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The Use of Zeatin to Initiate in Vitro Cultures of *Vaccinium* Species and Cultivars

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Abstract. Explants of mature pot-grown *Vaccinium corymbosum* L. cultivars were tested for initiation of new shoots using two growing conditions and four cytokinin treatments. Initiation tests with 12 genotypes showed significantly higher rates of new shoot growth on modified woody plant (MWPM) medium with 4 mg zeatin/liter at 25C under low light intensity than on any other treatment. Explants at 25C in light with 10 or 15 mg 2iP/liter initiated at a moderate rate, but significantly lower rates were found for all controls and at 4C in darkness. To determine the utility of zeatin for initiation of diverse genotypes, 96 *Vaccinium* accessions from the National Clonal Germplasm Repository, representing 22 species and 44 cultivars, were screened using 25C and low light intensity. Initiation rates higher than 60% were achieved for 89 of 96 accessions tested. Chemical name used: N⁶-[2-isopentenyl]adenine (2iP), 6-[4-hydroxy-3-methylbut-2-enylamino]purine (zeatin).

The National Clonal Germplasm Repository in Corvallis, Ore., maintains a collection of >600 *Vaccinium* accessions. To develop a backup in vitro collection encompassing the variability of the genus, standard conditions must be determined for successful initiation and growth of a wide range of species and cultivars. Usually only two plants of each genotype are available for explants, so high rates of initial shoot growth are important in culture establishment. Tissue culture propagation methods for some *Vaccinium* spp. and cultivars have been described in the literature (Chandler and Draper, 1986; Cohen, 1980; Frett and Smagula, 1983; Hosier et al., 1985; Lyrene, 1980; Nickerson, 1978; Orlikowska, 1986; Scorza et al., 1984; Wolfe et al., 1983; Zimmerman and Broome, 1980),

but many others have not been studied. In addition, specific reports on methods to initiate new shoot growth from axillary buds of explants are rare.

Some workers routinely use a 4C dark treatment with 2iP for initiation of new growth from explants (Orlikowska, 1986). Hu and Wang (1983) recommend low incubation light intensity for initiation of most plants. In a recent study of four highbush blueberries in a growth room, Eccher and Noe (1989) compared mixtures of zeatin and 2iP for improving shoot initiation. Mixtures of the two cytokinins were less phytotoxic to new explants than 2iP alone and produced higher initiation rates. In another study, new shoot growth was initiated on only two of 222 uncontaminated explants of *V. angustifolium* using 2iP at 49.2 and 22.8 μM (Brissette et al., 1990). This lack of new shoot growth can make initiation the limiting step in establishing *Vaccinium* cultures.

Because published studies of initiation have been limited to a few cultivars and species, more information is needed on conditions that will produce a high level of initial shoot growth

from explants of diverse collections of germplasm. Our study compared the effects of zeatin and 2iP and two growing conditions on explants of 12 *Vaccinium corymbosum* genotypes. The best treatment from the *V. corymbosum* study was then used to screen a wide range of species and cultivars to determine if the technique could be successfully used by those who manage germplasm collections or work with diverse species.

Explants of 5-year-old screenhouse or shadehouse pot-grown *Vaccinium* plants were taken in Aug. 1988 and 1989. Stem sections were cut into 5-cm pieces, disinfested with 0.52% (v/v) sodium hypochlorite with 1 ml Tween 20/liter (polyoxyethylene sorbitan monolaurate, Sigma, St. Louis) added, shaken on a rotary shaker for 15 min, and rinsed twice in sterile distilled water for 5 min each time. Stem sections then were cut into 1-cm single node pieces and planted in 16 \times 100-mm glass tubes on 5 ml of woody plant medium (Lloyd and McCown, 1980) modified by doubling the $\text{Ca}(\text{NO}_3)_2$ concentration (MWPM) and containing either no cytokinin, zeatin at 4 mg-liter⁻¹, or 2iP at 10 or 15 mg-liter⁻¹. The medium was adjusted to pH 5.2, solidified with 6 g agar/liter (Difco-Bacto, Detroit), and the tubes were capped with Kim Caps (Kimble, Morton Grove, Ill.) and autoclaved.

The effect of cytokinins on 12 genotypes of *V. corymbosum* was tested under low light intensity (low light; 10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 16-h days, 25C) or in darkness (4C). After 1 month, all cultures were transferred to fresh medium of the original composition and all cultures that had been grown at 4C were placed at 25C in low light. Six explants of each of 12 genotypes were used for each of three replications. For screening, six explants each of 84 additional *Vaccinium* accessions were placed on zeatin-containing medium in low light (10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 16-h days, 25C).

Two months after initial culture, the number of explants with new axillary shoot growth was recorded and new shoots were transferred to MWPM with 2iP at 5 mg-liter⁻¹ in Magenta GA-7 boxes (Magenta Corp., Chicago) for continued growth. Contaminated cultures were discarded and not included in the data analysis. Data were analyzed using the G statistic as calculated in Sokal and Rohlf (1969). This test is equivalent to the F test in determining significant treatment differences but is more accurate for the smaller sample sizes used. Factorial analysis and Duncan's multiple range test were run using

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Table 1. Percentage of *Vaccinium corymbosum* explants exhibiting axillary shoot initiation after 8 weeks of growth under low light intensity at 25C or in darkness at 4C and on four cytokinin conditions.

NCGR ^z no.	Cultivar	Light ^y	Initiation* (%)			
			Control (0)	Cytokinin* (mg·liter ⁻¹)		Zeatin (4)
				10	15	
226	Bluejay-83	+	41	52	67	81
		-	88	50	47*	93
51	Burlington	+	23	44	63	100***
		-	22	31	15	14
78	Cabot	+	12	78***	44	59*
		-	17	33	39	33
95	Coville	+	33	50	59	73
		-	23	25	18	33
56	Evelyn	+	0	59***	25	80***
		-	18	20	0	39
91	Herbert	+	7	64**	70**	89**
		-	40	86**	63**	25
217	Northsky	+	15	67*	53	71*
		-	19	33	32	65*
312	O'Neal	+	56	59	74	78
		-	41	77	88**	73
88	Pemberton	+	41	65	82	83*
		-	25	24	31	44
68	Pioneer	+	19	53	32	63*
		-	20	38	40	36
75	Washington	+	33	43	43	85
		-	29	31	0*	44
35	<i>V. corymbosum</i>	+	22	20	14	78*
		-	8	50	0	50

^zIdentifying number of the National Clonal Germplasm Repository, Corvallis.

^y+, 10 μmol·m⁻²·s⁻¹ for 16 h·day⁻¹; -, darkness.

*Mean of three replicates of six explants each. Significance is based on the comparison of cytokinin treatments with the control (no hormone) treatment within a cultivar and condition, using the G statistic (Sokal and Rohlf, 1969).

***Significant at *P* = 0.05, 0.01, or 0.001, respectively.

Table 2. Mean number of *Vaccinium corymbosum* explants producing new shoot growth following 8 weeks under two growth conditions on four cytokinin treatments.

Cytokinin	Condition ^z	
	4C, darkness	25C, light
No cytokinin	5.0 c ^y	5.4 c
2iP 10 mg·liter ⁻¹	6.7 bc	9.7 b
2iP 15 mg·liter ⁻¹	6.0 c	9.4 b
Zeatin	7.5 bc	14.1 a

^zMean of 18 explants each of 12 accessions; light = 10 μmol·m⁻²·s⁻¹; 16 h·day⁻¹.

^yMean separation in columns by Duncan's multiple range test, *P* = 0.05. LSD = 2.8.

MSTAT software (Michigan State Univ., East Lansing).

The best initiation rates (percentage of explants showing new shoot growth) for the 12 genotypes ranged from 31% to 100% for the eight treatments (Table 1). As in other studies (Eccher and Noe, 1989; Zimmerman and Broome, 1980), many differences exist between genotypes. New shoot production depended on the interaction of genotype with cytokinin and growth condition (*P* ≤ 0.001) (data not shown).

The type and concentration of cytokinin significantly affected the rate of initiation in individual cultivars of *V. corymbosum*. Eight of 12 genotypes exhibited significantly higher initiation (*P* ≤ 0.05) on medium containing zeatin than on control medium (Table 1). Chandler and Draper (1986) found that zeatin stimulates multiplication in *V. corym-*

bosum cultures. In low light, 'Cabot', 'Evelyn', 'Herbert' and 'Northsky' demonstrated significantly better (*P* ≤ 0.05) initiation on 2Pi at 10 mg·liter⁻¹ and 'Herbert' on 2Pi at 15 mg·liter⁻¹ as compared with the control (no hormone) medium. For 'Bluejay-83', 'Coville', 'O'Neal', and 'Washington', none of the cytokinin treatments was significantly better than the control.

In darkness at 4C, significant (*P* ≤ 0.05) increases in initiation over the controls were obtained for 'Herbert' and 'O'Neal' on 2iP at 15 mg·liter⁻¹ for 'Northsky' on zeatin, and for 'Herbert' on 2iP at 10 mg·liter⁻¹. However, significant decreases were obtained for 'Bluejay' and 'Washington' on 2iP at 15 mg·liter⁻¹ compared with controls. 'Herbert' initiated at higher rates on five of six treatments containing a cytokinin than in an earlier study (Orlikowska, 1986).

Mean initiation for the 12 *V. corymbosum* accessions tested was greatest on explants grown on zeatin in low light (Table 2). Explants grown in low light on medium with either 2iP level initiated at a moderate frequency. Initiation, however, was significantly lower for explants grown without a cytokinin. There were no significant differences among those held at 4C in darkness.

Although most studies of shoot initiation have been conducted in growth rooms (Brisette et al. 1990; Eccher and Noe, 1989), Orlikowska (1986) observed better new shoot growth for cultures initiated in darkness at 4C. In our study, with all cytokinin treatments pooled, six of the 12 *V. corymbosum*

genotypes responded differently (*P* ≤ 0.05) between the two conditions, with 25C and low light being better than 4C in darkness in all cases (data not shown).

Guided by the results of the 12 *V. corymbosum* genotypes, we screened 84 additional *Vaccinium* accessions using the most successful treatment, 4 mg zeatin/liter with low light (16-h days, 25C). Among the 84 accessions tested, the initiation rate of the 22 species included ranged from 23% to 100% for the six replicates planted. Nine species were 100% successful (*V. caesariense* Mackenzie, *V. constablaei* A. Gray, *V. delavayi* Franch., *V. moupinense* Franch., *V. pallidum* Ait., *V. parvifolium* J.E. Smith, *V. simulatum* Small, *V. uliginosum* L., and *V. vitis-idaea* L.) and only three species (*V. angustifolium* Ait., *V. myrtilloides* Michx., and *V. sempervirens* D. Rayner and J. Henderson cv. Bloodstone) initiated at <60%. The remaining 10 species displayed intermediate initiation rates (*V. ashei* Reade, *V. australe* Small, *V. corymbosum* L., *V. crasifolium* Andr., *V. elliotii* Chapm., *V. myrtilus* L., *V. ovatum* Pursh, *V. padifolium* Ait., *V. smalii* A. Gray, and *V. stamineum* L.).

Individual *V. corymbosum* cultivars also produced a range of initiation rates with 10 of 27 at 100% (cultivars Bluehaven, Burlington, Crabbe IV, Earliblue, Elizabeth, Grover, Harrison, Jersey, Shirley, and Washington) and only two <60% (cultivars Blueray and Bluetta). Intermediate rates were obtained with an additional 15 (cultivars Berkeley, Bluecrop, Bluejay 83, Cabot, Collins, Coville, Croatan, Evelyn, Herbert, Johnson, Laniera, Northsky, Patriot, Pemberton, and Pioneer).

Rabbiteye blueberries (*V. ashei* Reade cvs. Black Giant, Centurion, Climax, Early May, Owen, Satilla, Ethyl, Suwannee, Tifblue, and Walker) and cranberries (*V. macrocarpon* Ait. cvs. A.J., Cropper, and Pilgrim) had high initiation levels, with nine of 13 at 100% and all others >50%. Two hybrids, 'Avonblue' and 'Sharpblue' (*V. corymbosum* x *V. ashei* and *V. darrowi*), produced new shoots on 50% of the explants.

These results differ markedly from initiation levels found by Orlikowska (1986) on medium with 15 mg 2iP/liter, where only 5% of 'Bluecrop' (75% on zeatin in the present study), 20% of 'Jersey' (100%), and 50% of 'Herbert' (83%) explants grew. Initiation rates found by Eccher and Noe (1989) with higher concentrations of mixtures of zeatin and 2iP were similar or in some cases much lower than those observed in the present study with low levels of zeatin alone: 67% of 'Bluecrop' (75% in the present study), 90% of 'Bluehaven' (100%), 35% of 'Collins' (75%), and 50% of 'Earliblue' (100%). *V. angustifolium* initiated at a rate of 33% with zeatin in the present study compared with 0.9% found by Brissette et al. (1990) on 2iP.

Initiation of explants is often best in early summer when the new growth is slightly hardened but not yet influenced by dormancy (Eccher and Noe, 1989). The timing of the present study indicated that excellent rates

of initiation are possible at midsummer.

The initiation of new growth on *Vaccinium* explants is often a limiting step for in vitro culture (Lyrene, 1980; Orlikowska, 1986). Efforts to produce initial growth from explants in one case resulted in successful initiation from only two of >200 explants used to multiply a new selection (Brissette et al., 1990). Zeatin at 4 mg·liter⁻¹ and low light intensity initiated new shoot growth and reduced such problems in this study. The high rates of initiation obtained in the screen of species and cultivars indicate that this method is successful for a wide range of *Vaccinium* germplasm. Because of the great variability within this genus, some species and cultivars would still require further study to optimize multiplication media. Adding zeatin to the *Vaccinium* initiation medium produced more new shoots, allowed a broad range of genotypes to become established, and reduced the time and labor involved. The high cost of zeatin precludes extensive use as a general growth hormone; however, at low levels, zeatin can be used to initiate explant growth for research and commercial purposes.

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