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ORIGINAL PAPER

Micropropagation of *Populus trichocarpa* 'Nisqually-1': the genotype deriving the *Populus* reference genome

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Abstract *Populus* serves as a model tree for biotechnology and molecular biology research due to the availability of the reference genome sequence of *Populus trichocarpa* (Torr. & Gray) genotype 'Nisqually-1'. However, 'Nisqually-1' has been shown to be very recalcitrant to micropropagation, regeneration and transformation. In this study, a highly efficient micropropagation protocol from greenhouse-grown shoot tips of 'Nisqually-1' was established. The optimal micropropagation protocol involves growing in vitro shoots in plant growth regulator-free Murashige and Skoog (MS) basal medium supplemented with 3% sucrose, 0.3% Gelrite® and 5–10 g L^{-1} of activated charcoal. Plants grown on this medium were significantly longer, and contained significantly higher concentrations of chlorophyll. This highly effective protocol provides a consistent supply of quality leaf and stem materials throughout the year for transformation experiments and other in vitro manipulations, therefore eliminating inconsistency due to seasonal and greenhouse environmental variations and the need for repetitive tissue sterilization.

Keywords Activated charcoal · Cytokinin · Gelling agent · Gelrite · Tissue culture · Poplar

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Abbreviations

AC	Activated charcoal
BA	6-Benzyladenine
Chl *a*	Chlorophyll *a*
MS	Murashige and Skoog
PGR	Plant growth regulator

Introduction

Populus species are widely used for wood, paper, as an energy source and for other purposes worldwide. They are now considered by the US Department of Energy to be the leading choice for dedicated woody bioenergy crops due to their fast growth, wide adaptation and ease of propagation (Tuskan 1998; Wullschleger et al. 2002). *Populus* is also regarded as the model for studying woody plant gene function because of the available reference genome of *Populus trichocarpa*, genotype 'Nisqually-1' (Tuskan et al. 2006). However, 'Nisqually-1' can be very recalcitrant to transformation with in vitro plant tissue (Ma et al. 2004), and protocols using greenhouse plants require the use of a vigorous sterilization procedure and are subject to limitation of a year-round supply of materials (Song et al. 2006). In addition, seasonal variation may lead to inconsistent plant materials which may produce variant results (Song et al. 2006).

In the process of establishing 'Nisqually-1' in vitro for regeneration and transformation research, we have encountered persistent difficulty in maintaining even basic growth of this genotype in various media used for other *Populus* species in culture (Dai et al. 2003; Ma et al. 2004). Despite its ease to root and propagate in the greenhouse and field, in vitro shoots remain green for only about 1 week and

then begin to turn chlorotic and necrotic. This limits growth and proliferation and ultimately results in plant death. Rutledge and Douglas (1988) also failed to establish in vitro cultures of *P. trichocarpa* and were unable to conduct micropropagation. Furthermore, Nadel et al. (1992) reported severe shoot tip dieback of *P. trichocarpa* and leaf yellowing in three different media, though addition of Ca-gluconic acid and 2-[*N*-morpholino] ethanesulfonic acid (MES) transiently reduced the problem. The objective of this research was to develop an effective protocol for growing 'Nisqually-1' in vitro so consistent plant material can be produced year-round for transgenic research for poplar functional genomics research, particularly for creating a mutational library and validating gene function. We tested the effects of basal medium, cytokinin concentration, gelling agent and activated charcoal (AC) on microshoot growth of 'Nisqually-1' and thus developed an optimal medium for growing and maintaining this genotype in vitro. This protocol enables us now to produce consistent and year-round materials for regeneration, transformation and other in vitro manipulation.

Materials and methods

Plant material

In vitro cultures 'Nisqually-1' were established from young shoots of greenhouse-grown plants. Vigorously growing 5-cm shoot tips were excised and then soaked sequentially in a 1% Tween-20 solution for 5 min, 70% ethanol for 1 min and in a 0.525% sodium hypochlorite (Clorox®) solution for 15 min. Explants were triple rinsed with sterile water for 5 min each time. A 2-cm shoot tip was excised from the surface sterilized shoot and placed into a 200-mL baby food jar (Sigma–Aldrich, St. Louis, MO) containing 30 mL of Murashige and Skoog (MS) (1962) basal medium supplemented with MS vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS), 100 mg L^{-1} myo-inositol, 3% (w/v) sucrose, 4.4 μM N^6-benzylaminopurine (BA) and 0.8% (w/v) agar (Cat. No. BP1423, Fisher Scientific, Pittsburg, PA). The solution was adjusted to pH 5.8 prior to autoclaving at 120°C and 103.5 kPa for 20 min. This was the medium which was used to maintain in vitro aspen (*Populus* spp.) cultures in our laboratory (Dai et al. 2003). The cultures were maintained in a growth room at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 μmol m^{-2} s^{-1} as measured by a Licor LI-250 light meter (LI-COR Inc., Lincoln, Nebraska) held at the top of the culture vessels. All of the stock cultures in the following experiments were maintained under these conditions.

The effects of basal medium and cytokinin concentration on plant growth

To evaluate the effect of basal medium salt on plant growth, 'Nisqually-1' shoots were cultured aseptically on either MS, woody plant medium (WPM) (Lloyd and McCown 1981) or Driver and Kuniyuki walnut (DKW) (Driver and Kuniyuki 1984) medium. In each basal medium, three concentrations of BA were tested: 0.0, 2.2 and 4.4 μM (Table 1). All media were supplemented with 0.1% MS vitamins, 100 mg L^{-1} myo-inositol, 3% (w/v) sucrose and 0.8% (w/v) agar (Cat. No. BP1423, Fisher Scientific, Pittsburg, PA).

Table 1 Survival rate and performance rating of *Populus trichocarpa* 'Nisqually-1' after 4 weeks on three basal media with three concentrations of N^6-benzylaminopurine

Basal medium	BA concentration (μM)	Percentage of shoots surviving after 4 weeks	Performance rating[a,b]
MS	0	75.0	3.875 a
MS	2.2	50.0	2.750 b
MS	4.4	37.5	2.375 b
DKW	0	37.5	2.500 b
DKW	2.2	37.5	2.375 b
DKW	4.4	37.5	2.250 b
WPM	0	50.0	2.875 b
WPM	2.2	37.5	2.375 b
WPM	4.4	37.5	2.125 b

[a] Plants were rated on a 5-point scale: (5) plant survived, actively growing, no sign of senescence; (4) plant survived, limited growth, lower leaves showing senescence; (3) plant survived, no growth, showing moderate senescence; (2) plant nearly dead; and (1) plant completely dead. For each treatment, the average value was given as the performance rating

[b] The same letters in the different rows indicate that there is no significant difference ($P \leq 0.05$)

Table 2 The effect of gelling agent and N^6-benzylaminopurine concentration on the survival of *Populus trichocarpa* 'Nisqually-1' after 4 weeks

Gelling agent	BA concentration (μM)	Percentage of shoots surviving after 4 weeks	Performance rating[a,b]
Gelrite	0	100.0	4.875 a
Gelrite	2.2	62.5	3.250 bc
Gelrite	4.4	50.0	3.125 bc
Agar	0	75.0	3.875 b
Agar	2.2	50.0	2.750 c
Agar	4.4	37.5	2.375 c

[a] Plants were rated on a 5-point scale: (5) plant survived, actively growing, no sign of senescence; (4) plant survived, limited growth, lower leaves showing senescence; (3) plant survived, no growth, showing moderate senescence; (2) plant nearly dead; and (1) plant completely dead. For each treatment, the average value was given as the performance rating

[b] The same letters in the different rows indicate that there is no significant difference ($P \leq 0.05$)

The effect of gelling agent on plant growth

Once the effects of basal medium and cytokinin concentration on plant growth were evaluated, MS medium was selected as the basal medium for determining the effect of gelling agent on the growth of 'Nisqually-1' shoots. Two gelling agents were tested: 0.8% (w/v) agar (Fisher Scientific) and 0.3% (w/v) Gelrite® (PlantMedia, Dublin, OH). The culture medium contained either 0.0, 2.2 or 4.4 μM BA (Table 2). The surviving 'Nisqually-1' shoots from previous experiments were used in this experiment.

The effect of activated charcoal on plant growth

To evaluate the effect of AC on plant growth, 'Nisqually-1' shoots were cultured on MS basal medium without plant growth regulators (PGRs) and with 0.3% (w/v) Gelrite®. Four AC concentrations, 0, 3, 5 and 10 g L^{-1}, were tested (Fig. 1). AC was added to the culture medium after adjusting the pH to 5.8, prior to autoclaving.

Experimental design, data collection and statistical analysis

In the first two experiments (the effects of basal medium and cytokinin concentration on plant growth and the effect of gelling agent on plant growth), four 200-mL baby food jars containing one shoot per jar were used for each treatment, and both experiments were repeated three times. Plant performance was evaluated at 4 weeks for survival rate, leaf chlorosis and overall appearance according to the following numeric criteria: (5-points) plant survived, actively growing, no sign of senescence; (4-points) plant survived, limited growth, lower leaves showing senescence; (3-points) survived, but no growth, showing senescence; (2-points) nearly dead; and (1-point) completely dead. For each treatment, the average of the replicates was given as the performance rating. An additional observation was made at 6 weeks, but data are not shown because many plants died during the last 2-week period. In the experiment that tested AC on plant growth, the explants were aseptically grown in 9-cm diameter × 14-cm high (1,000 ml) culture vessels (PhytoTechnology Laboratories). Four explants were used for each treatment and the experiment was repeated three times. The length of each plant was measured every 2 weeks for 6 weeks. The experimental design was a Completely Randomized Design (CRD), and the performance rating and length data were evaluated by analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc, Cary, NC).

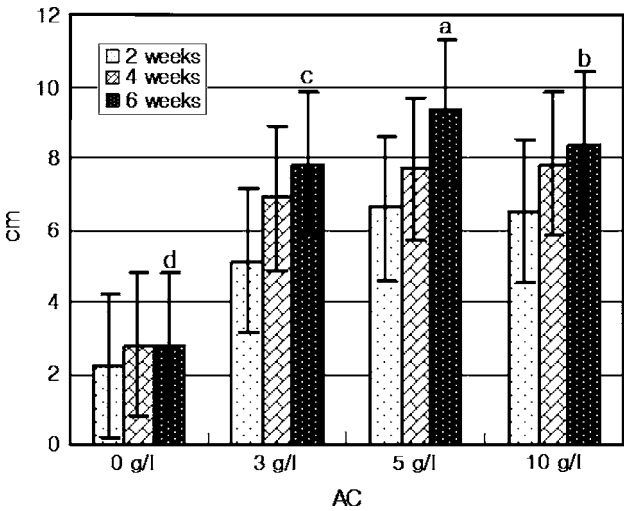

Fig. 1 Mean length of 'Nisqually-1' shoots grown on MS PGR-free Gelrite medium supplemented with either 0, 3, 5 or 10 g L^{-1} activated charcoal (AC) after 2, 4 and 6 weeks. Shoots were grown in 9 × 14 cm (1,000 ml) plastic vessels in sterile conditions at 25°C and 16 h photoperiod. Data are the means of 16 shoots. The *bar* represents the standard error of the mean. The *different letters above the bars* at 6 weeks (the final data collection date) indicate the separation of the treatment means

Chlorophyll content analysis by high-performance liquid chromatography (HPLC)

Since the plants grown in the medium supplemented with AC were significantly longer and appeared much healthier and greener in color than those grown in media without AC, leaf tissues were analyzed for chlorophyll content. Four plants from each of the four in vitro treatments were sampled. We also analyzed the chlorophyll content of plants grown on potting mix without AC in a growth chamber under fluorescent and incandescent lights. 'Nisqually-1' leaf tissues were lyophilized for no less than 48 h (Model 12 L FreeZone; LabConCo, Kansas City, MO) and stored at −80°C prior to extraction and analysis according to Kopsell et al. (2004) and analyzed according to Emenhiser et al. (1996). Briefly, a 0.1-g sample from each homogenate was re-hydrated with 0.8 mL of ultra pure H_2O and placed in a water bath set at 40°C for 20 min. After incubation, 0.8 mL of the internal standard ethyl-β-8′-apo-carotenoate (Sigma) was added to determine extraction efficiency. After sample hydration, there was an addition of 2.5 mL of tetrahydrofuran (THF) stabilized with 25 mg L^{-1} of 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT). Samples were then homogenized in a Potter–Elvehjem (Kontes, Vineland, NJ) tissue grinding tube. During homogenization, the tube was immersed in ice to dissipate heat. The tube was then placed into a clinical centrifuge for 3 min at $500\times g_n$. The supernatant was decanted and the sample pellet was re-suspended in 2 mL THF and homogenized again with the same extraction technique. The procedure was repeated for a total of four extractions per each sample to obtain a colorless supernatant. The combined sample supernatants were reduced to 0.5 mL under a stream of nitrogen gas (N-EVAP 111; Organomation Inc., Berlin, MA), and brought up to a final volume of 5 mL with methanol (MeOH). A 2-mL aliquot was filtered through a 0.2-µm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, DE.) using a 5-mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) prior to HPLC analysis.

An Agilent 1200 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA) was used for pigment separation. Chromatographic separations were achieved using an analytical scale (4.6 mm i.d. × 250 mm) 5 μm, 200 Å polymeric C_{30} reverse-phase column (ProntoSIL, MAC-MOD Analytical Inc., Chadds Ford, PA), which allowed for effective separation of chemically similar pigment compounds. The column was equipped with a guard cartridge (4.0 mm i.d. × 10 mm) and holder (ProntoSIL), and was maintained at 30°C using a thermostatted column compartment. All separations were achieved isocratically using a binary mobile phase of 11% methyl *tert*-butyl ethanol (MTBE), 88.9% MeOH and 0.1% triethylamine (TEA) (v/v). The flow rate was 1.0 mL min^{-1}, with a run time of 55 min, followed by a 2 min equilibration prior to the next injection. Eluted pigments and chemically similar pigment compounds from a 10 µL injection were detected at 453 (carotenoids and internal standard) and 652 [chlorophyll *a* (Chl *a*)] nm, and data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies). Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection using external standards (ChromaDex Inc., Irvine, CA).

Results

Basal medium and cytokinin effects

Shoots grown on MS medium without BA had higher survival rates at 4 weeks than those grown in MS medium with 2.2 or 4.4 µM BA, or in WPM or DKW medium with or without BA (Table 1). Shoots grown on MS medium without BA also appeared healthier and greener and became chlorotic and necrotic about 2 weeks later than those in other media (photographs not shown).

Gelling agent effect

It was clear that significantly more shoots grown on PGR-free medium with Gelrite as the gelling agent survived to week four and had significantly higher performance ratings than those grown in the medium solidified with agar (Table 2). The shoots remained green and produced new growth (photographs not shown). In Gelrite-containing media without BA, shoot survival rates were significantly higher than those grown on medium with either 2.2 or 4.4 µM BA, confirming that shoots grew better on PGR-free medium which was more favorable than BA-containing medium.

Activated charcoal effect

Although the shoots grown on MS basal medium solidified with Gelrite had higher survival rates, growth was still limited and not sustained. Addition of AC into Gelrite-containing medium significantly improved the growth of 'Nisqually-1'. In the medium without AC, all 'Nisqually-1' shoots survived to 4 weeks, but at 6 weeks survival was reduced to 50%. Shoots grew to an average of 2.2 and 2.8 cm at 4 and 6 weeks, respectively (Fig. 1). In contrast, all of the 'Nisqually-1' shoots grown on AC-containing media survived to week 6, and they grew significantly more than those on the AC-free medium (Fig. 1). At week 6, the 'Nisqually-1' shoots cultured on the medium with 3, 5 and

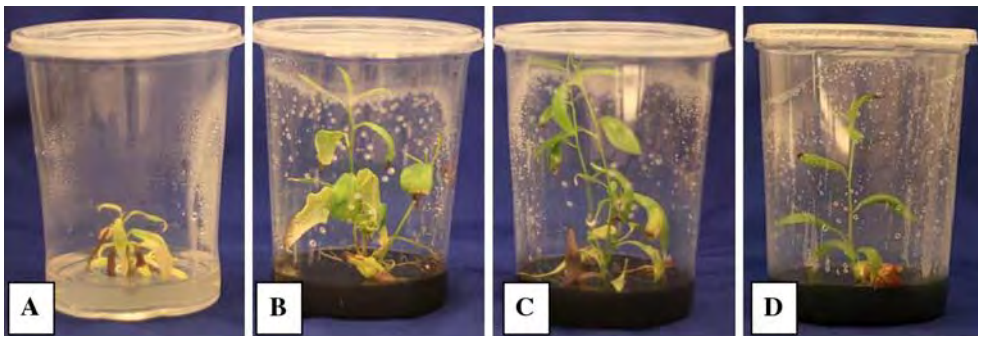

Fig. 2 'Nisqually-1' grown on MS basal medium, solidified with Gelrite and supplemented with either **a** 0, **b** 3, **c** 5 or **d** 10 g L^{-1} activated charcoal. Cultures were maintained in a growth room at 25°C and a 16-h photoperiod where fluorescent light intensity was 125 µmol $m^{-2}s^{-1}$. *Photographs* were taken at 6 weeks

10 g L^{-1} AC grew to 7.8, 9.3 and 8.4 cm, respectively (Fig. 1). All of the 'Nisqually-1' plants grown on AC-containing medium produced two to three green and healthy shoots (Fig. 2). Using this medium, we have maintained the 'Nisqually-1" shoots for more than 2 years (data not presented).

HPLC assay of chlorophyll a

'Nisqually-1' grown on basal MS medium with all AC-containing media appeared to have greener leaves (Fig. 2). This observation correlated with the chl *a* concentration of the leaves (Table 3). 'Nisqually-1' leaves from shoots grown on the medium containing 10 g L^{-1} AC had the highest concentration of chl *a* [1056.95 µg chl *a* g^{-1} dry weight (DW)], much higher than those grown on the medium containing 5 g L^{-1} AC (779.73 µg chl *a* g^{-1} DW), both being maintained in a growth room with 125 µmol m^{-2} s^{-1} fluorescent light. The control plants grown without AC in potting mix in a growth chamber had 507.68 µg chl *a* g^{-1} DW (data not presented), which was significantly less than those grown on media containing 5 or 10 g L^{-1} AC, but higher than those grown on culture media with 0 and 3 g L^{-1} AC.

Discussion

Although many *Populus* species are generally relatively easy to grow and propagate in the greenhouse as well as in tissue culture (Son et al. 2000; Dai et al. 2003), we have encountered extreme difficulty in maintaining the in vitro culture of the genotype 'Nisqually-1' of *P. trichocarpa* in a common medium that contains cytokinin and is solidified with agar (Dai et al. 2003). In previous reports with *P. trichocarpa*, extensive difficulty of in vitro propagation has also been reported (Rutledge and Douglas 1988; Nadel et al. 1992). Rutledge and Douglas (1988) failed to initiate shoots from *P. trichocarpa* meristems and were unable to perform micropropagation. Nadel et al. (1992) was able to reduce leaf yellowing and meristem dieback of *P. trichocarpa* in culture by growing on half strength medium supplemented with Ca-gluconic acid and 2-[*N*-morpholino] ethanesulfonic acid (MES), but the effect was transient only for a few subculture cycles. In this research, we have developed an effective and efficient method to propagate this important genotype of which the whole genome is sequenced. There are three key factors which contributed to the optimization of the protocol, namely, use of PGR-free MS medium, use of Gelrite as the gelling agent and addition of 5 or 10 g L^{-1} AC.

The basal medium type can affect the performance of woody plants in vitro (Mackay and Kitto 1988; Nadel et al. 1992; Cheng et al. 2000; Dai et al. 2005). Our results showed MS basal medium was more suitable than WPM and DKW for growing 'Nisqually-1'. The MS medium is known for its rich macro- and micro-elements, particularly nitrogen (Murashige and Skoog 1962). Although WPM was developed for micropropagating woody plants (Lloyd and McCown 1981) and DKW medium was developed for

Table 3 Visible absorption spectra of Chlorophyll *a* of 'Nisqually-1' by HPLC

AC treatment (g L^{-1})[a]	Sample dry wt (g)	HPLC % recovery	Recovered chlorophyll *a* (µg/g dry weight)
0	0.2812	0.91	48.91
3.0	0.0653	0.74	182.36
5.0	0.0736	0.79	779.73
10.0	0.0484	0.82	1056.95
Control (on soil, without AC)	0.0276	0.83	507.68

[a] Plants were grown on PGR-free MS basal medium, solidified with Gelrite, and supplemented with either 0, 3, 5 or 10 g L^{-1} activated charcoal (AC). Cultures were placed in a growth room where light intensity was 125 µmol m^{-2} s^{-1} provided by fluorescent light. The control plants were grown on soil without AC in a growth chamber where light intensity was 221 µmol m^{-2} s^{-1} provided by fluorescent and incandescent lighting

propagating walnut (*Juglans nigra*) (Driver and Kuniyuki 1984), both of these media were not well-suited for growing 'Nisqually-1'. Nadel (1992) also reported that MS medium was a suitable medium, although less effective than ½-strength MS.

Gelling agents can significantly affect the performance of in vitro culture (MacCrae and Van Staden 1990; Cheng and Shi 1995). The performance of 'Nisqually-1' in agar and Gelrite media were similar to that observed with Siberian elm [*Ulmus pumila* (Cheng and Shi 1995)], where shoots in agar-solidified medium deteriorated in 1 week, but fully recovered when transferred to Gelrite medium, while those in Gelrite medium deteriorated in 1 week after being transferred to agar-gelled medium. This deterioration phenomenon appears to be specific to 'Nisqually-1' because many other *Populus* species have been grown in agar-containing medium without this problem (Rutledge and Douglas 1988; Nadel et al. 1992; Son et al. 2000; Dai et al. 2003). In other species, agar medium was more favorable than Gelrite medium for shoot subculture of French tarragon [*Artemisia dracunculus* (Mackay and Kitto 1988)] and Asian white birch [*Betula platyphylla* (Cheng et al. 2000)]. Such a dramatic inhibitory effect may be attributed to the species-specific over-sensitivity of 'Nisqually-1' to microelements such as copper (Debergh 1983; Cheng and Shi 1995) in unpurified agar.

Activated charcoal clearly had a significant effect on improving growth of 'Nisqually-1'. Activated charcoal can improve development and growth of many plant species in vitro (Pan and Staden 1998), for example, establishment of lisianthus (*Eustoma grandiflorum*) protoplast culture (Kunitake et al. 1995; Teng 1997), spruce (*Picea abies*) somatic embryo development (Pullman et al. 2005), lily (*Lilium longiflorum*) bulb formation (Han et al. 2004) and embryogenesis of *Brassica oleracea* (da Silva Dias 1999). The beneficial effect of AC is thought to be attributed to its adsorption of inhibitory substances in the culture medium (Fridborg et al. 1978; Weatherhead et al. 1978, 1979). The positive effect of AC is also considered to be due to a reduction of the toxic effects of cytokinins and auxins in some cases (Weatherhead et al. 1978; Zaghmout and Torello 1988; Pan and Staden 1998), therefore, altering ratios of culture medium components and influencing plant growth in vitro (Johansson 1983; Druart and Wulf 1993). However, the enhancement of AC on 'Nisqually-1' is unlikely due to such an effect because our best performing medium lacks of exogenous hormones. One of the likely reasons for enhanced growth may be due to the higher content of chl *a*, responsible for greener leaves. Genomic analysis has revealed signaling functions among chlorophyll biosynthetic pathway intermediate compounds which regulate transcriptional production of light-harvesting chlorophyll-binding proteins such as carotene and xanthophyll carotenoids (Lohr et al. 2005). Furthermore, the biosynthesis of chlorophyll molecules is linked to the occurrence and production of light-harvesting complex polypeptides (Xu et al. 2001). Together, genomic and analytical data demonstrate the close connections between chlorophyll and carotenoid biosynthetic pathways. It is widely held that there is a positive correlation between the chlorophyll in leaves and the growth rate of plants (Gupta and Durzan 1984). Moreover, positive correlations between chlorophyll and carotenoid pigment concentrations in plants have been established (Kopsell et al. 2004). Since carotenoid pigments function in light-harvesting and photoprotection, it is also possible that elevated chlorophylls and carotenoids could impart greater fitness to the micropropagated plants. The dark environment provided by AC may also contribute to enhanced growth by promoting early root growth, thus allowing shoots to absorb nutrients early because 'Nisqually-1' produced more adventitious roots and produced roots early (data not presented).

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