots were described for cultures of four *Hosta* species (8) and two *Phlox* species (6) and for the woody perennials, *Viburnum lentago* (7) and *Prunus cerasifera* 'Thundercloud' (10). Growth regulator requirements for the stimulation of embryoid formation in *Asclepias tuberosa* (9) and for the production of shoots from callus of *Hemerocallis* (4)

have also been explained.

Cultured shoots develop adventitious roots either on the basal medium or on media containing IBA in the range 0.1 to 2.0 mg/l. Of the 50 species listed in Table 1, 20 formed adventitious roots best on media lacking growth regulators, while 29 required IBA and one used IAA. Although NAA can be effective in stimulating root initiation, it seems to inhibit root growth. The addition of IAA has been generally ineffective in the rooting process and 2,4-D stimulates callus and inhibits shoot growth.

RESPONSE OF PLANTS PRODUCED BY TISSUE CULTURE

Greenhouse and field plantings of the hundreds of thousands of plants produced by the Walters Gardens tissue culture laboratory have proven highly successful. Fantastic growth characteristics are typical of tissue culture-derived plants. Increases in branching and in the production of side shoots have been noted for many crops. Large, multi-branched *Gypsophila paniculata* plants with increases in the number of flowering shoots contrast sharply with those produced by cuttings which are usually weak and spindly. Propagation *in vitro* of disease-free baby's breath has also eliminated crown-gall problems. Numbers of side shoots used for divisions have increased many times in plantings of asters and chrysanthemums. Similarly, field plantings of *Bergenia cordifolia, Petrorhagia Saxifraga* (Syn.: *Tunica saxifraga*), *Chrysanthemum maximum, Hosta ventricosa,* and other species have yielded excellent results.

Genetic stability has also been typical of the species listed in Table 1. Some plants, including *Gypsophila paniculata, Aster novi-belgii,* and *Hosta* spp. have been subcultured many times without obvious changes in phenotype.

Changes in leaf variegation characteristics, however, have been noted in *Ajuga reptans.* Under high axillary shoot multiplication rates, certain *Ajuga* cultivars lose variegated characters. For example, the yellow-green cultivar 'Variegata' can revert to solid green, while the mixed yellow-purple-green cultivar 'Burgundy Glow' reverts to bronzy-green. This problem, however, can be eliminated by reducing the medium cytokinin concentration, thereby slowing the shoot multiplication rate.

COST ANALYSIS

Preliminary analysis of cost data indicates that production of herbaceous perennials by tissue culture can be competitive with conventional propagation techniques. Based on a "per plant" basis, transfer labor costs consist of 52% of total ex-

penses, while media materials and preparation (9%), transplanting (15%), overhead (12%) and other labor (12%) comprise the remainder. The benefits of tissue culture, such as improvement in quality and rapid availability of large quantities of material, however, are initially hard to analyze cost-wise, but certainly must be included in any final economic projects.

CONCLUSIONS

High quality herbaceous perennials can be produced efficiently by tissue culture techniques. *In vitro* propagation can be applied to a wide range of plant species and is economically viable. Production of shoots directly from the explant and their multiplication by the proliferation of axillary shoots has been used for most species. The proper rooting environment enables success after transplanting. The final products exhibit vigorous growth characteristics. Tissue culture is an excellent technique for the propagation of herbaceous perennials.

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DICK ZIMMERMAN: I would like to comment on your agar concentrations. Your use of high agar concentrations is going in just the opposite direction of the work in Europe. Agar is a relatively expensive item and they are doing very well with concentrations as low as 4 grams per liter. In some cases they are using 2 grams and supplementing this with pectin.

MARK ZILIS: High agar levels are useful in acclimatizing

plants from the *in vitro* environment to the soil environment. With the high production rates in tissue culture we are not concerned about material costs. In a cost study we found that media costs consumed about 9% of total expenditures. We feel that you get a higher percentage of transplants with higher agar.

BRUCE BRIGGS: You mentioned the use of GA. Have you noticed any slowing up in rooting of your plants, as we have found with woody material?

MARK ZILIS: We have found that it increased axillary bud break in asters but have not noticed any significant root inhibition.

ROOT AND SHOOT GROWTH RATE RELATIONSHIPS OF TWO JAPANESE HOLLY CULTIVARS DURING PROPAGATION

WILLIAM MERTENS

Department of Horticulture
Virginia Polytechnic Institue and State University
Blacksburg, Virginia 24061

Abstract. Cuttings of *Ilex crenata* Thumb. 'Helleri' and 'Rotundifolia' were rooted and grown in polyvinyl chloride pipe sections from which longitudinal sections could be removed for root observations. Plants were fertilized at either 150 or 300 ppm N with a 20N-8.7P-16.5K soluble fertilizer. Rate of root and shoot growth was determined through 2 to 3 flushes of growth following rooting by taking weekly measurements of shoots and roots. Root growth of both cultivars usually preceded a shoot growth flush by 1 to 2 weeks. This growth pattern was observed at both fertility levels.

The propagation of woody nursery plants in small containers is widely practiced in the nursery industry. Following rooting the plants are fertilized and grown in these containers until they are large enough to transplant to the field or larger containers. A well balanced root and shoot system is necessary if these plants are to survive transplanting shock. Gilliam and Wright (3) demonstrated that fertilizer treatments of rooted cuttings which encourage top growth may limit root growth. This indicates that some control over root and shoot growth is possible. Before control can begin, however, some knowledge of root and shoot growth patterns during and following rooting are required.

This study was made to determine the pattern of root and shoot growth during and following rooting of *Ilex crenata* 'Helleri' and 'Rotundifolia' grown at two fertilizer levels following rooting.