Effects of Absciscic Acid on ex vitro Acclimatization of *Aronia arbutifolia* (L.) Pers.

Wilfredo Colón-Guasp¹, Terril A. Nell², Michael E. Kane³, and James E. Barrett²

Department of Environmental Horticulture, University of Florida, Gainesville, FL 32611

Additional index words. leaf carbon assimilation, micropropagation, nonstructural carbohydrates, woody plant

Abstract. The use of absciscic acid (ABA) as an in vitro prehardening treatment to enhance ex vitro acclimatization of Stage III *Aronia arbutifolia* plantlets was explored. Effects of ABA (0-4 mg-liter⁻¹) pretreatment on ex vitro shoot growth, leaf carbon assimilation (LCA) and nonstructural carbohydrate content were evaluated during plantlet acclimatization under two photosynthetic photon flux (PPF) levels (450 and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Stage III plantlets rooted in the presence of ABA exhibited both shoot growth inhibition and transient negative LCA rates at time of transfer ex vitro. Regardless of treatment, maximum LCA rates were achieved by day 20 post-transplant. Pretreatment with ABA had no effect on stem or leaf starch content at time of transplant, however, leaf and stem soluble sugar content was higher in ABA treated plantlets than controls. Further suppression of shoot growth and alteration in the pattern of stem starch utilization occurred at the higher irradiance level. These results indicate that ABA pretreatments provide no physiological advantage that would facilitate ex vitro acclimatization of *Aronia* plantlets.

The ultimate success of shoot culture (in vitro propagation) depends on the ability to transfer and reestablish vigorously growing plants from in vitro to ex vitro conditions. This involves acclimatizing or hardening-off plantlets to conditions of lower relative humidity and higher light levels. During the acclimatization process, tissue cultured plantlets undergo changes in both leaf anatomy (Capellades et al., 1990) and physiology (Grout and Millam, 1985), which confer the plants with a greater potential for survival ex vitro. Even when acclimatization procedures are followed, transplant survival can be low due to the inability of plantlets to maintain adequate water relations or fully transition from a mixotrophic to a photoautotrophic mode of nutrition (Grout and Aston; 1978, Wardle et al., 1983).

A composite of anatomical, morphological, and physiological features, characteristic of plants cultured in vitro under low light intensity and high relative humidity, contribute to the decreased survival of plantlets often observed immediately following transplanting. These features include reduction in leaf epicuticular wax deposition, abnormal stomate function, poorly developed stem to root vascularization, and limited photosynthetic competence (Brainerd and Fuchigami, 1982; Preece and Sutter, 1991). The modifications in leaf development and photosynthetic competence that typically occur during ex vitro acclimatization appear to be inducible. In vitro culture of plantlets under conditions of higher light levels and lower relative humidity conditions induce anatomical modifications of foliar epicuticular wax, stomata, and epidermal cells similar to those produced on acclimatized greenhouse-grown plants (Capellades et al., 1990). However, the physiological mechanisms regulating acclimatization remain obscure.

Induction of similar changes in leaf developmental patterns following absciscic acid treatment in vivo (Zeevaart and Creelman, 1988) and in vitro (Jarret and Gawel, 1991; Kane and Albert, 1989) suggest a possible role of endogenous ABA in the acclimatization process. In a previous study (Colón et al., 1990) we reported that

plantlets of the woody shrub *Aronia arbutifolia* (Rosaceae), produce leaves with morphological and anatomical features similar to greenhouse-grown plants when cultured in vitro in the presence of ABA. Conceivably, medium supplementation with ABA during microcutting rooting (Stage III) could serve as an in vitro prehardening treatment to prematurely induce developmental and physiological changes, which decrease water loss, increase photosynthetic capacity and thus increase the ability of the plantlet to survive ex vitro.

The consequence of ABA pretreatment in vitro on subsequent transpiration, nonstructural carbohydrate content, and leaf carbon assimilation rates (LCA) of rooted microcuttings during ex vitro acclimatization is unknown. Transient reductions in transpiration and photosynthesis have been reported for plants following ABA application in vivo (Arteca et al., 1985). In some cases, greater reductions in transpiration than net photosynthetic rate have been observed in ABA-treated plants (Blake et al., 1990). Where examined, sucrose/starch synthesis ratios are not significantly affected by ABA treatment in vivo (Sharkey et al., 1985). The objective of this study was to characterize the effects of ABA pretreatment in vitro on subsequent changes in shoot growth, LCA, and nonstructural carbohydrate content of *A. arbutifolia* plantlets during acclimatization.

Materials and Methods

Stage 1/11: Culture establishment and shoot multiplication. Stems with lateral buds of *A. arbutifolia* were cut from actively growing and sexually mature plants, divided into 15-mm lengths with two to three lateral buds and rinsed in tap water for 1 h. Lateral buds were surface sterilized by repeated immersion in 50% (v/v) ethanol for 1 min and then in 1.05% (v/v) sodium hypochlorite for 12 min, followed by three 5-min rinses in sterile deionized water (Kane et al., 1991). The sterilized nodal explants were transferred to 25 × 150-mm culture tubes containing 15 ml of medium consisting of woody plant medium (WPM), salts and vitamins (Lloyd and McCown, 1980), 3% (w/v) sucrose, 2 mg-liter⁻¹ N⁶-benzylaminopttrine (BA) and solidified with 1.0% (w/v) TC agar (JRH Biosciences, Lenexa, Kan.) The medium pH was adjusted to 5.5 with 0.1 N KOH before autoclaving at 1.2 kg-cm⁻² pressure for 20 min at 121 C. All cultures were placed under a 16-h photoperiod provided by cool-white fluorescent lamps at 45 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Air temperature was maintained at 25 ± 2C.

Stock cultures were maintained by subdividing shoots (15 mm long bearing three axillary buds with attached subtending leaves)

Received for publication 7 Apr. 1995. Accepted for publication 19 Sept. 1995. Florida Agricultural Experiment Station Journal Series no. R-004463. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

¹Current address: Escuela Agrícola Panamericana, P.O. Box 93, Tegucigalpa, Honduras.

²Professor.

³Associate professor. To whom reprint requests should be addressed.

and transferring them onto fresh WPM every 5 to 7 weeks.

Stage III: Microcutting rooting and treatments. Rooted microcuttings were prepared by cutting 5-week-old shoots into 10-mm stem segments consisting of two or three nodes. Fifteen microcuttings were transferred into 473-ml clear polypropylene culture vessels (Better Plastics, Kissimmee, Fla.) containing 100 ml of agar-solidified WPM supplemented with 2 mg·liter⁻¹ of indole-3-butyric acid (IBA) but without BA. Synthetic ABA (90% mixed isomers, Sigma Chemical, St. Louis) was prepared as a concentrated aqueous stock solution and sterilized by Millipore filtration (pore size: 0.22 µm) before being added to molten (40C) sterile medium. All cultures were maintained under the aforementioned conditions.

Stage IV: Ex vitro establishment. After 30 days, ABA treated and control Stage III rooted microcuttings were transplanted into plug trays (50 cells per tray) containing sterile Rootcubes (Smithers-Oasis, Kent, Ohio). Rooted microcuttings were fertilized weekly with a 20N-8.8P-16.6K soluble fertilizer (200 mg·liter⁻¹ N). Trays were placed in a walk-in growth room at 24 ± 2C in a 16-h photoperiod provided by metal arc High Intensity Discharge (HID) lamps (GTE, Manchester, N.H.). Rooted micro-cuttings were placed under two PPF treatments (450 µmol·m⁻²·s⁻¹ and 650 µmol·m⁻²·s⁻¹). PPF attenuation was provided by polypropylene shade cloths. At transplanting, humidity domes were placed over the trays and progressively opened until fully removed at 10 days. Plants were watered as needed to maintain substrate moisture.

Absciscic acid levels of 0, 2, or 4 mg·liter⁻¹ were applied in vitro in Stage III culture in a randomized complete block design with four replications. Ten plants were chosen as subsamples for determination of LCA. LCA rates were determined at 0, 10, 20, and 27 days after transfer with a Clark-type oxygen electrode (Hanastech, Norfolk, England). Immediately before measurement, leaves were cut and transferred to a leaf cuvette. Oxygen evolution was recorded using an oxygen electrode mounted in a cooling jacket (LD2; Hansatech) connected to a temperature controlling bath and light was supplied by a red diode light source (600 rim). Carbon dioxide was maintained at a constant level by adding 1 ml 1 M Na₂HCO₃ to the mat under the leaf disc (Delieu and Walker, 1983). The leaf material was illuminated for 4 min at a PPF of 450 µmol·m⁻²·s⁻¹ and oxygen evolution was measured when steady state was reached.

In another experiment 2 mg·liter⁻¹ IBA and ABA at 0, 1, 2, or 3 mg·liter⁻¹ were incorporated into the medium in Stage III culture in a randomized complete block design with five replications. At the end of 30 days of in vitro growth and at 5, 10, 20, and 30 days post transplant, rooted microcuttings were assayed for nonstructural carbohydrate content (leaf and stem soluble sugars and starch) and shoot growth.

Soluble sugars and starch content of leaf and stem tissue were extracted and analyzed with the phenol-sulfuric technique (Dubois et al., 1956). Whole plants were collected at 1500 h and placed in an oven at 60C. Dried and ground leaf and stem material (0.01 g) was extracted with 8 ml of 80% (v/v) ethanol at 95C for 20 min. After cooling, the samples were filtered through glass microfibre filters (Whatman, Maidstone, England). The dried pellet recovered from the filtrate was hydrated with 2 ml of 0.1 M acetate buffer (pH 5.6) in a water bath at 85C for about 1 h. After cooling to 37C, 3 ml of enzyme solution (31 units alpha amylase/ml with 25.5 units of amyloglucosidase/-ml, and 0.44 mg CaCl₂/ml) was added and incubated in a shaking water bath for 24 h at 37C. Samples were diluted to obtain a soluble sugar concentration between 15 and 25 µg·ml⁻¹. An aliquot of 1 ml was taken and 1 ml of 5% phenol was added and agitated. Finally, 5 ml of concentrated sulfuric acid was

added and the samples were placed for 20 minutes in a water bath at 25C. Standard solutions of glucose ranging in concentrations from 10 to 70 µg·ml⁻¹ were prepared and treated in the same manner. The amount of soluble sugars present in the plant extracts was determined spectrophotometrically (Lambda 3A; Perkin-Elmer, Norwalk, Conn.) at 490 nm.

Chlorophyll determinations were made following the procedure outlined by Bruinsma (1963). After 30 days of in vitro growth, fully expanded leaves were excised, weighed, dried in a freeze drier, pulverized, and added to 10 ml of 80% acetone. Solutions were placed in a refrigerator in the dark at 4C for 24 h, after which optical densities were measured at 663, 645, and 652 nm for chlorophyll a, b, and total, respectively. Carotenoid content was determined spectrophotometrically at a wavelength of 480 nm.

Data were analyzed by analysis of variance by SAS General Linear Model procedure. First (linear) or second (quadratic) order polynomials were fitted to the data by regression analysis.

Results and Discussion

There was no significant PPF × ABA interaction on shoot growth or nonstructural carbohydrate content of planted during ex vitro acclimatization (Tables 1 and 2). Plantlets grown in vitro for 30 days on ABA-supplemented medium exhibited reduced shoot growth ex vitro compared to control plants (Table 1). Shoot growth was further inhibited at the higher irradiance level. Root growth was also reduced in the presence of ABA (data not shown). Plantlets cultured on ABA-free medium exhibited positive but very low LCA at the time of transfer to ex vitro conditions (Fig. 1 A and B). This indicates a capacity for rapid conversion to the photoautotrophic state upon transfer since *Aronia* plantlets exhibit negative LCA during in vitro culture (Colon et al., 1990). Leaf carbon assimilation rates were initially negative in plantlets pre-cultured with ABA (Fig. 1 A and B). However, ABA-induced suppression of LCA was transient. With respect to LCA, maximum acclimatization was achieved by day 20 regardless of treatment. Survival of *Aronia* plantlets ex vitro was 100% regardless of initial LCA at time of transplant.

The physiological basis for the greater initial suppression of LCA in ABA-treated plantlets is not apparent. Low photosynthetic capacity of plantlets in vitro is attributed to several factors including low chlorophyll content, reduced ribulose biphosphate carboxylase activity and non-stomatal inhibition of photosynthesis following starch accumulation in chloroplasts (Capellades et al., 1991; Grout and Aston, 1978). No significant differences in chlorophyll or carotenoid content were observed between control and ABA-treated plantlets (data not shown).

Depression of LCA in ABA treated plantlets may be the consequence of increased stomatal resistance resulting from ABA-induced stomatal closure. Stomata in leaves produced in vitro are often nonfunctional but become responsive following 4 to 5 days acclimatization to reduced relative humidity (Brainerd and Fuchigami, 1981, 1982; Shackel et al., 1990). Leaves produced in vitro on *Aronia* plantlets cultured in the presence of ABA have epidermal features, including stomata, that are structurally similar to those produced on greenhouse-grown plants (Colon et al., 1990). Conceivably, these stomata are functional but initially closed during acclimatization. However, a direct affect of ABA on carbon fixation can not be ruled out (Zeevaart and Creelman, 1988).

Absciscic acid treatment had a significant main effect on plantlet leaf and stem soluble sugar content and leaf starch content over the acclimatization period (Table 2). There was also a significant main effect of PPF level on leaf and stem soluble sugar content and on stem starch content (Table 2). After 30 days in vitro

Table 1. Effects of ABA (0, 1, 2, and 3 mg·liter⁻¹) on shoot length (mm) of *Aronia arbutifolia* rooted microcuttings grown ex vitro under 450 and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF for 5, 10, 15, 20, 25, and 30 days.

Treatment		Shoot length (mm)						
PPF	ABA	Days post-transplant						
($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	(mg·liter ⁻¹)	0	5	10	15	20	25	30
450								
Linear	0	15	18	23	28	37	59	93
	1	14	17	21	24	30	49	75
	2	13	14	17	19	24	38	61
	3	13	14	17	19	23	39	61
		*	**	**	**	**	**	**
Quadratic		NS	NS	NS	NS	NS	NS	NS
650								
Linear	0	16	20	24	28	35	46	61
	1	12	13	16	17	20	30	44
	2	12	13	15	16	20	26	35
	3	11	12	15	16	20	29	41
		**	**	**	**	**	**	**
Quadratic		*	**	**	**	**	**	*
PPF $\times$ ABA		NS	*	NS	NS	NS	NS	NS
PPF (<i>t</i> test)		NS	NS	*	*	*	*	*

NS,*,** Significant or nonsignificant at $P \leq 0.05$ or 0.01, respectively.

(0 days after transfer ex vitro), ABA pretreatments had no significant effect on leaf or stem starch content (Fig. 2 A and B and Fig. 3 A and B) as determined at the time of transfer. Starch content rapidly increased before and then declined after day five post-transplant in both leaf and stem tissues. The accumulation of starch from new photosynthate is unlikely since LCA were low or negative during this period. The possibility of carbohydrate translocation from the roots was not examined. Stem starch utilization was delayed in plantlets pre-cultured with ABA and maintained at the higher PPF (Fig. 3B).

Leaf (Fig. 2 C and D) and stem (Fig. 3 C and D) soluble sugar levels were greater in ABA-treated plantlets than in control plantlets at the time of transfer. Under the higher PPF, transient elevations in soluble sugar levels were observed in both leaf (Fig. 2D) and stem (Fig. 3D) tissues of ABA-treated plantlets. However, no differences between

treatments were observed by 20 days after transfer. Beyond 10 days post-transplant the increased shoot growth observed (Table 1) was perhaps due to mobilization of carbohydrate from leaf and stem carbohydrate reserves to areas of active growth.

Based on starch utilization, Stage III *Aronia* rooted plantlets remain highly dependent on carbohydrate reserves for sustained growth and development of photosynthetically competent leaves during initial acclimatization. Starch reserves became depleted within the first 20 days. Lack of sufficient energy reserves for both adventitious root formation and growth maintenance would explain the poor ex vitro survival of unrooted Stage II *Aronia* microcuttings (Kane et al., 1991). Treatments that increase stem starch content might facilitate ex vitro survival of more problematic species. While in vitro application of ABA induced starch deposition in other plants (Smart and Trewavas, 1983), no such

Table 2. F values from GLM of ABA (0, 1, 2, 3 mg·liter⁻¹) and PPF (450 and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) two factor experiment on leaf and stem soluble sugar and starch content (mg·g⁻¹ dry weight) after transfer from an in vitro to an ex vitro environment.

Plant part	Sugar				Starch			
	Model R^2	Source	df	F value	Model R^2	Source	df	F value
Leaf	0.67**	ABA	3	11**	0.77**	ABA	3	7.53**
		PPF	1	16**		PPF ²	1	0.28 ^{NS}
		DAT ³	4	30**		DAT ³	4	82**
		PPF $\times$ ABA	3	0.55 ^{NS}		PPF $\times$ ABA	3	0.58 ^{NS}
		DAT $\times$ PPF	4	2.68*		DAT $\times$ PPF	4	0.82 ^{NS}
		DAT $\times$ ABA	12	3.02**		DAT $\times$ ABA	12	1.34 ^{NS}
Stem	0.53**	ABA	3	5**	0.76**	ABA	3	1.05 ^{NS}
		PPF	1	29**		PPF	1	6*
		DAT	4	6**		DAT	4	74**
		PPF $\times$ ABA	3	0.67 ^{NS}		PPF $\times$ ABA	3	0.49 ^{NS}
		DAT $\times$ PPF	4	8**		DAT $\times$ PPF	4	4**
		DAT $\times$ ABA	12	1.56 ^{NS}		DAT $\times$ ABA	12	1.00 ^{NS}

²Photosynthetic photon flux.

³Days after transfer.

NS,*,** Significant or nonsignificant at $P \leq 0.05$, or 0.01, respectively.

Fig. 1. Effect of ABA(0–4 mg·liter⁻¹) pre-treatment on subsequent leaf carbon assimilation (LCA) of rooted *A. arbutifolia* plantlets during acclimatization under 450 (A) and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (B) PPF for 27 days. Values are mean $\pm$ SE.

response was observed in *Aronia*.

Although development of cutinized greenhouse-type leaves are induced in vitro in the presence of ABA, the suppression of shoot growth and LCA that occur provide no physiological benefit that would facilitate ex vitro acclimatization of *Aronia* plantlets. However, studies of the ABA effects on acclimatization of more problematic species could prove useful.

Literature Cited

- Arteca, R.N., D. Tsai, and C. Schlagnhauser. 1985. Absciscic acid effects on photosynthesis and transpiration in geranium cuttings. *HortScience* 20:370–372.
- Blake, T.J., W. Tan, and S.R. Abrams. 1990. Antitranspirant action of absciscic acid and ten synthetic analogs in black spruce. *Physiol. Plant* 80:365–370.
- Brainerd, K.E. and L.H. Fuchigami. 1981. Acclimatization of aseptically cultured apple plants to low relative humidity. *J. Amer. Soc. Hort. Sci.* 106:515–518.
- Brainerd, K.E. and L.H. Fuchigami. 1982. Stomatal functioning of in vitro and greenhouse apple leaves in darkness, mannitol, absciscic acid, and CO₂. *J. Expt. Bot.* 33:388–392.
- Brunisma, J. 1963. The quantitative analysis of chlorophylls a and b in plant extracts. *Photochem. Photobiol. (Chlor. Metabol. Symp.)* 2:241–249.
- Capellades, M., R. Fontarnau, C. Carulla, and P. Debergh. 1990. Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa multiflora*. *J. Amer. Soc. Hort. Sci.* 115:141–145.
- Capellades, M., R. Lemeur, and P. Debergh. 1991. Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured in vitro. *Plant Cell Tissue Organ Cult.* 25:21–26.
- Colon, W., M. E. Kane, and D. L. Ingram. 1990. Effects of absciscic acid on photosynthesis, growth and development of Stage III *Aronia arbutifolia* (Rosaceae). *Proc. Fla. State Hort. Soc.* 103: 178–182.
- Delieu, T.J. and D.A. Walker. 1983. Simultaneous measurement of oxygen evolution and chlorophyll fluorescence from leaf pieces. *Plant Physiol.* 73:534–541.
- Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric methods for determination of sugars and related substances. *Analytical Chem.* 28:350–356.
- Grout, B.W.W. and M.J. Aston. 1978. Transplanting of cauliflower plants regenerated from meristem culture. II. Carbon dioxide fixation and the development of photosynthetic ability. *Hort. Res.* 17:65–71.
- Grout, B.W.W. and S. Millam. 1985. Photosynthetic development of micropropagated strawberry plantlets following transplanting. *Annu. Bot.* 55:129–131.
- Jarret, R.L. and N. Gawe. 1991. Absciscic acid-induced growth inhibition of sweet potato (*Ipomea batatas* L.) in vitro. *Plant Cell, Tissue, and Organ Culture.* 24:13–18.
- Kane, M.E. and L.S. Albert. 1989. Absciscic acid induction of aerial leaf development in *Myriophyllum* and *Proserpinaca* species cultured in vitro. *J. Aquatic Plant Mgt.* 27:102–111.
- Kane, M. E., B. Dehgan, and T.J. Sheehan. 1991. In vitro propagation of Florida native plants: *Aronia arbutifolia*. *Proc. Fla. State Hort. Soc.* 104:287–290.
- Lloyd, G. and B. McCown. 1980. Commercially-feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot-tip culture. *Proc. Intl. Plant Prop. Soc.* 30:421–427.
- Preece, J.E. and E.G. Sutter. 1991. Acclimatization of micropropagated plants to

Fig. 2. Leaf soluble sugar and starch content of *A. arbutifolia* rooted plantlets pre-treated in vitro with ABA (0, 1, 2, and 3 mg·liter⁻¹) and grown under 450 (A and C) and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (B and D) PPF during a 30 day growing period after transfer from in vitro. Values are mean $\pm$ SE.

Fig. 3. Stem soluble sugar and starch content of *A. arbutifolia* rooted plantlets pre-treated in vitro with ABA (0, 1, 2, and 3 mg·liter⁻¹) and grown under 450 (A and C) and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (B and D) PPF during a 30 day growing period after transfer from in vitro. Values are mean $\pm$ SE.

- greenhouse and field, p. 71–93. In: P.C. Debergh and R. H. Zimmerman (eds.). *Micropropagation Technology and Application*. Kluwer Acad. Publ., Boston.
- Shackel, K.A., V. Novello, and E.G. Sutter. 1990. Stomatal function and cuticular conductance in whole tissue-cultured apple shoots. 1990. *J. Amer. Soc. Hort. Sci.* 115:468–472.
- Sharkey, T.D., J.A. Berry, and K. Raschke. 1985. Starch and sucrose synthesis in *Phaseolus vulgaris* as affected by light, CO₂, and absciscic acid. *Plant Physiol.* 77:617–620.
- Smart, C.C. and A.J. Trewavas. 1983. Absciscic-acid-induced turion formation in *Spirodela polyrrhiza* L. 1. production and development of the turion. *Plant Cell Environ.* 6:507–514.
- Wardle, K., E.B. Dobbs, and K.C. Short. 1983. In vitro acclimatization of aseptically cultured plantlets to humidity. *J. Amer. Soc. Hort. Sci.* 108:386–389.
- Zeevaert, J.A.D. and R.A. Creelman. 1988. Metabolism and physiology of absciscic acid. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:439–473.