Comparing Micropropagation Protocols for a Herbaceous Perennial, a Woody Shrub, and a Conifer.

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Abstract

Micropropagation offers opportunities to reproduce plants when conventional propagation methods are unfeasible or inappropriate. At the University of Idaho Forest Research Nursery Micropropagation Unit, three types of plants, a herbaceous perennial (*Hackelia venusta*), a woody shrub (*Purshia tridentata*), and a conifer (*Pinus monticola*), have all been successfully micropropagated. We describe and contrast the protocols for each species, all of which yielded plants that performed well after culture. Our discussion provides land managers a basis to evaluate if micropropagation is appropriate for their revegetation needs and give estimates of the time and subsequent cost associated with producing micropropagated stock.

Keywords

Hackelia venusta, *Purshia tridentata*, *Pinus monticola*, reintroduction, tissue culture

Introduction

Vegetative propagation is useful for producing native plants in nurseries. The most common methods include root cuttings, hardwood cuttings from dormant tissue, softwood cuttings from actively growing tissue, grafting, and layering. For many native plants, these techniques offer the only successful method (from either a plant or economic perspective) for producing new plants. Micropropagation, another type of vegetative propagation, involves sterilizing explants (e.g., seeds, shoots, or buds), inducing shoot growth in sterile culture, causing shoots to form buds, inducing those buds to elongate into shoots, rooting elongated shoots, and acclimatizing the new plantlets

to conditions outside the laboratory. Once acclimatized, plantlets can be grown under standard nursery culture to the size and viability (quality) required for outplanting.

Our focus over the past few years has been off-site conservation of rare, threatened, or endangered species and reintroduction of these species into former and/or protected habitats as advocated by Maunder (1992), and mass-propagation of outstanding clonal selections of more common species. Land managers coping with problems associated with threatened and endangered plants should consider micropropagation as a means for off-site conservation and eventual reintroduction because the technique is fast, uses small amounts of plant material (i.e., seed or shoots), and may succeed when other methods fail (Fay 1992). As might be expected, micropropagation protocols can be as diverse as the species being propagated, but often objectives of specific protocols are similar. In this paper, we examine three species: *Hackelia venusta* (Piper) St. John, *Purshia tridentata* (Pursh) DC, and *Pinus monticola* Dougl. ex. D. Don., three species for which the actual micropropagation protocols vary considerably.

The Plants

Hackelia venusta (showy stickseed) is an endangered herbaceous perennial (CPC 1991) endemic to the Washington Cascade Range of the interior northwestern United States. The taxon consists of white-flowered and blue-flowered populations. Before a reintroduction of micropropagated plants in 1995, fewer than 100 white-flowered plants existed, threatened by road construction, plant collection, introduction of competitor species, rock fall, and fire (Edson et al. 1996).

Overgrazing, fire, and introduced grasses have reduced the abundance of Purshia tridentata (antelope bitterbrush) (Ferguson and Medin 1983), a genetically diverse rosaceous woody shrub and important ungulate browse species of rangelands and forests in the western United States (Nord 1965, Welch et al. 1983, Winward and Findley 1983). Natural and artificial regeneration is often limited by low seed yield, complex seed dormancy, and rodent predation (Young and Evans 1981). Edson et al. (1997) concluded vegetative propagation could help regenerate unreproductive populations. Since some genotypes sprout after fire (Martin and Driver 1983), appropriate habitat could be revegetated with fire-resistant clones. Unfortunately, stem cuttings do not root readily (Everett et al. 1977).

Pinus monticola (western white pine) is a large conifer of the northern Rocky Mountains. Introduction of blister rust (*Cronartium ribicola* Fischer) from Europe decimated stands. Breeding efforts resulted in seed orchards yielding progeny with varying resistance to blister rust and additional orchards are planned (Howe and Smith 1994). Zobel (1992) concludes vegetative propagation can be an important factor in tree improvement programs. Although *P. monticola* can be propagated via conventional cuttings from juvenile donor plants (ortets)(Power and Libby 1986, Edson et al. 1994), a lack of cutting orchards means that plantlets micropropagated from seeds could be produced faster than rooted cuttings from seedling ortets.

Comparing Protocols

When explants are placed "*in vitro*" (literally cultured "*in glass*"), the section of plant in contact with the basal medium begins to grow into an undifferentiated, tumor-like mass of tissue called callus. Many adventitious buds can form on callus and form new shoots (microshoots), a process known as organogenesis. However, because buds arise from an undifferentiated mass, genetic variation (mutation) can be introduced (commonly called somaclonal variation). In conventional micropropagation, somalclonal variation is usually of little concern, and adventitious budding is often desired because a clone can be "multiplied" (more microshoots available from the original explant) in a shorter amount of time. However, when working with small populations of threatened and endangered plants or plants from very specific habitats, the objective is to avoid somaclonal variation, select explants from as large a group of genotypes as possible, and produce relatively few clones of each genotype. Somaclonal variation may be suppressed by reducing or eliminating the amount of plant hormones (auxin and cytokinin) in the basal medium. Explants then produce nominal callus with fewer adventitious buds. To multiply the clone, micropropagators

Table 1. Micropropagation protocols for *Hackelia venusta*, *Purshia tridentata*, and *Pinus monticola*.

	Hackelia venusta		*Purshia tridentata*		*Pinus monticola*	
	Culture	wks	Culture	wks	Culture	wks
Explant	Shoot tip or node		Shoot tip		Non-stratified embryos	
Microshoot proliferation	Murashige & Skoog (1962)(MS) + 0.04 μM BA	8	Woody Plant Medium (Lloyd & McCown (1980) + 0.44 μM BA & 0.54 μM NAA	12	Litvay et al. (1981)(Lt) + 45 μM BA 1/2 strength Lt + 0.2% charcoal Lt	4 4 20
Microshoot elongation	Occurs during proliferation		Occurs during proliferation		Quoirin & LePoivre (1977)	12
Microshoot rooting	*in vitro* MS + 2 μM IAA MS + 2 μM IAA (recalcitrant clones)	4 (8)	*ex vitro* 0.1% NAA or 0.1% IBA No auxin	5 (8)	*in vitro* 1/2 -strength Gresshoff & Doy (1972)(GD) + 50 μM IBA 1/2 -strength GD *ex vitro* 0.3% IBA	(1) (11) 12
Acclimation	Progressively decrease relative humidity from 88% to 40% to ambient. Increase sunlight from 60% shade to 30% shade to normal greenhouse sunlight	4	Progressively decrease relative humidity from 88% to 40% to ambient. Increase sunlight from60% shade to 30% shade to normal greenhouse sunlight	4	Progressively decrease relative humidity from 88% to 40% to ambient. Increase sunlight from 60% shade to 30% shade to normal greenhouse sunlight	4
Nursery culture	20:20:20 (N:P:K) at 200 ppm N	8	7:40:17 (N:P:K) at 50 ppm N 20:20:20 (N:P:K) at 200 ppm N 5:25:35 (N:P:K) at 60 ppm N	4 12 12	7:40:17 (N:P:K) at 50 ppm N 20:20:20 (N:P:K) at 200 ppm N 5:25:35 (N:P:K) at 60 ppm N	8 12 12
Final size	10 cm tall 10 cm wide 15 cm long, firm root plug Container volume = 170 ml		25 cm tall 3+ mm stem diameter 15 cm long, firm root plug Container volume = 170 ml		10-15 cm tall 2.5+ mm stem diameter 15 cm long, firm root plug Container volume = 90 ml	
Total culture time		24		49		88
Reference	Edson et al. 1996		Edson et al. 1997		Edson and Wenny 1997	

Abbreviations: 6-benzyladenine (BA), indoleacetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA), micromole (μM; 10^{-6}).

Table 2. Assuming 20 initial explants, the number of microshoots proliferated per cycle, and the weeks required to proliferate microshoots to produce 500 plantlets for outplanting of *Hackelia venusta*, *Purshia tridentata*, and *Pinus monticola*. Micropropagation costs for 500 plantlets assumes a viable protocol is known, labor is $10 per hour, and that after initial mircoshoot proliferation, subsequent shoot multiplication cycles require only 4 weeks. Nursery production costs assume *H. venusta* and *P. tridentata* grown in Copperblock 77/170 (366 plants per m^2, 170 ml; 34 plants per ft^2, 20 in^3), and *P. monticola* grown in Copperblock 160/90 (764 plants per m^2, 90 ml; 71 plants per ft^2, 5.5 in^3).

	Initial explants	Microshoots per explant	Microshoots proliferated per cycle			Weeks required to proliferate 500 microshoots	Micropropagation costs	Nursery production costs	Cost per plant
			1	2	3				
Hackelia venusta	20	2.3	46	106	244	20	$600	$75	$1.35
Purshia tridentata	20	5	100	500	2500	20	$300	$175	$0.95
Pinus monticola	20	40	800	32000	1280000	40	$1900	$85	$3.97

must then rely completely on axillary buds, which form above the callus on the explant.

Because our objective was to produce true-to-type *H. venusta* and *P. tridentata* clones, we used very low levels of growth hormones *in vitro*, and low numbers of axillary buds limited the number of possible microshoots (low multiplication of clones). In contrast, our objective with *P. monticola* was mass *in vitro* propagation of superior selections (ones less affected by blister rust) through organogenesis (high multiplication of clones). The multiplication method affects how rapidly explants can be converted into plantlets for outplanting, but it is not the only factor. Other factors include how quickly microshoots elongate, elongated microshoots produce roots, and rooted microshoots grow *ex vitro* (not in culture) to outplantable size and viability (Table 1). Microshoot survival at each step is another important factor. Moreover, the more time required to multiply clones, elongate microshoots, root microshoots, and grow them in the nursery, the higher the production cost (Table 2). Even plant growth form influences the protocol and resulting production costs. The major cost of micropropagation is associated with transfers. In our system, explants and microshoots of all three species were transferred from old medium to fresh medium every 3 to 4 weeks because of water loss from the medium, a decrease in nutrient concentrations due to plant uptake, and buildup of toxins.

Micropropagation time varies with species (Table 1). The herbaceous perennial *H. venusta* can be cultured and outplanted in as little as 6 months. Microshoots can be proliferated and elongated in the same step, elongated microshoots root quickly *in vitro*, and acclimated plantlets grow rapidly under nursery conditions. The woody shrub *Purshia tridentata* requires about twice as much time as *Hackelia* from onset of culture until a plant is ready for outplanting. Like *H. venusta*, microshoot proliferation and elongation occur in the same step. Rooting of elongated microshoots takes about 2 months, and acclimated plantlets require about 8 months in the nursery to grow large enough for outplanting. The conifer *P. monticola* requires nearly 2 years from onset of culture until a plantable product is ready. Unlike *H. venusta* and *P. tridentata*, *P. monticola* requires a lengthy two-step process to proliferate and elongate microshoots. *Pinus monticola* also takes nearly twice as long to form roots on elongated microshoots.

Although *H. venusta* propagates quickly *in vitro*, axillary bud yield is low. In contrast, *P. monticola* produces adventitious shoots at a high rate (Table 2), but requires 500% more time for microshoots to proliferate and elongate than *H. venusta* (8 weeks vs. 40 weeks;

Table 1). Starting with equal numbers of explants and proliferating 500 plantlets of each species, the low microshoot fecundity of *H. venusta* results in a culture time nearly 50% of that required for *P. monticola* (Table 2).

Depending on species, microshoots are rooted either *in vitro*, *ex vitro*, or sometimes can be rooted both ways (Table 1). *Ex vitro* rooting, where microshoots are stuck into a soil-less medium commonly used in nurseries, is the most cost effective method (requires fewer transfers to fresh medium), especially if the rate of rooting is high. Nearly 90% of elongated *H. venusta* microshoots rooted successfully, but only *in vitro* (Edson et al. 1996), a more costly method than *P. tridentata* which had nearly 90% rooting *ex vitro* (Edson et al. 1997). Although *P. monticola* does not root at a particularly high rate (68%; Edson and Wenny 1997), it does have the advantage of rooting *ex vitro*.

Once microshoots are rooted (plantlets), most species can be acclimated similarly (Table 1), and grown under normal nursery conditions (e.g., Wenny and Dumroese 1992). A herbaceous perennial like *H. venusta* grows rapidly in the greenhouse and can be outplanted in as little as 2 months (Table 1). Woody plants like *P. tridentata* and *P. monticola* require a longer nursery cultural phase to reach outplanting size and viability, similar to traditional container seedlings grown for reforestation or conservation (e.g., Wenny and Dumroese 1987).

Outplanting Performance

Eight weeks of nursery culture produced robust *H. venusta* plantlets (Table 1). We outplanted 296 plants in 1994 on two different sites in the Wenatchee National Forest, Washington. First year survival averaged 60% on two different sites. Most (86%) of the surviving reintroduced plants were reproductive the spring following planting (Edson et al. 1996).

Clones from selected *P. tridentata* populations were successfully micropropagated. In the nursery, 98% of micropropagated plants survived and all flowered during the second year of growth. Under conventional nursery culture, plants grew well (Table 1), averaged about 25 cm in height, and were outplanted in spring on the Ochoco National Forest, Oregon. Survival that fall was 99% and one year later was 96%.

With *P. monticola*, the objective of mass clonal production is hampered by the time consuming and laborious process of proliferating, elongating, and rooting microshoots. However, the high adventitious bud fecundity helps ameliorate those shortcomings (Table 2). Rooted microshoots grow well in the nursery, much like seedlings, and we expect outplanting performance to be similar.

Summary

A variety of plants can be micropropagated, and this technique may be particularly useful for land managers with threatened and endangered populations, plants with particular growth characteristics not reliably reproduced by other propagation techniques, and when demand for a particular genotype exceeds plants available from conventional propagules. Although micropropagation protocols vary with species, longer micropropagation cycles generally result in higher production costs. Factors that affect cost include time *in vitro* and subsequent number of transfers, microshoot proliferation technique (axillary vs. adventitious), rootability of elongated microshoots, and time necessary to grow plantlets to outplantable size. Fortunately, acclimated rooted microshoots are easily grown with standard nursery procedures, and outplanting success is high.

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