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Rapid Propagation of Blueberry Plants Using ex Vitro Rooting and Controlled Acclimatization of Micropropagules

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Abstract. **A protocol is presented that enables a propagator to produce field-sized blueberry transplants within 6 months of obtaining microshoots from tissue culture. The protocol involves subjecting microshoots to ex vitro rooting in a fog chamber under 100 μmol·m^{-2}·s^{-1} photosynthetic photon flux for 7 weeks, transferring plants to a fog tunnel for 2 weeks, then to a greenhouse for 7 more weeks. Plant survival and rooting of cultivars Berkeley (*Vaccinium corymbosum* L.) and Northsky (*Vaccinium angustifolium* ×*corymbosum*) were near 100% under these conditions. Plantlets in fog chambers receiving 100 μmol·m^{-2}·s^{-1} grew rapidly, while those at lower irradiance levels grew more slowly, and supplemental CO_2 enhanced growth only at 50 μmol·m^{-2}·s^{-1}. Growth rates slowed when plants were moved into the fog tunnel; but by the end of 16 weeks, plants that were under high irradiance in the fog chamber had root systems that were 15 to 30 times larger than plants under low irradiance. Within 6 months, these plants were 30 to 60 cm tall and suitable for field planting.**

Blueberry hectarage is expanding rapidly throughout much of the world (Eck, 1988), which has led to an increased demand for planting stock. Conventional propagation has limitations in meeting this demand. For example, propagation by stem cuttings is slow, and many genotypes do not respond to root-inducing growth regulators (Nickerson, 1978). Lyrene (1978) observed only 7% rooting of stem cuttings, but 95% rooting of tissue-culture-derived microcuttings. Consequently, micropropagation has become increasingly popular for rapid multiplication of virus-indexed cultivars (Wolfe et al., 1983).

Micropropagation facilities are expensive (Dunstan and Turner, 1984; Kozai and Iwanami, 1987); therefore, steps that can reduce costs will make micropropagation more attractive to nursery operators. Rooting blueberry microcuttings ex vitro (in open air rather than within sealed glass containers) can reduce costs (Read and Fellman, 1985; Simmonds, 1983; Zimmerman, 1987), but rooting is often slower than in vitro (Wolfe et al., 1986).

Enhanced growth from an accelerated net assimilation rate may help plantlets survive rapid ex vitro acclimatization (Mousseau, 1986). For several plant species, improved vigor during acclimatization has been achieved by CO_2 enrichment and supplemental irradiance (Brissette et al., 1990; Desjardins et al., 1987, 1990; Hurd and Thornley, 1974; Infante et al., 1989; Lakso et al., 1986).

Rooting and survival of microcuttings also can be limited by their ability to regulate water loss through leaves (Brainerd and Fuchigami, 1982; Desjardins et al., 1987; Grout and Donkin, 1987). Consequently, microcuttings moved from in vitro culture conditions must be acclimatized gradually to ambient conditions to avoid mortality that might otherwise occur under an abrupt change in relative humidity, temperature, or irradiance.

This investigation was conducted to identify environmental conditions that would accelerate rooting and acclimatization and improve survival of ex vitro blueberry microcuttings.

Materials and Methods

Microshoot cultures of 'Berkeley' and 'Northsky' blueberries were obtained from Briggs Nursery, Olympia, Wash., and Hartmann's Plantation, Grand Junction, Mich. The cultures were multiplied on McCown's woody plant medium (Lloyd and McCown, 1980) supplemented with (mg·liter^{-1}) 1 thiamin, 0.5 nicotinic acid, 0.5 pyridoxine, 5 *N*-(3-methyl-2-butenyl)-1*H*-purin-6-amine (2iP), 2 glycine, 100 myo-inositol, plus 30 g sucrose and 7 g agar (Difco, Bactoagar)/liter. The pH of the medium was adjusted to 4.5 with KOH or HCl before dispensing into baby food jars with B-caps (Magenta Corp., Chicago) and autoclaving for 18 min at 121C. Culture vessels were sealed with parafilm and incubated in a culture room maintained at 24 ± 2C and receiving 50 μmol·m^{-2}·s^{-1} for 16 h/day. Axillary shoot cultures were subdivided and transferred to fresh medium every 4 weeks.

When sufficient microshoots were obtained, they were excised from the initial explant and placed on the rooting medium ex vitro. This medium consisted of 4 moist peat : 1 perlite (v/v) in number 128 flat trays (TLC Plymouth, Plymouth, Minn.) measuring 40 × 30 × 2.5 cm. Each 2.5 × 2.5 × 3.8-cm plug held a single microcutting.

The design consisted of a factorial arrangement of treatments, with two CO_2 levels (450 and 1200 ppm) and three irradiance levels (18 h of photosynthetically active radiation at 30 ± 5, 50 ± 10, and 100 ± 20 μmol·m^{-2}·s^{-1}). The experiment was conducted twice; a total of 160 microcuttings was used for each treatment combination.

The fog chamber was 3.6 × 2.4 × 0.56 m with six 1.2 × 1.2-m compartments. Carbon dioxide gas flowed through a flow rate meter, and concentration was monitored by a Horiba CO_2 monitor (Horiba Instruments, Irvine, Calif.). Irradiance was supplied by six 2.4-m General Electric Cool White Power Groove or Sylvania Cool White/Very High Output fluorescent tubes with a timer. The two lower irradiance levels were obtained by placing several layers of Saran shadecloth on top of the chambers.

Fog was generated by four 7.6-liter household ultrasonic humidifiers (Sunbeam, Hattiesville, Miss.). Cycling was adjusted to achieve 94% to 100% relative humidity, depending on the irradiance level. Temperature was manually controlled by a heater or air conditioner in the room in which the fog chamber was located.

A fog tunnel facilitated the transition from the fog chamber to greenhouse conditions. The fog tunnel was a raised wooden frame (3.5 × 1.0 × 0.75 m) on a greenhouse bench covered with clear polyethylene sheeting on three sides. Three 60-W incandescent lights were used to maintain a constant 16-h photoperiod throughout the year. Fog was generated at one end using three ultrasonic humidifiers as described previously. The end farthest from the fogger was open, thus generating a humidity gradient. Two layers of Saran shadecloth at the high humidity end, and one layer at the other, provided an irradiance gradient. Plants were moved through the fog tunnel during 2 weeks, from one end with low irradiance and high relative humidity, to the other with near ambient greenhouse conditions.

Ten microcuttings were selected randomly and harvested every 2 weeks, except during the second replication of 'Berkeley' when five were harvested. Roots were washed and separated from shoots. Leaves were separated from stems and their leaf area measured by a LI-COR leaf area meter (model LI-3100; Lincoln, Neb.). Plant parts were oven-dried at 75C for 2 to 4 days and dry weights measured.

Rooted microcuttings were potted into 0.75-

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liter (10 cm) standard plastic pots filled with 4 peat : 1 perlite (v/v). Plants were grown from Sept. 1992 to May 1993 in a greenhouse at Cornell Univ. Days in the greenhouse averaged 21C and nights 17C, and the photoperiod was maintained at 16 h.

A general-purpose N20–P20–K20 Peters soluble fertilizer (Grace-Sierra, Fogelsville, Pa.) at 2 g·liter^{-1} was applied to flats in the fog chamber at 2-week intervals. In the greenhouse, fertilizer was applied monthly. Irrigation was applied when necessary.

Dry weight data were transformed to the natural logarithm and analyzed by a linear model or two-way analysis of variance, as appropriate.

Results

Microcuttings rooted quickly ex vitro (>99%) and performed well during the 126-day observation period. Root primordia formed within 7 days and typical fibrous roots after 14 days. Significant treatment differences in plant dry weight and leaf area occurred by 42 days ($P < 0.03$), with higher irradiance levels associated with higher plant dry weight (Fig. 1). No significant irradiance effect for root dry weight was found for 'Berkeley' until the time of transfer to the fog tunnel, at day 56. Plantlets that were rooted under the highest irradiance level (100 μmol·m^{-2}·s^{-1}) had extensively branched roots, compared to other treatments. No significant main effect of CO_2 was detected over the 126-day observation period; however, an interaction developed upon transfer to the fog tunnel for plants rooted under 50 μmol·m^{-2}·s^{-1}. Plants rooted at this irradiance level at the higher CO_2 concentration grew at the same rate as plants exposed to the higher irradiance level (Fig. 1).

Relative growth rates tended to decrease upon transfer to the fog tunnel at day 56; during this period, mature leaves abscised and new leaves did not form. Growth resumed within 2 weeks in the greenhouse, but not at the same rate as before (Fig. 2). The effect of fog chamber irradiance was still measurable in the greenhouse, but growth rates in the greenhouse did not vary significantly.

A comparison of growth over the entire 126-day period indicated that plants of both cultivars at 100 μmol·m^{-2}·s^{-1} and plants at 50 μmol·m^{-2}·s^{-1} with supplemental CO_2 grew equally well (Table 1). 'Berkeley' plants grown at 30 μmol·m^{-2}·s^{-1} had root dry weights that were 15 to 30 times less than plants grown at the highest irradiance level (data not shown). Even though plants were small at low irradiance levels, percent survival was high across all treatments, ranging from 97% to 100%. Within 6 months, plants that were rooted and grown under high irradiance levels and acclimated to greenhouse conditions were between 30 and 60 cm tall.

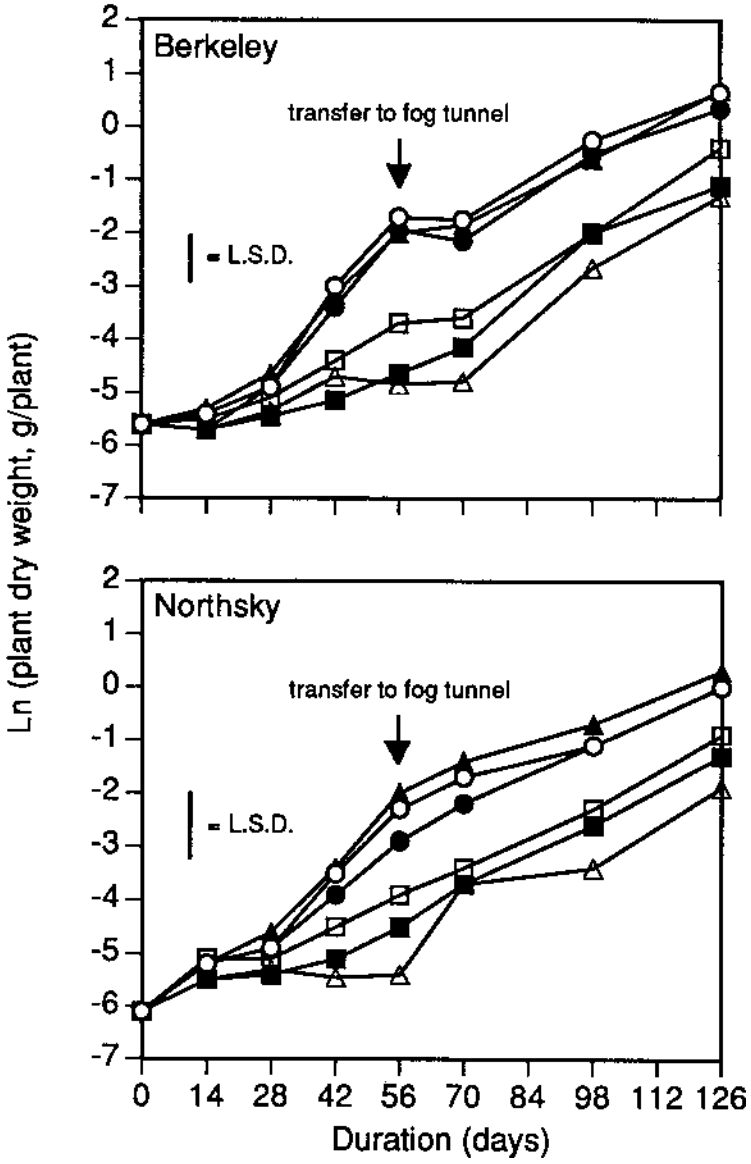

Fig. 1. Dry weight of 'Berkeley' and 'Northsky' plantlets subjected to various levels of irradiance and CO_2 for the first 56 days: 450 ppm CO_2 and (■) 30, (▲) 50, and (●) 100 μmol·m^{-2}·s^{-1} and 1200 ppm CO_2 and (■) 30, (▲) 50, and (●) 100 μmol·m^{-2}·s^{-1}.

Discussion

Ex vitro rooting and subsequent acclimatization of blueberry plantlets were achieved successfully by rooting plantlets in a fog chamber with controlled light, humidity, and elevated CO_2; passing them through a fog tunnel containing a gradient of light and humidity; and into a greenhouse with ambient light, humidity, and CO_2. Rooting percentage ex vitro was near 100%, but subsequent growth depended on irradiance level in the fog chamber. Our previous work suggested that blueberry microcuttings would not tolerate initial irradiance levels of >200 μmol·m^{-2}·s^{-1} (unpublished data). In the study under discussion, 100 μmol·m^{-2}·s^{-1} provided the best environment for growth, but this level induced leaf discoloration, whereas lower levels did not. We were able to alleviate the red discoloration temporarily with fertilization, suggesting that nutrient levels must be higher under higher light to optimize growth.

The effect of irradiance on plant growth is likely mediated through an increase in photoautotrophism and, hence, more photoassimilate. If stomata are functional and irradiance is high, elevated CO_2 can increase photosynthesis and improve water-use efficiency. The absence of a CO_2 effect in our experiment at 100 μmol·m^{-2}·s^{-1} suggests that stomata may not be fully functional for several weeks after growing in vitro, or that light levels were sufficiently low such that 450 ppm CO_2 was not limiting. The absence of a CO_2 effect has been observed in other species (Desjardins et al., 1987; Kramer, 1981). The enhanced performance of plants in the presence of supplemental CO_2 at 50 μmol·m^{-2}·s^{-1} photosynthetic photon flux may have been an artifact, because at a lower irradiance level, no effect was observed, and at a higher level, plants exposed to lower CO_2 (450 ppm) tended to grow better.

Previous acclimatization protocols under low irradiance with blueberry (Wolfe et al., 1983) and other species (Grout and Aston, 1978; Wardle et al., 1983; Ziv, 1986) resulted in good survival, but encountered problems

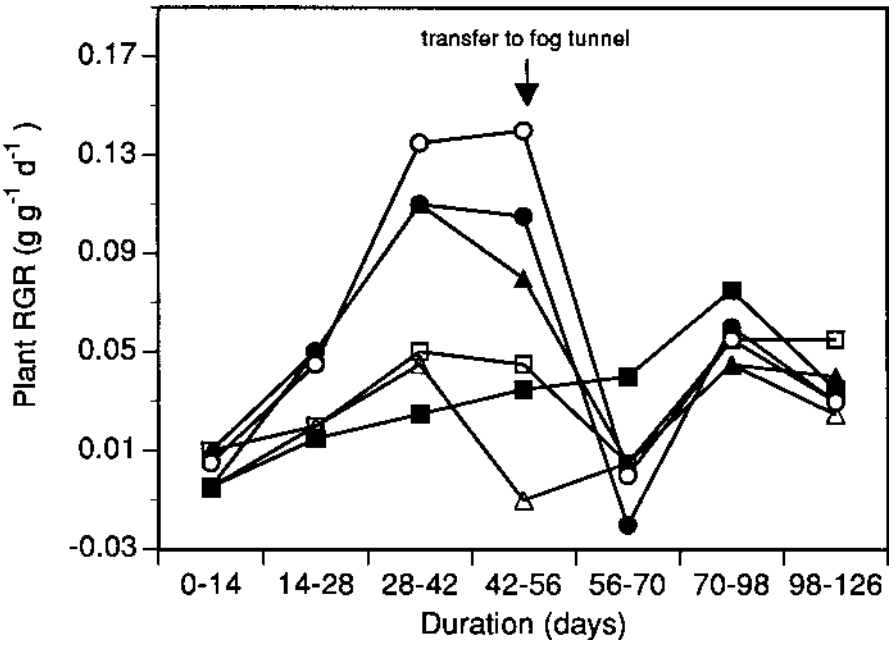

Fig. 2. Relative growth rate of 'Berkeley' plantlets subjected to various levels of irradiance and CO_2 for the first 56 days: 450 ppm CO_2 and (■) 30, (▲) 50, and (●) 100 μmol·m^{-2}·s^{-1} and 1200 ppm CO_2 and (■) 30, (▲) 50, and (●) 100 μmol·m^{-2}·s^{-1}.

Table 1. Relative growth rates by 126 days for total plant dry weight, leaf area, and root dry weight for 'Northsky' and 'Berkeley' for various combinations of irradiance and CO_2.

		Cultivar					
		Northsky			Berkeley		
Irradiance (μmol·m^{-2}·s^{-1})	CO_2 (ppm)	Total dry wt[z]	Leaf area[y]	Root dry wt[z]	Total dry wt[z]	Leaf area[y]	Root dry wt[z]
30	450	0.039 b[x]	0.045 b	0.041 b	0.039 b	0.038 b	0.046 a
	1200	0.032 a	0.038 a	0.034 a	0.034 a	0.035 a	0.042 a
50	450	0.040 b	0.045 b	0.041 b	0.041 b	0.045 c	0.053 b
	1200	0.049 c	0.051 c	0.049 c	0.052 c	0.051 d	0.062 c
100	450	0.052 c	0.055 d	0.060 d	0.052 c	0.050 d	0.059 c
	1200	0.049 c	0.051 c	0.050 c	0.054 c	0.051 d	0.063 c
$LSD_{0.05}$		0.003	0.002	0.002	0.003	0.002	0.004

[z]Dry weight = g·g^{-1}/day.
[y]Leaf area = cm^2·cm^{-2}/day.
[x]Mean separation within columns at $P \leq 0.05$.

with low vigor. With our protocol, high vigor was expressed by blueberry plants grown under 100 μmol·m^{-2}·s^{-1}, and plants produced multiple shoots at an early stage.

Our results demonstrate the consistency and efficiency of a fog chamber in facilitating rooting and achieving high growth rates. For example, using our protocol, a plant propagator could root and produce >1000 plants/m^2 in a fog chamber in <2 months. In another 4 months, these plants could be acclimated to ambient light and humidity in a fog tunnel and greenhouse. Therefore, within 6 months a propagator could produce blueberry plants between 30 and 60 cm tall, sufficiently large for direct field planting, according to commercial standards. Obtaining plants this large can take 2 or more years through conventional propagation and 1 year with currently used micropropagation protocols.

Lyrene (1981) attributed the ease of rooting blueberries in vitro to a reversion to a juvenile condition. If this is true, and the juvenile period persists for several years, then productivity could be reduced in micropropagated plants. Therefore, micropropagated blueberry plants should be planted in the field with conventionally propagated plants to determine the long-term effects of propagation method on plant performance.

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