Leaf explants from 1-, 2-, and 3-week-old shoot cultures were prepared by dissecting perpendicular to the midrib of two to three of the youngest, fully-expanded leaves to remove the petiole and the distal third portion of the blade. The remaining proximal portion of the leaf blade was placed abaxial side up on sterile filter paper in 100 × 15-mm petri dishes saturated with pH 5.4 liquid pretreatment medium #1 (PT1) containing 0.01 M ethylene-dinitrol-tetraacetic acid ferric-sodium salt and 0.1 M thiamine·HCl, and without K₂SO₄, CaCl₂, FeSO₄, and Na₂EDTA. The medium also contained 15 mM sucrose, Difco Bacto-agar (5.5 g·L⁻¹), 24.6 M 2iP, and 9.1 M zeatin. The pH was adjusted to 5.2 before autoclaving at 121 °C at 131 kPa for 15 min; the medium was then dispensed into glass jars that were sealed with plastic wrap. Shoot cultures were incubated at 23 °C with a 16-h photoperiod provided by cool-white fluorescent lights at a PPF of 40 µmol·m⁻²·s⁻¹.

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supplemented with either 0.5, 1.0, or 5.0 µM TDZ or 20 µM ZR. Explants were incubated in dark for 1 week, and then incubated at 23 °C, under a 16-h photoperiod provided by cool-white fluorescent lights at a PPF of 55 ± 5 µmol·m⁻²·s⁻¹. Explants were transferred to fresh medium every 2 weeks. After 6 weeks, explants were transferred to the same medium without growth regulators for 10 d and then scored for percentage of regenerating explants and for mean number of shoots per explant.

Data collection and statistical analyses. Petri dishes were arranged in a completely randomized design. Regeneration experiments were replicated a minimum of three times with 10 leaf explants per treatment per replication. Data for number of shoots per explant were analyzed by analysis of variance techniques using the generalized linear model procedures of SAS (version 6.12) (SAS Institute, 1997). Differences among means were tested using the LSD test, P ≤ 0.05.

Results and Discussion

Influence of pretreatment. The two-step growth regulator pretreatment significantly enhanced the efficiency of shoot organogenesis from leaf explants of 'Bluecrop' (Figs. 1 and 2). Pretreatment vs. no pretreatment experiments found pretreatment to be a significant factor in 10 of 12 treatments (all except 1-week cultures on either 0.5 or 5 µM TDZ) (Fig. 1). Similar to previous results (Cao and Hammerschlag, 2000), no shoot regeneration occurred when 'Bluecrop' leaf explants were dissected and transferred directly to shoot regeneration medium. Pretreatments have also been effective for increasing shoot organogenesis in Rubus and Malus (Swartz et al., 1990) and in Prunus (Antonelli and Druart, 1989), but in these cases, shoot cultures received the pretreatment prior to explant excision. In the present study, we investigated the effect of a pretreatment on shoot regeneration because we found the same pretreatment to be important for the early stages of blueberry transformation using Agrobacterium (Cao et al., 1998), and because in Agrobacterium-mediated transformation experiments, explants generally receive some type of pretreatment prior to placement on regeneration medium. Our PT1 medium was based on a pretreatment (cocultivation) medium used for apple transformations (Hammerschlag et al., 1997; Liu et al., 2001) and was also used as a pretreatment (cocultivation) medium for our studies on the early stages of blueberry transformation (Cao et al., 1998). For the blueberry transformation studies, we initially substituted ZR for TDZ in our blueberry cocultivation medium since ZR was shown to be more effective than TDZ in our blueberry regeneration studies (Cao and Hammerschlag, 2000). However, when TDZ was replaced by ZR, our GUS assays were negative (Cao and Hammerschlag, unpublished data); thus, we replaced ZR with TDZ for cocultivation and continued to use TDZ as a pretreatment in the present regeneration studies. PT2 was added as a pretreatment medium because it too is being used in our blueberry

![Fig. 1](image1.png)

![Fig. 2](image2.png)
transformation experiments (Cao and Hammerschlag, unpublished data). Although pretreatment of the cultures on PT1 and PT2 added one extra week of dark treatment, previous studies (Cao and Hammerschlag, 2000) have demonstrated that 1 or 2 weeks in the dark were equally effective for blueberry regeneration.

**Influence of age of explant source.** Age of shoot culture did not influence the number of ‘Bluecrop’ shoots regenerating per explant when a pretreatment was included in the regeneration experiments (Fig. 1). These results are similar to what was reported previously for regeneration from leaves of cvs. Duke, Georgiagem, Jersey, and Sierra in the absence of pretreatments (Cao and Hammerschlag, 2000). However, in contrast to these results, age of shoot culture was of significance for the early stages of transformation of cvs. Bluecrop, Duke, and Sierra, which includes an explant pretreatment (Cao et al., 1998).

**Influence of TDZ and ZR.** Following the two-step pretreatment, percentage of regeneration on medium with 1 µM TDZ was about double (100%) that on medium containing either 0.5, or 5 µM TDZ or 20 µM ZR (Table 1). Also, significantly more shoots per explant were produced on this medium than on any of the other media (Fig. 2). In early highbush blueberry regeneration studies (Billings et al., 1988; Callow et al., 1989), 2iP was the preferred growth regulator and a maximum of seven shoots per regenerating leaf segment was achieved for cvs. Berkley and Bluehaven. Rowland and Ogden (1992) initiated studies to examine the effects of ZR on regeneration from leaf explants of highbush cvs. Sunrise, Duke and Bluecrop based on the studies with tomato (Tatchell and Binns, 1986) and potato (Sheerman and Bevan, 1988) that reported that the cytokinin conjugate ZR was superior to other cytokinins for shoot regeneration from leaf explants. ZR stimulated two and five shoots regenerating per explant of ‘Bluecrop’ on PT1 and PT2, respectively. TDZ = thidiazuron; ZR = zeatin riboside.

In summary, a simple, reliable protocol for high efficiency shoot regeneration from leaf explants of ‘Bluecrop’ is reported that relies on a two-step pretreatment and regeneration on TDZ medium. This protocol will facilitate using genetic transformation and tissue culture technologies to improve commercially important ‘Bluecrop’ and may be potentially useful for other recalcitrant woody species.

**Table 1. Mean shoot regeneration frequency of pretreated leaf explants of highbush blueberry cv. Bluecrop as a function of growth regulators in the regeneration medium and age of explant source.**

| Growth regulator (µM) | Age of culture (weeks) | 1 | 2 | 3 |
|-----------------------|------------------------|---|---|---|
| 0.5 TDZ               | 50 ± 18                | 55 ± 15 | 55 ± 13 |
| 1.0 TDZ               | 100 ± 00               | 98 ± 2.5 | 93 ± 5 |
| 5.0 TDZ               | 58 ± 18                | 48 ± 10 | 40 ± 12 |
| 20 ZR                 | 60 ± 12                | 60 ± 12 | 68 ± 10 |

*Each value represents the mean ± SE of a minimum of three replications with 10 explants per treatment per replica. TDZ = thidiazuron; ZR = zeatin riboside.*

previous and present studies because earlier studies (Rowland and Ogden, 1992) demonstrated this to be the optimum concentration for shoot regeneration from leaf explants of blueberry cv. Sunrise. The present study demonstrates the importance of a pretreatment and TDZ for ‘Bluecrop’ shoot regeneration. Studies with other Vaccinium species have demonstrated the importance of TDZ for shoot regeneration (Shibli and Smith, 1996), and this growth regulator has been shown to induce either organogenesis or embryogenesis of many woody fruit crop genera (Heutteman and Preece, 1993; Murthy et al., 1998).

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**Literature Cited**

Antonelli, M. and Ph. Druart. 1989. The use of a 2,4-D pretreatment to induce leaf regeneration on Prunus canescens. Abstract 20.1 Intl. Symp. In Vitro Cult. Hort. Breed., Cesena, Italy.

Billings, S.G., C.K. Chin, and G. Jelenkovic. 1988. Transformation experiments (Cao and Hammerschlag, 2000) have demonstrated that 1 or 2 weeks in the dark were equally effective for blueberry regeneration. Although pretreatment of the cultures on PT1 and PT2 added one extra week of dark treatment, previous studies (Cao and Hammerschlag, 2000) have demonstrated that 1 or 2 weeks in the dark were equally effective for blueberry regeneration. Influence of age of explant source. Age of shoot culture did not influence the number of ‘Bluecrop’ shoots regenerating per explant when a pretreatment was included in the regeneration experiments (Fig. 1). These results are similar to what was reported previously for regeneration from leaves of cvs. Duke, Georgiagem, Jersey, and Sierra in the absence of pretreatments (Cao and Hammerschlag, 2000). However, in contrast to these results, age of shoot culture was of significance for the early stages of transformation of cvs. Bluecrop, Duke, and Sierra, which includes an explant pretreatment (Cao et al., 1998). Influence of TDZ and ZR. Following the two-step pretreatment, percentage of regeneration on medium with 1 µM TDZ was about double (100%) that on medium containing either 0.5, or 5 µM TDZ or 20 µM ZR (Table 1). Also, significantly more shoots per explant were produced on this medium than on any of the other media (Fig. 2). In early highbush blueberry regeneration studies (Billings et al., 1988; Callow et al., 1989), 2iP was the preferred growth regulator and a maximum of seven shoots per regenerating leaf segment was achieved for cvs. Berkley and Bluehaven. Rowland and Ogden (1992) initiated studies to examine the effects of ZR on regeneration from leaf explants of highbush cvs. Sunrise, Duke and Bluecrop based on the studies with tomato (Tatchell and Binns, 1986) and potato (Sheerman and Bevan, 1988) that reported that the cytokinin conjugate ZR was superior to other cytokinins for shoot regeneration from leaf explants. ZR stimulated two and five shoots regenerating per explant of ‘Bluecrop’ on PT1 and PT2, respectively. TDZ = thidiazuron; ZR = zeatin riboside.

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