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Micropropagation of Lowbush Blueberry from Mature Field-grown Plants

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Abstract. **Bud cultures from nonjuvenile field clones of lowbush blueberry *(Vaccinium angustifolium* Ait.) were established on Z-2 medium with 59 µM 2iP. Reversion to juvenile characteristics with small and rounder leaves occurred only on two explants after 19 weeks in culture. These shoots grew vigorously and could be easily subcultured. The number of shoots of one clone doubled every 23.3 days. Reducing the 2iP concentration to 12.3 and 24.6 µM reduced shoot proliferation, but permitted better shoot elongation. After 5 weeks in a mix of 3 peat : 2 vermiculite : 1 perlite, shoots >20 mm rooted better than shoots measuring 10 to 20 mm. Chemical names used: N-(3-methyl-2-butenyl)-1-H-purine-6-amine (2iP); 1H-indole-3-acetic acid (IAA).**

Annual yields of lowbush blueberry are variable in the northern area of its distribution. Losses by cold injury can reach 50% in some years. This problem could be partially overcome by the culture of selected cultivars. The propagation of highly productive cultivars is also possible by cuttings, but tissue culture may be better to increase rapidly the quantity of clones needed for release. Tissue culture plants of lowbush blueberry are known to spread faster than those of the same clones propagated by cuttings.

Tissue culture techniques have been developed for highbush *(Vaccinium corymbosum* L.) (Cohen, 1980; Zimmerman and Broome, 1980; Wolfe et al., 1983; Grout and Read, 1986) and rabbiteye *(Vaccinium ashei* Reade) (Lyrene, 1978, 1980) blueberry. Apical and lateral buds of juvenile and mature plants can be used to establish the cultures, but the age of the stock plants influences micropropagation. For example, multiple shoot formation from shoot tips taken from juvenile rabbiteye blueberry was easy and rapid (Lyrene, 1980), but growth of mature shoot explants was slow, and reversion to juvenile characteristics with vigorous growth was only occasional (Lyrene, 1981).

Shoot formation from hypocotyl or excised cotyledons of lowbush blueberry was reported by Nickerson (1978). Plantlets from bud cultures of a field-selected clone were obtained by Frett and Smagula (1983), but the percentage of rooting was only 44%. As shoot length plays an important role in rooting highbush blueberry shoots in vitro (Wolfe et al., 1983), insufficient shoot elongation may be the cause of low rooting percentages of lowbush blueberry. Shoot length is inversely related to the 2iP concentration in the culture medium of highbush blueberry (Zimmerman and Broome, 1980; Billings et al., 1988). Also, reduced levels of cytokinin (24.6 µM 2iP) permit elongation of rabbiteye blueberry microshoots (Lyrene, 1978). In the case of lowbush blueberry, Frett and Smagula (1983) predicted a maximum shoot length at 59 µM 2iP. The objectives of our research were to obtain rejuvenated clones from field-grown stock plants and to study the effect of 2iP concentration on shoot length and on subsequent rooting.

For the initiation phase, actively growing shoot tips (30 to 50 mm long) were selected from plants growing in a commercial field located in Ste Marguerite-Marie (lat. 48°47'N, long. 72°18'W) near St. John's Lake in the province of Quebec in June 1986. The shoots were sectioned into 20-mm segments and surface-disinfected for 10 min in 6% calcium hypochlorite. The segments were rinsed a few seconds in sterile distilled water and soaked in a sterile solution containing 7.5 mg citric acid and 5 mg ascorbic acid in 100 ml of water. Finally, the segments were rinsed twice for 10 min each time with sterile distilled water. Both ends of the segments were recut to obtain single-bud explants 6 mm long, including a leaf and a portion of stem. A total of 475 explants were placed on 20 ml of Z-2 medium (Zimmerman and Broome, 1980) in 20-mm test tubes (1 explant/tube). The pH was adjusted to 4.8 before adding sucrose (3%) and agar (1%). Growth regulators IAA (11.4 and 22.8 µM) and 2iP (49.2 and 73.8 µM) were added to the medium in a factorial combination for the first 6 weeks in culture. The cultures were grown at 23 ± 2C on a shelf unit under 16-hr photoperiod (30 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at the level of the explants) using cool-white fluorescent tubes.

For the multiplication phase, only 2iP (59

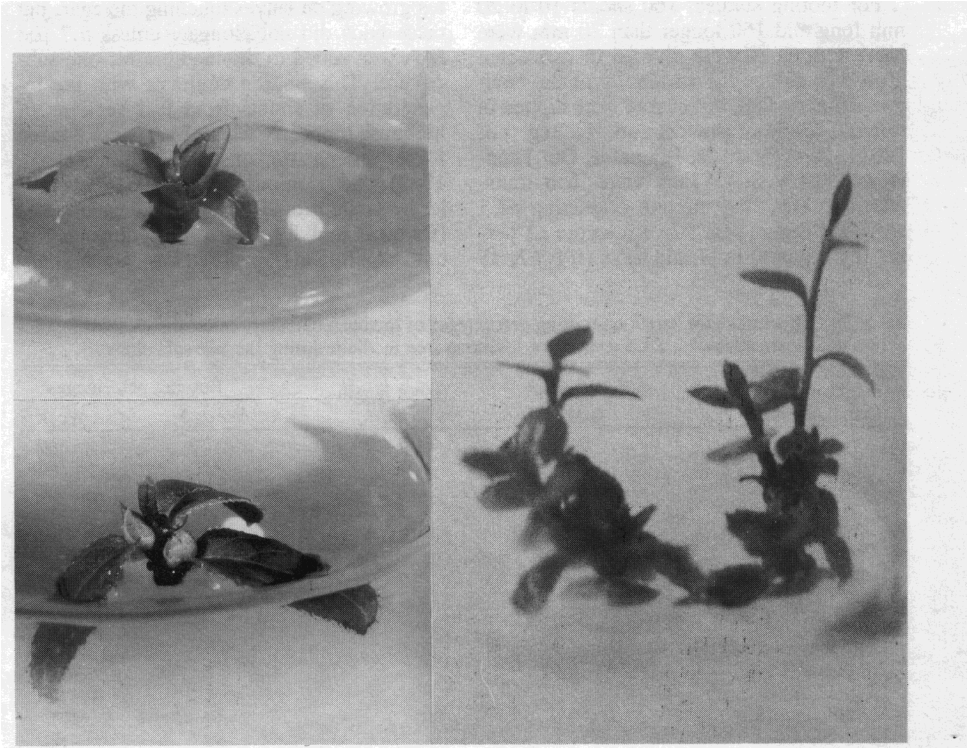

Fig. 1. **(upper left)** The opened axillary bud of a non-juvenile explant of lowbush blueberry after 6 weeks (× 4). **(lower left)** Three expanded leaves of mature appearance; there is no stem elongation and the apex is dead. Two axillary buds were produced **(** × 4). **(right)** Juvenile shoots proliferating by axillary budding (× 3).

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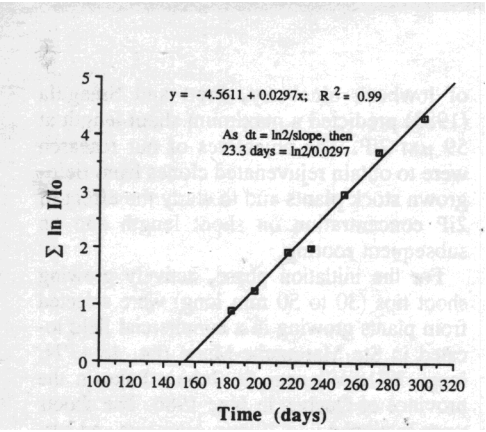

Fig. 2. Regression curve calculated from the summation of the natural logarithms of the number of shoots measured at the beginning (I_o) and at the end (I) of each subculture of lowbush blueberry on Z-2 medium with 59 µM 2iP, and estimating the shoot doubling time.

µM) was added to the basal medium. Subcultures were done at 3-week intervals. The environmental conditions were similar to the initiation phase. The number of shoots was measured at the beginning and at the end of each subculture to estimate the shoot doubling time by the method of Flegman and Wainwright (1981).

For the elongation phase, shoots (5 mm long) were placed on Z-2 medium with reduced cytokinin concentration (0, 12.3, and 24.6 µM 2iP). The culture test tubes were maintained at room temperature under the same light regime as in the initiation phase or under high-pressure sodium lamps (400 W HPS) at 140 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

For rooting studies, 160 shoots 10 to 20 mm long and 160 longer than 20 mm were harvested. They were divided in two equal groups' to reduce the variability of the environmental factors. All shoots were dipped in sterile Stim-Root powder (no. 1; Reg. no. 12011, Plant Products, Bramalea, Ont.) containing 0.1% IBA. They were then transferred to a potting mixture consisting of 3 moist sphagnum peat : 2 vermiculite : 1 perlite (by volume) in plastic trays (IPL 67, 45 cm^3 each hole) and placed randomly in a rooting chamber where they were misted manually three times a day. The shoots were grown at 20 ± 2C under a 18-hr photoperiod (68 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at the level of the trays) using cool-white fluorescent tubes. The microcuttings were evaluated for percent rooting after 5 and 8 weeks. The data were analyzed by analysis of variance.

In the first 3 weeks after culture initiation, 222 explants out of 475 in culture were contaminated and many explants browned or became covered with callus. The leaves of 33 axillary buds expanded during the first 6 weeks. Their shape was oblong, such as mature leaves. Their development was very slow and there was virtually no internode elongation (Fig. 1, upper left). Death of the shoot apices was frequently observed, followed by the formation of axillary buds (Fig. 1, lower left). Those buds often browned before opening or again produced oblong leaves with no internode elongation. After 19 weeks, thin stems bearing small and rounder leaves were produced on two explants. One explant was initiated on the medium containing 73.8 µM 2iP and 22.8 µM IAA, and the other was initiated on the medium containing 49.2 µM 2iP and 11.4 µM IAA. The measurements of the leaves (3 mm wide, 4 mm long) and stems (0.48 mm) were similar to the measurements of tissue culture shoots of rabbiteye blueberry (Lyrene, 1981). Shoots that closely resemble new seedlings are juvenile. They were sectioned and subcultured on the multiplication medium, where they proliferated by axillary budding (Fig. 1, right). Adventitious buds were also present on callus growing on leaves touching the agar, but these buds did not elongate unless 5.7 µM IAA was added to the medium for one subculture. This finding contrasts with the regeneration of shoots from leaf sections of highbush blueberry where IAA is not needed for the regeneration of shoots (Billings et al., 1988). Adventitious budding on detached leaves could provide high multiplication rates (Dweikat and Lyrene, 1988); however, in our experiments, proliferation by axillary budding was preferred over adventitious budding on leaf callus because the genetic stability of the adventitious system has not been verified (Cohen, 1980). The shoot doubling time of clone B was measured from week 26 to week 43 after the initiation of the cultures (Fig. 2). The number of shoots of lowbush blueberry doubled every 23.3 days. This result compares to the best doubling times obtained with raspberry (Desjardins and Gosselin, 1987).

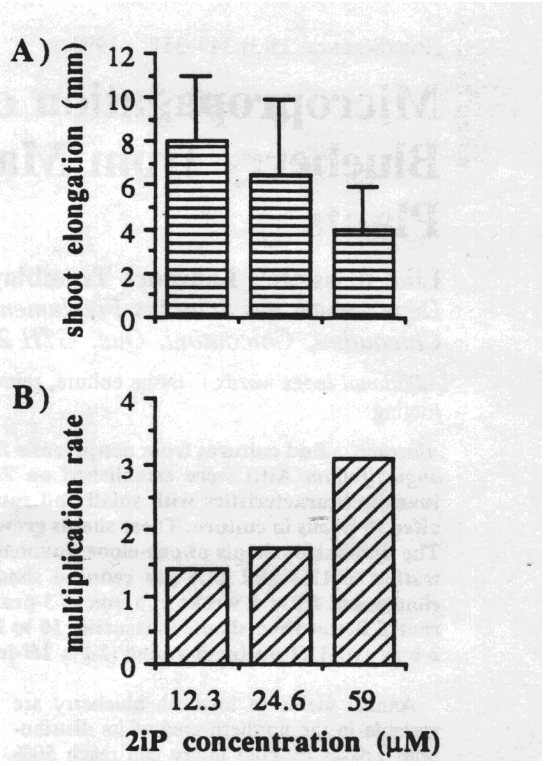

Fig. 3. Effect of 2iP concentration on **(A)** shoot elongation and **(B)** multiplication rate of lowbush blueberry. Data **(A)** are means (±SD) for 20 shoots.

Shoot elongation was promoted by reducing the concentration of 2iP to 24.6 µM, but the microshoots were not long enough to be rooted after one subculture (3 weeks). When shoots were left on the same medium without transfer for 3 to 6 additional weeks, proliferation slowed down and the shoots elongated. In the absence of 2iP, the microshoots elongated, but proliferation stopped. After 3 weeks, yellow and red spots were present on leaves, and the shoots appeared to be unhealthy. In another experiment, 12.3 µM 2iP was compared to 24.6 and 59 µM 2iP for their effects on shoot elongation (Fig. 3). The first one was the best concentration tested to obtain longer shoots rapidly, without the problem of yellowing of cultures observed in the hormone-free medium.

The microshoots were rooted in June 1987. The best results were obtained with shoots pretreated under high light intensity, on 24.6 µM 2iP, and >20 mm (Table 1). High light intensity influenced the rapidity of rooting, as percentages > 90% could be obtained after 5 weeks, whereas the same percentages were obtained only after 8 weeks when the shoots

Table 1. Effect of shoot length on rooting percentages of lowbush blueberry microshoots grown under two light intensities with 24.6 µM 2iP or hormone-free medium during the last subculture.

Light intensity ($\mu mol \cdot s^{-1} \cdot m^{-2}$)	2iP concn (µM)	Shoot length (mm)	Rooting percentages[z]	
			Week 5	Week 8
140	0	10–20	52	67
		>20	81	87
	24.6	10–20	81	95
		>20	98	100
30	0	10–20	52	79
		>20	83	94
	24.6	10–20	65	85
		>20	69	88
Significance				
Light intensity (LI)			*	NS
2iP concn (C)			*	*
LI × C			*	NS
Shoot length (SL)			**	*
LI × SL			NS	NS
C × SL			*	NS
LI × C × SL			NS	NS

[z]Rooting percentages were measured 5 and 8 weeks after dipping the microshoots in 0.1% IBA.
[NS,*,**]Not significant or significant at $P = 0.05$ or 0.01, respectively.

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Fig. 4. Rooted plantlets 4 months after transfer to soil (× 0.5).

did not receive a high light intensity pretreatment. The concentration of 2iP in the medium at the last subculture should be low to obtain longer shoots which root more easily. The plantlets grew well after they were rooted (Fig. 4). They were put in a cold frame during Fall 1987 and transferred to a greenhouse in the spring. They produced normal flowers and fruits in Summer 1988.

Growth of mature explants of lowbush blueberry in vitro was rare, but, when reversion to juvenile characteristics occurred, proliferation was rapid. At 59µM, 2iP was good for shoot proliferation, but the microshoots remained too short' for rooting. Reduction of the cytokinin level in the last subculture before rooting permitted shoot elongation and facilitated rooting. Efforts should be directed toward the improvement of the initiation phase, as this is the limiting step.

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Observations on the Relationships among Seed Number, Fruit Calcium, and Senescent Breakdown in Apples

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Abstract. **In a population of 'Delicious' apples *(Malus domestica* Borkh.) with varying seed number at harvest, fruit size and Ca concentration in fruit increased with seed number. Neither K nor Mg concentration in fruit was related to seed number. In another population of 'McIntosh' apples from 50 commercial orchard blocks, the percentage of fruit that developed senescent breakdown, a Ca-deficiency disorder, decreased linearly as seed number per fruit increased. Low seed number is probably a factor contributing to Ca deficiency in apple fruit.**

Previously, we reported that spraying 'McIntosh' apple trees with gibberellins A_{4+7} (GA_{4+7}) and N-(phenylmethyl)-1H-purine-6-amine (BA) 17 days after full bloom resulted in decreased Ca concentrations in mature fruit as growth regulator concentrations increased (Greene et al., 1982). Looney (1979) reported similar findings with 'Spartan' apples and attributed the lower Ca concentration to the diluting effect of increased fruit size. In our study, we recorded increasing numbers of seedless fruit and increasing incidences of senescent breakdown (a Ca-deficiency disorder), along with decreasing Ca concentration, as GA_{4+7} and BA concentrations increased. Since parthenocarpic apples tend to be Ca-deficient (Bangerth, 1976), seed number may be a factor in determining Ca concentration in fruit at harvest. However, because our fruit had been sampled for mineral analyses without regard for seed number and with avoidance of fruit with breakdown, interrelationships among seed number, fruit Ca, and senescent breakdown could not be tested.

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