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Use of a Cytokinin Conjugate for Efficient Shoot Regeneration from Leaf Sections of Highbush Blueberry

Lisa J. Rowland and Elizabeth L. Ogden
Fruit Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, Beltsville, MD 20705

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Abstract. **Conditions for improving the efficiency of shoot regeneration from leaf sections of highbush blueberry *(Vaccinium corymbosum* L.) were investigated. Effectiveness of tissue culture medium supplemented with the cytokinin conjugate zeatin riboside or the cytokinin zeatin at 10, 20, or 30 µM was compared with medium supplemented with the optimum 2iP concentration of 15 µM. Use of 20 µM zeatin riboside resulted in the most shoots per leaf section, » 6-fold higher than the number of shoots produced on 2iP medium. The number of shoots produced on medium supplemented with zeatin was not significantly higher than the number of shoots produced on 2iP medium. Consequently, we concluded that the cytokinin conjugate zeatin riboside was more effective than either of the free cytokinins, 2iP or zeatin, in promoting shoot regeneration from leaf sections of highbush blueberry. Chemical names used: 6-(y,y-dimethylallylamino)-purine (2iP); 6-(4-hydroxy-3-methyl-but-2-enylamino)purine (zeatin).**

Interest in producing transgenic blueberry plants using an engineered strain of *Agrobacterium tumefaciens* has prompted us to investigate ways of improving the efficiency of shoot regeneration from leaf sections. The number of transformed cells recovered after inoculation with *Agrobactetium* is proportional to the number of cells capable of regenerating shoots. Therefore, production of many shoots per leaf section is critical.

Several laboratories have reported the ability to regenerate shoots from leaf sections of cultivated highbush blueberry (Billings et al., 1988; Callow et al., 1989; Dweikat and Lyrene, 1988). Callow et al. (1989) examined shoot regeneration from leaf sections of 'Bluecrop' blueberry on tissue culture medium supplemented with 5, 25, 50, or 100 µM 2iP and reported the greatest number of meristematic nodules and buds per leaf were induced at 25 µM. They also reported the ability to produce shoots from leaf explants of 'Bluejay' and 'Jersey' blueberries on medium supplemented with 5 to 25 µM 2iP. Billings et al. (1988) examined shoot regeneration from leaf sections of 'Berkeley' and 'Bluehaven' blueberries on medium containing 0, 5, 10, 15, or 20 µM 2iP and found the optimal 2iP concentration for shoot regeneration was 15 µM.

Medium containing the cytokinin conjugate zeatin riboside has been reported to be superior to cytokinins for shoot regeneration from leaf explants of tomato (Tatchell and Binns, 1986) and potato (Sheerman and Bevan, 1988). The effectiveness of conjugates of plant growth regulators is apparently due to the slow, steady release of free growth regulator from the medium (Hangarter et al.,

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Fig. 1. Representative leaf sections of 'Sunrise' blueberry on shoot regeneration medium containing 15 μM 2iP (A) and 10, 20, or 30 μM zeatin riboside (B-D, respectively). Photographs were taken after 11 weeks in culture.

Table 1. Comparison of 2iP and zeatin riboside (ZR) for effectiveness in promoting shoot regeneration from leaf sections of 'Sunrise' blueberry.[z]

Cytokinin	Concn (μM)	Live sections at 13 weeks (no.)	Buds/live section (no.)	Shoots/live section[y] (no.)	Shoot length[y] (mm)
2iP	15	22	7.1 b[x]	3.7 b	1.3 b
ZR	10	18	5.1 b	6.1 b	2.6 a
	20	22	18.1 a	20.8 a	2.6 a
	30	22	17.6 a	8.8 b	1.4 b

[z]$LSD_{0.05}$ value for number of buds was 6.39. $LSD_{0.05}$ values for the transformed data, square root of number of shoots and log of shoot length, were 1.35 and 0.17, respectively.
[y]Means were back-transformed.
[x]Mean separation within columns by LSD, $P = 0.05$.

Table 2. Comparison of 2iP and zeatin for effectiveness in promoting shoot regeneration from leaf sections of 'Sunrise' blueberry.[z]

Cytokinin	Concn (μM)	Live sections at 13 weeks (no.)	Buds/live section (no.)	Shoots/live section[y] (no.)	Shoot length[y] (mm)
2iP	15	13	19.1 a[x]	3.1 a	1.2 b
Zeatin	10	11	25.9 a	7.8 a	2.8 a
	20	19	28.1 a	11.8 a	2.3 a
	30	16	34.1 a	12.9 a	2.5 a

[z]$LSD_{0.05}$ value for number of buds was 15.65. $LSD_{0.05}$ values for the transformed data, square root of number of shoots and log of shoot length, were 1.97 and 0.21, respectively.
[y]Means were back-transformed.
[x]Mean separation within columns by LSD, $P = 0.05$.

1980). We present a comparison of shoot regeneration from leaf explants of three highbush blueberry varieties on medium containing the determined optimal 2iP concentration of 15 μM (Billings et al., 1988) and on medium containing zeatin riboside at 10, 20, or 30 μM. Results from a later comparison of shoot regeneration on medium containing 2iP and zeatin are also presented.

Leaves for these experiments were collected from established tissue cultures of 'Bluecrop', 'Duke', and 'Sunrise'. They were established from shoot explants by growing cultures at 23C for a 16-h photoperiod (40 to 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on woody plant medium (WPM) (Lloyd and McCown, 1980) modified as follows ($mg \cdot liter^{-1}$): 684 $Ca(NO_3)_2 \cdot 4H_2O$, 190 KNO_3, 73.4 $C_{10}H_{13}FeN_2NaO_8$, and 0.1 thiamine HCl. Not included were K_2SO_4, $CaCl_2$, $FeSO_4$, and Na_2EDTA, Medium also contained 0.5% sucrose, 0.55% agar, 24.6 μM 2iP, and 9.12 μM zeatin. The final pH was adjusted to 5.2. Explants were prepared from tissue-cultured leaves by cutting perpendicular to the midrib to remove apical and basal portions of the leaf. The remaining central portions of the leaves were then placed abaxial side up on the surface of shoot regeneration medium in 100 × 15-mm petri plates.

To verify the optimum 2iP concentration for subsequent comparisons to zeatin and zeatin riboside, a preliminary experiment was performed in which shoot regeneration of 'Sunrise' was compared on medium supplemented with three concentrations of 2iP. Shoot regeneration medium consisted of WPM modified as described above, 2% sucrose, 0.55% agar, pH 5.2, and 2iP concentrations of 5, 15, or 25 μM. Twenty leaf sections were placed on medium containing each concentration of 2iP. Use of 15 μM 2iP resulted in the most shoots per section-4.7 and 3.5 times higher than the number of shoots per section produced on 5 μM 2iP and on 25 μM 2iP, respectively.

Shoot regeneration was then compared on medium supplemented with 15 μM 2iP and 10, 20, or 30 μM zeatin riboside. Five plates were prepared per cytokinin concentration for each variety. Each petri plate contained five leaf sections for a total of 25 sections per treatment for each variety. Petri plates were placed in the dark for 1 week at 23C and then provided with a 16-h photoperiod (20 to 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 23C for the remaining time in culture. The number of regenerating leaf sections and buds per leaf section were determined after 5 weeks in culture. After 7 weeks in culture, all explants were transferred to modified WPM with 2% sucrose, 24.6 μM 2iP, and 9.12 μM zeatin. After 13 weeks in culture, regenerated shoots were excised and counted. Shoots were also measured and the mean shoot length calculated for each leaf section.

To determine if differences in the efficiency of shoot regeneration on medium containing zeatin riboside vs. 2iP were due to the presence of a different cytokinin (zeatin rather than 2iP) or to the presence of a cytokinin conjugate rather than a free form of cytokinin, regeneration was also compared on medium containing 15 μM 2iP and 10, 20, or 30 μM zeatin. This comparison was identical to the previous one, with the exception that a total of 20 leaf explants per variety rather than 25 was tested at each of the four cytokinin concentrations.

In the comparisons of 2iP with zeatin and zeatin riboside, the mean number of buds per surviving section, number of shoots per surviving section, and shoot length were calculated for each plate. Data were subjected to an analysis of variance (ANOVA) after testing the assumptions of variance homogeneity and normality and transforming data as necessary to conform to assumptions. Transformation was necessary for number of shoots (square root) and for shoot length (log) but not for number of buds. The analysis was run as a one-way, completely randomized design with four or five plate replicates per treatment, depending on the experiment. When the ANOVA indicated that significant differences existed between treatment means, the least significant difference test ($P = 0.05$) was performed.

From the comparison of 2iP with zeatin riboside, we noted that 'Sunrise' exhibited excellent regeneration ability (Table 1), producing several shoots per section, whereas 'Duke' and 'Bluecrop' produced no shoots on any of the media tested (data not shown). Representative leaf sections of 'Sunrise' after 11 weeks in culture and started on medium containing 15 μM 2iP or 10, 20, or 30 μM zeatin riboside are shown in Fig. 1. Leaf sections on medium supplemented with 20 or 30 μM zeatin riboside produced the most buds per surviving section after 5 weeks in culture, which was significantly higher than for leaf sections on medium supplemented with either 2iP or 10 μM zeatin riboside (Table 1). After 13 weeks in culture, buds had developed into shoots and had elongated sufficiently to count. Leaf sections started on medium containing 20 μM zeatin riboside produced significantly more shoots than did all the other sections. Mean number of shoots per surviving section was 5.6 times higher on medium supplemented with 20 μM zeatin riboside than on medium supplemented with 2iP. Also, shoots were about twice as long on medium supplemented with 20 μM zeatin riboside than on 2iP medium (Table 1).

The comparison of 2iP with zeatin revealed that the number of buds and shoots per surviving section of 'Sunrise' did not differ significantly among treatments although the extreme values differed by factors of 1.8 and 4.1, respectively (Table 2). However,

shoots on medium containing zeatin were about twice as long as those on 2iP medium (Table 2). Chandler and Draper (1986) have previously reported that use of zeatin increased proliferation of shoot tips two to four times more than 2iP in three highbush blueberry clones. Again, 'Bluecrop' and 'Duke' produced no shoots on any of the media tested. Regeneration efficiency has been found to vary among varieties of many other species; thus, finding regeneration efficiency to be genotype-dependent in blueberry is not surprising. Several of the shoots produced on zeatin and zeatin riboside media were later transferred to 50% perlite : 50% peat, where they easily rooted.

In summary, our results show first that the cytokinin conjugate zeatin riboside is more effective in promoting shoot regeneration from leaf sections of highbush blueberry than the free cytokinin 2iP. Our results also suggest that this improvement in shoot regeneration is not entirely due to the presence of a different cytokinin (zeatin vs. 2iP) but, rather, to the presence of a cytokinin conjugate vs. free cytokinin. Furthermore, although zeatin and zeatin riboside are considerably more costly than 2iP, zeatin riboside may be well worth the additional cost under certain circumstances, specifically, *Agrobacterium*-mediated transformations. Recently, successful use of *Agrobacterium tumefaciens* to transform apple *(Malus domestica* Borkh.) was reported (James et al., 1990). Transformation efficiency was very low; 400 leaf explants gave rise to three transgenic shoots. The average number of expected shoots per leaf explant, without selection, was six. If the number of expected shoots per explant had been, for example, 3-fold higher, then, theoretically, one-third of the leaf explants (in this case, 133 vs. 400) would have given rise to the same number of transgenic shoots. In the case of blueberry, by using zeatin riboside in the medium, we have obtained five to six times as many shoots per leaf explant as with 2iP. Therefore, the conditions we describe are probably more suitable than use of 2iP for recovering transgenic shoots after inoculation of leaf sections with *Agrobacterium.*

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