In vitro Sugar and Water Use in Diploid and Tetraploid Genotypes of Daylily (*Heemerocallis* spp.) in Liquid Medium as Affected by Density and Plant Growth Regulators

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Abstract. Two tetraploid and two diploid genotypes of *Heemerocallis* spp. were micropropagated on an orbital shaker in Murashige and Skoog liquid medium in a factorial combination of two sucrose concentrations (90 mM and 180 mM), two 6-benzylaminopurine (benzyladenine) concentrations (0.32 μ M and 3.2 μ M), at two densities (57 explants/L and 171 explants/L), in the presence (0.32 μ M) and absence of α -cyclopyl- α -[4-mehtoxyphenyl]-5-pyrimidinmethanol (ancymidol). There were linear relationships between fresh weight and water use ($R^2 = 0.800$, $P < 0.0001$), dry weight and sucrose use ($R^2 = 0.636$, $P < 0.0001$), and relative dry weight (dry weight/fresh weight = relative dry weight) to concentration of sucrose residual in medium after culture ($R^2 = 0.553$, $P < 0.0001$). Eighty-five percent of the water used and 74% of the sucrose used were incorporated as plant fresh weight and dry weight, respectively. A 1% increase in percent sucrose residual (mass/volume in spent medium) was correlated to an increase of 1.8% relative dry weight over the range 7% to 22% relative dry weight. In vessels with 90 mM initial sucrose, where the most growth had occurred (>15 g fresh weight), sucrose was depleted (<0.2% sucrose) and plantlets had the lowest relative dry weight ($\approx 6.9\%$). In vessels from 180 mM initial sucrose, with similarly high fresh weight, plantlets had 12.0% relative dry weight with 2.1% sucrose residual in medium. Fresh weight, dry weight, or relative dry weight of plantlets in the laboratory did not correlate with subsequent survival or growth in the greenhouse. Plantlets grown without ancymidol at the lower benzyladenine concentration acclimatized to the greenhouse with 93% survival. However, greenhouse survival of plants grown with ancymidol and a higher level of benzyladenine was only 4%. ‘Barbara Mitchell’ was the largest plant in the laboratory, but often had poorest growth in the greenhouse. When optimizing a liquid micropropagation protocol for larger vessels, sucrose and water requirements may be directly related to targeted biomass yield, but each genotype needs to be handled independently with ex vitro validation of plant vigor.

Virtually the entire U.S. micropropagation industry relies on agar-based medium (R. Strode, Agri-Starts I; Mount Dora, Fla.); yet, many micropropagated plants grow better in liquid medium than on agar (Adelberg, 2004; Berthouly and Etienne, 2005; Takayama and Akita, 2005). Liquid systems offer advantages of reduced media costs, lower labor costs, and greater efficiencies for scale-up

(Etienne and Berthouly, 2002; Gross and Levin, 1999; Paek et al., 2005). Several inexpensive plastic bioreactors have been introduced for micropropagation that lessen barriers to adapt liquid-based systems. Bioreactor vessels designed for micropropagation include The Israeli LifeGuard (Ziv 1999), the French RITA (Etienne and Berthouly, 2002), BIT twin-flask in Cuba (Escalona et al., 1999), and the American Liquid Laboratory Rocker (Adelberg and Simpson, 2004). Shaker cultures are used to transition plants from agar-based culture to larger bioreactor systems, but shakers have high costs for numbers of plants produced (Takayama and Akita, 2005). Geophytes, and other herbaceous perennials with storage roots, have been most readily adaptable to bioreactor environments.

The proportion of explants to media volume and vessel size must be optimized to produce the greatest numbers of high-quality shoots from plant bioreactors (Adelberg, 2005; Berthouly and Etienne, 2005; Hahn and Paek, 2005). Plants in liquid systems grew faster than plants on agar as a result of greater exchange rates of sugar and water at the plant–medium interface (Ibaraki and Kurata, 1998). In liquid shaker culture of *Hosta tokudama* Tratt ‘Newberry Gold’, initial sucrose concentrations in media were correlated to endogenous sugar concentrations, plant dry weight (DW), and subsequent growth in the greenhouse (Gollagunta et al., 2004). Sugar and water use estimates for targeted crops would be useful to guide scale-up to larger bioreactor vessels.

Plant growth regulators influenced nutritional requirements for plants in bioreactors (Paek et al., 2005) and are used in micropropagation to promote desired morphology. For example, 6-benzylaminopurine (benzyladenine; BA) is the plant growth regulator most often used during stage II of micropropagation (multiplication) to promote axillary branching and to break apical dominance. Plant growth regulators that inhibit gibberellin synthesis were useful in bioreactor culture to improve morphology and anatomy of several geophyte crops (Ziv, 1992). Ancymidol (ANC) and paclobutrazol in liquid-based micropropagation systems reduced water uptake and vacuolization while decreasing internode length and leaf size (Ziv, 2005). Ancymidol and paclobutrazol in liquid also reduced plant size and increased the number of divisions in small, shaken flasks of *Musa acuminata* Colla ‘Grand Naine’ (Albany et al., 2005). Ancymidol reduced plant size and increased occurrence of divisions of *Hosta* ‘Blue Vision’ over a prolonged period of the culture cycle while in small, shaken jars (Maki et al., 2005). Because there is less leaf and root tissue to be cut away and discarded, smaller plants are handled more quickly during aseptic transfer processes, lowering the costly input of hood labor (Adelberg and Toler, 2004; Adelberg et al., 2005; Albany et al., 2005).

Daylilies (*Heemerocallis* spp.) are one of the most popular perennial landscape plants in the United States and are among the most valuable commercially micropropagated plants. *Heemerocallis* spp. is typically grown on agar-gelled Murashige and Skoog (MS) medium with 30 g·L⁻¹ sucrose and BA. More than 40,000 hybrid cultivars derived from an original genetic base of ≈ 16 interfertile species make up the daylily germplasm pool (Tomkins et al., 2001). Furthermore, hybridizers have used colchicine to convert many diploid cultivars, and then used the altered material as tetraploid breeding stock. As a result, the modern germplasm pool is a mix of both diploid and tetraploid cultivars.

Four daylily cultivars (two diploid and two tetraploid) with dissimilar phenotypes and pedigrees were selected for this study based on their diverse growth characteristics

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and genetic divergence as evaluated within the daylily germplasm pool using molecular genetic markers amplified fragment length polymorphism (AFLPs) (Tomkins et al., 2001). While propagating in vitro stock cultures of daylily in liquid media in small shaker vessels, the main and interactive effects of four genotypes (two diploid and two tetraploid) at two plant densities, with two sucrose concentrations, and at two concentrations of the plant growth regulators—BA and the gibberellin-antagonist α -cyclopyl- α -[4-mehtoxyphenyl]-5-pyrimidinmethanol (ancymidol; ANC)—were observed on growth and nutrient use in vitro and subsequent performance of the plantlets in the greenhouse. The exploratory factorial was designed to establish the relative importance of several factors, and their interactions, before future work in larger vessels. We present the most significant treatment effects and establish correlative effects, valid over a wide range of conditions as a guide to a rationally approach scale-up for larger vessels.

Materials and Methods

Four cultivars (genotypes) of daylily [two tetraploid ('Mary's Gold' and 'Heart of a Missionary') and two diploid ('Barbara Mitchell' and 'Brocade Gown')] were selected based on dissimilar pedigrees and phenotypes. Sterile explants were obtained by dissecting unexpanded buds in crown tissue in solution of 100% commercial bleach solution (5.25% NaOCl) for 2 min. Buds were plated on MS medium containing 1 μ M BA and 3% sucrose, and surface sterilization was repeated if infestation persisted. Buds were propagated in 180-mL baby food jars with 35 mL liquid medium, and were placed on an orbital shaker at 90-rpm, under 35 μ mol·m⁻²·s⁻¹ photosynthetically active radiation supplied by cool white fluorescent tubes in 16 h·d⁻¹, at 23 $\pm$ 2 °C. Subcultures were repeated about every 6 weeks for a minimum of 6 months.

When adequate quantities of tissue were available, the four genotypes were micro-propagated in modified MS liquid medium containing high or low sucrose concentrations (90 mM and 180 mM sucrose), high or low BA concentrations (0.32 μ M and 3.2 μ M), at high or low plant densities (57 explants/L and 171 explants/L), in the presence (0.32 μ M) or absence of ANC. A control group of six jars of medium without plants accompanied the experimental vessels on the shaker to determine the water loss and to act as a control for nutrient concentrations after autoclave.

After 35 d in culture, plants were removed and counted. The volume of remaining medium was determined for each jar. One milliliter of medium was then removed for nutrient analysis, and the sucrose concentration was determined using a refractometer (Atago N10, Atago Instruments Ltd., Tokyo). Plants were blotted dry on paper towels and fresh weight (FW) was recorded. About 5 g tissue/vessel was placed in paper envelopes and dried in a convection oven at 60 °C for 48 h, at which time DW was recorded. Dry

weight was estimated as the product of FW and relative dry weight (RDW). Sucrose use was expressed as percent sucrose in initial formulation less percent sucrose in spent medium.

The remaining plants were moved to the greenhouse mist bed and planted in Fafard 3B potting media (Fafard, Anderson, S.C.). After 2 weeks under mist, plants were moved to a standard greenhouse bench and irrigated by hand as necessary for another 4 weeks. After a total of 6 weeks ex vitro, survival and whole plant FW was determined.

The experiment was a completely randomized design with four genotypes, two BA concentrations, two ANC concentrations, two sucrose concentrations, and two explant densities (4 $\times$ 2 $\times$ 2 $\times$ 2 $\times$ 2) replicated three times (three vessels of each) in a factorial arrangement. Data were analyzed in JMP as analysis of variance (version 3.2.6; SAS Institute, Cary, N.C.). Hypothesis testing was conducted at $P = 0.001$.

Results and Discussion

Genotypes varied in their FW, DW, and sucrose use. The diploid 'Barbara Mitchell' and the tetraploid 'Mary's Gold' had the greatest growth, 'Heart of a Missionary' (tetraploid) was intermediate, and the diploid 'Brocade Gown' had the least growth based on FW or DW (Table 1). 'Barbara Mitchell' produced the largest plants in the laboratory (as measured in FW per plant). The individual genotype greatly affected growth, but ploidy level was not an important factor.

With all four genotypes in all quantitative treatment combinations, the variation in tissue FW and media use showed a linear relationship ($R^2 = 0.80$, $P < 0.0001$), where FW increased 0.85 g for every milliliter of media used (Fig. 1A). Murashige and Skoog medium with 90 mM or 180 mM sucrose is $\approx$ 97% water by volume and has a calculated

Table 1. The main effects of genotype on plant growth and media use after 35 d of in vitro growth and 42 d of subsequent growth in a greenhouse.

Genotype	Ploidy	Fresh wt. (g/vessel)	Dry wt. (g/vessel)	% Dry wt	Sucrose use (% original)	Laboratory plant size ^a (no. observed)	Greenhouse plant size ^a (no. observed)
Barbara Mitchell	2 n	9.1 a	1.07 a	12.6 a	83 a	2.1 a (261)	2.7 b (85)
Mary's Gold	4 n	8.2 a	0.99 a	12.8 ab	67 ab	1.6 b (305)	4.6 ab (110)
Heart of a Missionary	4 n	6.4 b	0.82 b	14.5 ab	65 ab	1.3 b (268)	6.4 a (59)
Brocade Gown	2 n	4.8 b	0.64 c	14.7 b	56 b	1.1 b (242)	3.8 b (63)

^aPlant size was reported in grams per plant.

a, b, c represent difference of treatment means within columns that were significant by Tukey's HSD at $P = 0.05$.

Fig. 1. (A–C) Volume of media used, residual sucrose concentration, and quantity of sucrose used correlated with fresh, dry, and percent dry weight after 35 d of in vitro growth of four genotypes of daylily at two concentrations of benzyladenine, two concentrations of sucrose, and two plant densities.

density of 1.00 to 1.01 g·mL⁻¹ (dependent on sucrose concentration). Therefore, water use by plant (FW – DW) can be equated with media use on a volume basis (1 mL medium = 1 g FW – DW). After adjusting for the 2 mL of evaporated water/vessel during the culture period, 80% to 85% of the media volume used was incorporated as tissue FW. The 10% to 15% of media use that did not result in FW may have transpired as water, condensed on the vessel, or was otherwise uncounted. In the 5% of the vessels with greatest growth (>15 g FW), 47% of the media volume remained. Therefore, the volume of water supplied in media was ample to support growth of *Heimerocallis* spp. in the density ranges tested. The higher density of explants in media could be retained in scale-up.

There was a linear relationship between concentration of sucrose residual in remaining medium and percent RDW of plantlets at time of harvest ($R^2 = 0.55$, $P < 0.0001$; Fig. 1B). For *Heimerocallis* spp., each 1% increase in residual sucrose concentration was correlated to a 1.8% increase in RDW over the range of 7% to 22% (95% confidence interval). In the 5% of the vessels with the greatest growth (>15 g FW) and conventional (90 mM) sucrose concentration, less than 0.2% sucrose remained as residual, and tissue was 6.9% RDW. The vessels with greatest growth (>15 g FW) from high initial sucrose (180 mM) had 2.1% sucrose residual and 12.0% RDW. Therefore, in the vessels with the most vigorous growth, a higher sucrose formulation was required to maintain a high relative dry matter content (6.9% vs. 12.0% RDW). Because optimization proceeds to establish greater biomass in defined medium volumes, a higher sucrose concentration would be needed to maintain adequate plant quality.

Primarily, FW gain during heterotrophic plant culture is the result of the uptake of water, and the DW gain is mainly the result of the uptake of sugar and inorganic ions. Ibaraki and Kurata's (1998) heterotrophic growth model shows that water uptake and sugar uptake depends upon the water potential difference between the plantlet and medium. When sugar becomes depleted from medium, plants continue to grow by taking on more water relative to soluble solids. High initial sugar concentrations allowed high-density cultures to maintain high relative dry matter content while producing large amounts of biomass. Residual sugar concentration of in vitro medium was a good index of the plants water status at the time tissue was removed from the vessel.

Dry weight increased with sucrose use, and a 0.26 g/vessel increase in DW was correlated with each 1% of sucrose used (Fig. 1C). One percent sucrose has a mass of 10 g·L⁻¹ that, when assimilated, yielded 7.4 g·L⁻¹ in tissue DW. Therefore, *Heimerocallis* spp. in liquid medium fixed 74% of the sucrose mass as dry matter, with the remainder exhausted as CO₂ and water, or remaining otherwise uncounted. Plant cells have roughly 50% conversion efficiency of organic

carbon feed to final cell DW in bioreactor systems (Curtis, 1999), so we may conclude that the daylily plantlets in liquid MS medium were a relatively efficient system.

In vitro ANC treatment reduced the plantlet FW and the size of the leaf blades (Fig. 2). Reduced leaf size made it easier to divide plantlets, with less discarded materials speed-

ing the technician's processing time (Adelberg et al., 2005). There was no relationship between FW, DW, RDW, and the survival of plants in the greenhouse. Although plantlets of 'Barbara Mitchell' were largest in laboratory, greenhouse plants of 'Barbara Mitchell' that survived transfer were often smaller than the greenhouse-grown plants of

Fig. 2. Daylily cultivar Barbara Mitchell at (A) 57 explants/L (two explants/vessel) and (B) 171 explants/L (six explants/vessel) are shown after 35 d of culture on orbital shaker. Ancymidol treatments are to the right of the vertical bisector, high sucrose treatments are below the horizontal bisector, and high benzyladenine treatments are in the center of the diamond.

Table 2. Ancymidol and benzyladenine (BA) concentration during 35 d of in vitro growth affects the survival rate (percent) of plantlets in the greenhouse 42 d later.

	No ancymidol	Ancymidol (0.32 μ M)
0.32 μ M BA	93 a	52 b
3.2 μ M BA	60 b	4 c

a, b, c represent difference of treatment means that were significant by Tukey's HSD at $P = 0.05$.

the other three genotypes (Table 1). Plants of 'Mary's Gold', 'Brocaded Gown', and 'Heart of a Missionary' had more than tripled in mass in the greenhouse, whereas 'Barbara Mitchell' had increased only about 1.3 times in 6 weeks.

Ancymidol and BA had negative effects on greenhouse survival. Plants grown without ANC and a low concentration of BA had a good rate of survival (93%; Table 2). High BA concentrations, with ANC produced plantlets that were poorly suited for survival (4%). Treatment with ANC at low BA concentrations, or high BA concentrations in the absence of ANC produced intermediate levels of survival in the greenhouse (52% and 60% respectively). Ancymidol and higher BA concentrations also reduced the FW of greenhouse plants, the number of storage roots, and the length of the leaf blades (data not presented). In general, the adverse carry-over effects of BA on rooting of plantlets and their survival ability in the greenhouse should be carefully evaluated when selecting cytokinin (Kane, 2005). Ancymidol carryover also needs to be carefully assessed during micropropagation protocol development.

Multifactor experiments are useful at the exploratory stage of an investigation for examination of large numbers of factors and their interactions, with a reasonably sized trial. The limited amount of tissue available during scale-up to a bioreactor process is a circumstance during which small trials must address a large numbers of factors. Information gained during shaker culture bulk-up can rationally guide bioreactor-scaled process development. In this trial it was shown with *Hemerocallis* spp. that 1 mL medium is required for each 0.85 g FW of tissue harvest anticipated (bracketed with an appropriate confidence interval). Similarly, 1 g sucrose is required for each 0.74 g DW of tissue harvest anticipated. There were no direct relationships between FW, DW, or sucrose use, and subsequent greenhouse survival or growth. Certain factors like genotypic

variation need to be empirically determined. 'Barbara Mitchell' had the largest plantlets in the laboratory but grew poorly in the greenhouse. A scaled-up micropropagation bioreactor process should include greenhouse trials of each genotype, because varieties with superior laboratory performance may become problematic in the nursery.

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