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In Vitro Responses and Acclimatization of *Prunus serotina* with Paclobutrazol

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Abstract. Paclobutrazol (PBZ), a triazole growth retardant known to improve tolerance of various species to stress, was incorporated into the in vitro rooting medium of *Prunus serotina* var. *virens* at rates of 0.00, 0.15, 0.30, and 0.60 mg/L with and without 1.0 mg/L indolebutyric acid (IBA). PBZ significantly reduced shoot growth in vitro but increased/improved the quality and coloration. The percentage of water loss from detached leaves of in vitro plantlets was significantly reduced by PBZ and IBA. At 4 weeks after transfer to the greenhouse, survival was significantly improved by PBZ, IBA, and the combination. Incorporation of PBZ in vitro better enables *Prunus serotina* plantlets to withstand the stresses associated with acclimatization.

Acclimatization of tissue-cultured material is often difficult because of the anatomical and physiological changes induced by the in vitro environment (Donnelly 1990). When woody micropropagated plantlets are transferred to the greenhouse or field, this change in environment often results in low survival and reduced growth rates. These problems can be overcome for many species by commonly used acclimatization techniques such as misting and gradual hardening of plantlets in a greenhouse, a process that may require considerable time and expense (Anderson and Meagher 1978). Attempts to decrease this time by improving the hardiness of the plants before removal from culture by increasing sucrose or percentage of agar or by medium overlays have met with some success (Wardle et al. 1983, Ziv et al. 1983), but quite often the advantages, such as rapid growth, inherent in a micropropagation system are sacrificed (Donnelly 1990, Fujiwara et al. 1988).

Incorporation of triazole growth regulators into the medium during the in vitro rooting stage may allow the advantages of micropropagation to be retained while easing the acclimatization period that limits the commercial use of in vitro propagation for a wide variety of species. Studies using growth retardants on plantlets grown in vitro have been limited, but paclobutrazol (PBZ) [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(^{1}H-1,2,4-triazol-1-yl)pentan-3-ol], a triazole growth retardant known to harden plants to various stresses (Davis et al. 1988), offers the potential for improving acclimatization. It has been reported to ameliorate some of the desiccation associated with transfer to soil in micropropagated chrysanthemum (Smith et al. 1990) and grapevine (Smith et al. 1992). Plants that have been successfully treated with PBZ include *Asparagus officinalis* (Khunachak et al. 1987), *Prunus avium* (Snir 1988), and other *Prunus* spp. plantlets (Marino 1986).

The purpose of this research was to determine the effects of PBZ on *Prunus serotina* ssp. *virens* var. *virens* (western black cherry) (McVaugh 1951) shoot growth in vitro, the percent water loss from detached leaves, and subsequent survival in the greenhouse using a minimal acclimatization regime. *Prunus serotina* has been successfully micropropagated, but requires an extended, multistep acclimatization period (Kavanaugh 1987).

Materials and Methods

Tissue Culture Regime

Explant Sterilization. Bud explants from mature *Prunus serotina* twigs were rinsed for 60 min under running water. Benomyl at the rate of 1.5 mg/ml was used as a pre-explant soak for 10 min, followed by 20 min in a solution of 10% aqueous Clorox (v/v) and

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one to three drops of the surfactant Tween 20. Explants were then rinsed with sterile deionized water three times. Bud scales were removed and discarded. Explants were placed in commercial hydrogen peroxide (30%) for 10–30 s followed by three rinses in sterile distilled water (Tricoli et al. 1985).

Shoot Initiation. Explants were placed in 25 × 150 mm borosilicate glass tubes containing 25 ml of initiation medium and covered with Kaputs[R] plastic caps. Components of the initiation medium follow that as per Murashige and Skoog (1962) with the following: (in mg/L) 0.4 thiamine-HCl, 100 I-inositol, 1.0 benzyladenine (BA), 0.1 gibberellic acid (GA), 0.1 indole-3-butyric acid (IBA), and 30 g/L sucrose used. The pH of the medium was adjusted to 5.7 with 1*N* NaOH or 1*N* HCl prior to autoclaving the medium at 121°C and 15 psi for 15 min. Tubes containing explants and media were placed in a culture room under a 16-h photoperiod. A photosynthetic photon flux of 30 $\mu E\ m^{-2}\ s^{-1}$ was provided by cool white fluorescent lamps, and at a constant temperature of 25 ± 2°C.

Shoot Multiplication. Following establishment and elongation of the meristem over 3–4 weeks, the plantlets were transferred to a modified MS medium to induce multiplication. This medium had full-strength MS salts with reduced levels of BA (0.75 mg/L) and IBA (0.01 mg/L). The concentration of GA was increased from 0.1 to 0.2 mg/L and folic acid (0.001 mg/L) was added (Tricoli et al. 1985). At the end of a 4-week cycle, individual shoots 1.5–2.0 cm tall were excised from the shoot clump, and placed on a multiplication medium without any growth regulators (MS0) for another 3 weeks prior to their use in growth retardant evaluations.

Experiment 1: Optimization of Paclobutrazol Levels in Vitro

Individual shoots, 1.0–2.0 cm in length, were taken from the MS0 medium and then cultured in 120-ml baby food jars using a modified MS rooting medium with one-quarter levels of all of the macrosalts except NH_4NO_3. The rooting medium contained 0.00, 0.15, 0.30, and 0.60 mg/L PBZ with and without 1.0 mg/L indolebutyric acid (IBA). The pH of the medium was 5.5 and contained no BA or GA. PBZ and all the other constituents were added to the medium prior to autoclaving. PBZ is heat-stable and unchanged by autoclaving (Snir 1988).

The shoots were placed in the dark for 5 days, and then moved to a culture room with a low light level of 1.0–3.0 $\mu E\ s^{-1}\ m^{-2}$ used to induce rooting for the remainder of the 4-week period (Tricoli et al. 1985). At the end of this period, the plantlets were evaluated for stem height, number of roots, average root length, percentage rooting, average root width, shoot coloration, and moisture loss. The experimental design used was a randomized complete block with four levels of PBZ with and without IBA, factorially arranged (2 × 4) and replicated 10 times.

Percentage of Water Loss. Detached leaves were assayed for rate of water loss as an indicator of hardening and a predictor of subsequent survival of plantlets once removed from in vitro culture (Brainerd et al. 1981, Crane and Hughes 1990). Percent water loss was determined by collecting and weighing one leaf from the second to fourth node of one plantlet from each replication. Each leaf was then allowed to dry abaxial side up on aluminum pans at 25°C and 35% RH under fluorescent lights and weighed at 30-min intervals for 3 h. The leaves were oven-dried at 70°C for 24 h, allowed to equilibrate to room temperature and humidity conditions, and then reweighed to obtain dry weight. The percent water loss during the drying period was calculated as follows:

$$\text{Percent Water Loss at Tn} = \frac{(FW_{T0} - FW_{Tn})}{(FW_{T0} - DW)} \times 100$$

where

FW_{T0} = initial fresh weight (at 0 minutes)
FW_{Tn} = fresh weight (at n minutes)
DW = dry weight of the leaf

The experimental design used to measure water loss percentage was a split plot design with a factorial arrangement (2 × 4) of IBA levels and PBZ levels as main treatments and time as a split plot.

Experiment 2: Survival of PBZ-Treated Plantlets

Acclimatization. The best seven out of the 10 replicates from Experiment 1 were moved into flats with sterilized Promix BX soil previously drenched with Captan solution (3.75 g/L; Microflo, Lakeland, FL). The acclimatization flat consisted of a bedding plant contained with 72 cells (3.75 × 3.75 × 5.9 cm) inserted in three standard plastic flats (52.5 × 26.25 × 6.25 cm), which were placed one under the other with a plastic liner between the second and third and a capillary mat between the first and second to maintain the moisture required to avoid desiccation. The plantlets were rinsed free of agar, roots dipped in Captan solution, and then gently placed in the flat. While transferring the plants, care was taken to avoid drying of the plantlets by frequent misting with deionized-distilled water. When the flat was filled, it was misted with a solution of 0.1 mM KNO_3 (Kavanaugh 1987) and a transparent cover, the same size as the flat (propagation dome, Cassco, Montgomery, AL) was placed over the flat to cover the plantlets. Then, the whole assembly was moved into the growth room. Layers of cheesecloth were used to reduce the light intensities of the growth room to under 50 $\mu E\ m^{-2}\ s^{-1}$ for the first week. During the second to fourth weeks, light intensities were increased gradually up to 200 $\mu E\ m^{-2}\ s^{-1}$ by removing cheesecloth layers and decreasing humidity by propping open the clear plastic cover for increasing amounts of time. In the third week, the flats were moved to the greenhouse. Although the cover was left open slightly, cheesecloth was used once again to reduce the natural high-ambient-light intensities. This procedure was followed until all the plantlets were acclimatized, occurring within 4 weeks after transfer to ex vitro conditions.

All treatments were factorially arranged and placed in a randomized complete block arrangement within the flat.

Results and Discussion

Morphological Modifications

PBZ significantly reduced shoot height with and without IBA, although most of this effect was not due to the concentration level but due to the pres-

Table 1. Mean values for shoot growth and color and ANOVA mean square values for *Prunus serotina* as a function of PBZ and IBA.

	IBA 0.0		IBA 1.0	
Composition of the medium (mg/L)	Shoot height (cm)	Shoot color (1–5)[a]	Shoot height (cm)	Shoot color (1–5)
Paclobutrazol 0.00	2.76[b]	4.0	2.68	2.7
Paclobutrazol 0.15	1.77	4.3	1.92	3.3
Paclobutrazol 0.30	2.09	3.7	2.07	3.7
Paclobutrazol 0.60	1.89	3.9	1.73	4.1
Significance[c] Linear	***	***	**	***

Source	Degrees of freedom	Shoot height	Shoot color
REP	9	0.07279	9.0847***[c]
IBA	1	0.1653	5.5125***
PBZ	3	3.5444*	1.4792*
IBA × PBZ	3	0.0887	2.7125***
Error	63	0.9213	0.6435

[a] 1 = deep green; 3 = medium green; 5 = light yellow-green.
[b] For each value, $n = 10$.
[c] Significance: *, **, *** are significant at $p < 0.10$, $p < 0.05$ and 0.01, respectively.

ence or absence of PBZ alone (Table 1). In the absence of PBZ, the plantlets, with and without IBA, were taller and spindly due to low-light intensities needed for in vitro rooting. PBZ has been useful in maintaining the quality of foliage plants in low-light interior environments by preventing excessive spindly shoot growth (Davis 1987). Shorter stems and darker green leaves were also reported in *Prunus avium* in response to PBZ (Snir 1988).

Although PBZ had no significant effect on rooting percentage, number of roots, root length and width, visually, roots in the PBZ treatments typically appeared shorter, thicker, and more numerous as shown in Fig. 1. Increased rooting percentage, root width, and decreased root length were found by Marino (1986) but he used levels 0.0 and 0.2 mg/L IBA with PBZ concentrations of 0.0, 0.25, and 0.50 mg/L on *Prunus* species hybrid rootstock in vitro, whereas the IBA level used in this study was higher, 1.0 mg/L.

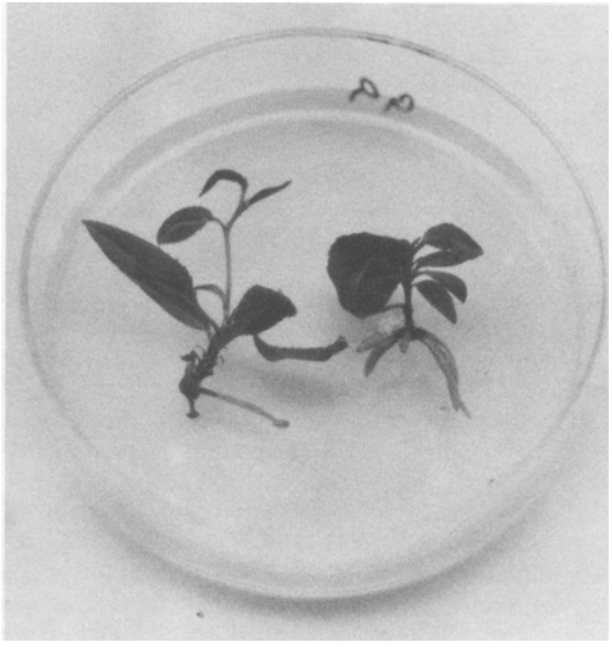

Fig. 1. The plantlet on the *right*, cultured on medium containing 0.30 mg/L PBZ and no IBA, exhibits the typical features of the treated plantlet—greater number of shorter, thicker roots, and a more compact shoot with compressed internodes. The plantlet on the *left* was cultured on medium containing no IBA or PBZ.

Water Loss Modifications

Water loss of excised leaves ranged from the low rates found in the greenhouse seedlings to much higher rates of tissue cultured controls (not treated with PBZ) (Fig. 2). In vitro grown plum and apple plantlets that had been hardened in the greenhouse showed similar patterns of water loss (Brainerd et al. 1981, Brainerd and Fuchigami 1981). Excised leaves from plantlets cultured on a medium containing PBZ showed a significant reduction both in magnitude and overall rate of water loss relative to the tissue-cultured controls. The longer the excised

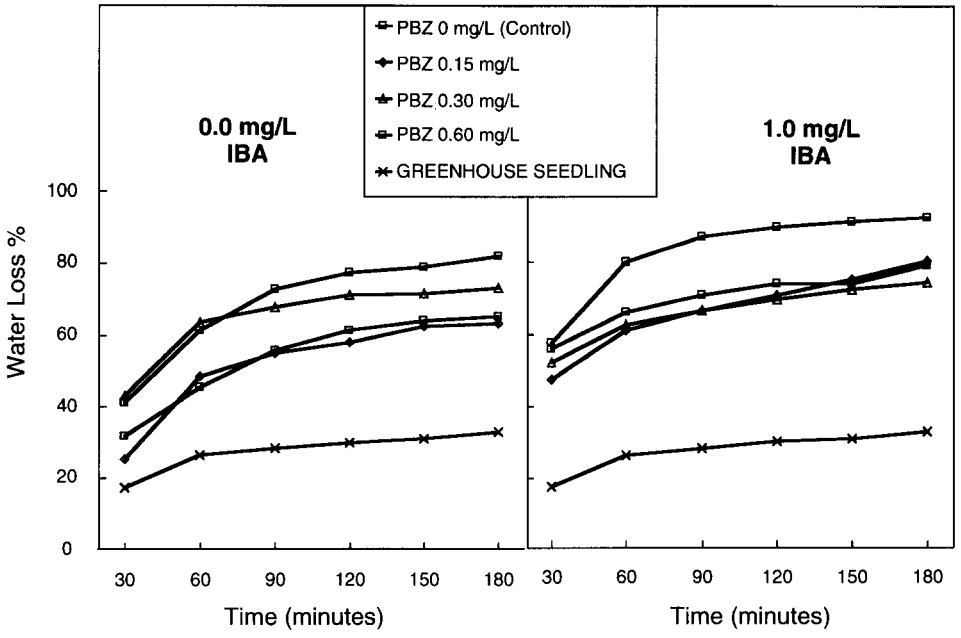

Fig. 2. Water loss from detached leaves as a function of PBZ concentration and time, with or without IBA. Seedling is only used as a reference to indicate water loss in leaves of typical greenhouse grown plants.

leaves were held, the greater the significance of treatment by PBZ became. Over time, IBA also had a significant effect on water loss, although the effect became less significant with increasing holding time (Table 2).

Increased resistance to wilting observed in chrysanthemum cultured with PBZ was associated with improved stomatal response (Smith et al. 1990). Water loss was also minimized in water-stressed plantlets in PBZ-cultured grapevine due to smaller stomatal apertures (Smith et al. 1992). These results are consistent with reports in the literature of reduced rate of water loss in apple seedlings (Steffens and Wang 1986). Reduced water loss in PBZ-treated plants is often also attributed to decreased leaf area (Davis et al. 1988, Wample and Culver 1983). Although PBZ is known to reduce leaf area (Orzolek 1987, Smith et al. 1990), micropropagated leaves tend to be smaller than their in vivo-grown counterparts (Donnelly 1990). Because of this, we chose not to use leaf area as a measurement of PBZ's effects in vitro. No differences in size were observed between PBZ-treated and control plantlets or among different concentrations of PBZ.

Table 2. Analysis of variance for percentage of water loss over time for detached leaves of *Prunus serotina*.

Source	DF	Mean square
IBA	1	6027.92
Paclobutrazol	3	4489.04
Interaction	3	1234.66
Error A	72	2668.57
Time	5	9612.45***
Time * IBA	5	124.15**
Time * PBZ	15	73.93*
Time * Interaction	15	55.16
Error B	360	48.37
Total	479	

PBZ = paclobutrazol.
*, **, *** Significant at 0.1, 0.05, and 0.01 levels of probability, respectively.

Survival of PBZ-Treated Plantlets

Four weeks after transfer to the ex vitro environment, survival was significantly greater for plantlets treated with all levels of PBZ and IBA (Fig. 3, Table 3). PBZ has been found to improve survival in vegetable transplants with minimum carry-over of growth retardation (Orzolek 1989). In that study, tolerance to environmental stress was imparted to treated transplants during the first 4 weeks, followed by a period of accelerated plant growth. Sankhla et al. (1991) also observed that PBZ greatly enhanced the quality of regenerated *Echinochola frumentacea* plantlets with respect to survival following transplanting to ex vitro conditions. Increased root number and thickness, shorter stems, and improved plant quality in response to PBZ in vitro may have facilitated replacement of water loss

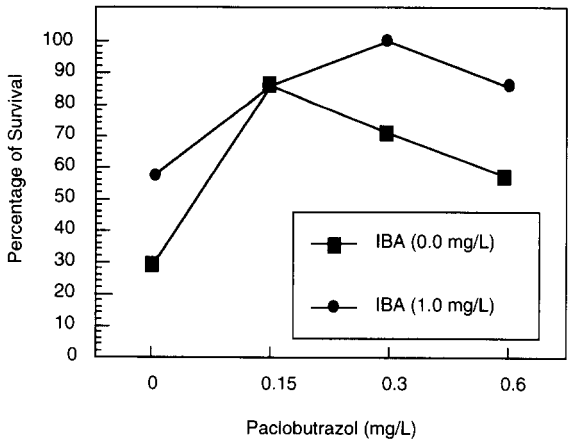

Fig. 3. Influence of PBZ incorporated in vitro on subsequent survival in ex vitro environment.

Table 3. Analysis of variance for survival and plant quality for PBZ/IBA experiment.

Source	Degrees of freedom	Mean squares	
		Survival	Plant quality
REP	6	0.1964	2.2838
IBA	1	0.6429*	1.4137
PBZ	3	0.5714**	1.8605
IBA × PBZ	3	0.0714	6.9867**
Error	26	2.0816	2.0816

*, ** Significant at 0.1 and 0.05 levels of probability, respectively.

by the leaves of transferred plantlets. This would reduce the desiccation associated with the high mortality rates normally seen during acclimatization. PBZ could have given the newly transferred plantlets an initial edge over the control plantlets with respect to reduced desiccation and this may have been coupled with increased in vitro rooting and/or repartitioning of assimilates from shoot to root (Davis et al. 1988). The repartitioning could have been responsible for the subsequent increased ex vitro rooting and root development (enhanced lateral root development was also observed at 4 weeks but not quantified). Thus, when growth resumed after PBZ inhibition of shoot growth had declined, a more robust plant was the result.

PBZ modifies the percentage of water loss in detached leaves of micropropagated *Prunus serotina* shoots and plants. It does this without phytotoxic effects or gross morphological alteration. PBZ tends to reduce shoot height, increase the depth of greening of in vitro plants kept at low light levels, increase the width, and decrease overall length of the roots found. Due to compactness, resistance to water loss, and better overall quality, plantlets treated with both PBZ and IBA in combination survived transfer to the greenhouse in greater numbers and were of a higher quality. Although PBZ's effects can be observed as quickly as at the end of the in vitro rooting stage, statistically there was little significant effect on the various growth characteristics measured and the most apparent effects occurred ex vitro during survival challenges. Using a lower level of IBA in conjunction with a PBZ concentration of 0.15–0.30 mg/L may increase the positive effects of in vitro hardening. PBZ's effects tend to be significant or the most apparent when the treated plants are subjected to stress conditions (Davis et al. 1988) and it is during the subsequent transfer to the greenhouse that the incorporation of PBZ into the tissue culture medium may prove to be the most worthwhile in producing plantlets that are better able to withstand stress.

References

Anderson C, Meagher GW (1978) Cost of propagating broccoli plants through tissue culture. HortScience 12:543–544

Brainerd KE, Fuchigami LH (1981) Acclimatization of aseptically cultured apple plants to low relative humidity. J Amer Soc Hort Sci 106:515–518

Brainerd KE, Fuchigami LH, Kwartkowski S, Clark CS (1981) Leaf anatomy and water stress of aseptically cultured "Pixy" plum grown under different environments. HortScience 16:173–175

Crane J, Hughes H (1990) Medium overlays for improved hardening of micropropagated potatoes. HortScience 25:794–795

Davis TD (1987) Interior performance of three foliage plant species treated with Paclobutrazol. Appl Agr Res 2:120–123

Davis TD, Steffens GL, Sankhla N (1988) Triazole plant growth regulators. Hort Rev 10:63–105

Donnelly DJ (1990) The culture-induced phenotype. Proc Plant Growth Reg Soc Am 17:47–60

Fujiwara K, Kozai T, Watanabe I (1988) Development of a photoautotrophic tissue culture system for shoots and/or plantlets at rooting and acclimatization stages. Acta Hort 230:129–135

Kavanaugh KL (1987) Acclimatization of *Prunus serotina* Ehrh. Propagation in vitro. MS Thesis. SUNY College of Environmental Science and Forestry, Syracuse, NY

Khunachak A, Chin DK, Gianfagna TL, Gianfagna T (1987) Promotions of asparagus shoot and root growth by growth retardants. Plant Cell Tissue Organ Culture 11:97–110

McVaugh R (1951) A revision of the North American black cherries (*Prunus serotina* Ehrh. and relatives). Brittonia 7(4): 279–315

Marino G (1986) The effect of Paclobutrazol on the in vitro rooting of a *Prunus* species hybrid rootstock. Riv Ortoflorofrutt It 70:267–275

Murashige T, Skoog F (1962) A revised medium for rapid growth

and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Orzolek MD (1987) Use of growth retardants for tomato transplant production. HortScience 22:1161 (Abstr)

Orzolek MD (1989) Growth retardants improve plant performance. Am Veg Grow 37:42–44

Sankhla A, Davis TD, Sankhla D, Sankhla N, Upadhyaya A, Joshi S (1991) Triazole plant growth regulators influence somatic embryogenesis and plantlet regeneration in tissue cultures of *Echinochloa frumentacea*. Proc Plant Growth Reg Soc Am 18:84–89

Smith EF, Roberts AV, Mottley J (1990) The preparation in vitro of chrysanthemum for transplantation to soil 2. Improved resistance to desiccation conferred by Paclobutrazol. Plant Cell Tissue Organ Culture 21:133–140

Smith EF, Gribaudo I, Roberts AV, Mottley J (1992) Paclobutrazol and reduced humidity improve resistance to wilting of micropropagated grapevine. HortScience 27:111–113

Snir I (1988) Influence of Paclobutrazol on in vitro growth of sweet cherry shoots. HortScience 23:304–305

Steffens GL, Wang SY (1986) Biochemical and physiological alterations in apple trees caused by a gibberellin biosynthesis inhibitor, Paclobutrazol. Acta Hort 179:433–442

Tricoli DM, Maynard CA, Drew AP (1985) Tissue culture propagation of mature trees of *Prunus serotina* Ehrh. I. Establishment, multiplication and rooting in vitro. Forest Science 31:201–208

Wample RL, Culver EV (1983) The influence of Paclobutrazol, a new growth regulator, on sunflowers. J Amer Soc Hort Sci 108:122–125

Wardle K, Dobbs EB, Short KC (1983) In vitro acclimatization of aseptically cultured plantlets to humidity. J Amer Soc Hort Sci 108:386–389

Ziv M, Meir G, Malevy AM (1983) Factors influencing the production of hardened glaucous carnation plantlets in vitro. Plant Cell Tissue Organ Culture 2:55–65