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Sucrose concentration in liquid media affects soluble carbohydrates, biomass and storage quality of micropropagated hosta

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Abstract

The effects of sucrose concentration (1, 3, 5, or 7% w/v) in liquid media, in the presence and absence of benzylaminopurine (BAP), on internal carbohydrate status and growth of *Hosta tokudama* Tratt.'Newberry Gold' during the multiplication phase (stage II) was investigated. Cultures from all treatment combinations were transferred to media containing 3% (w/v) sucrose during the rooting phase (stage III). At the end of the stage III, these micropropagules were subjected to 5 weeks of storage at 10 °C under low light (photosynthetic photon flux of 5 μmol $m^{-2}s^{-1}$). Endogenous concentrations of soluble sugars (glucose, fructose, and sucrose) in the plantlets increased linearly as the media sucrose concentration increased from 1% to 7% during stage II. Root and shoot biomass increased with increasing media sucrose concentration. BAP increased the biomass and multiplication rate but did not affect internal concentration of soluble sugars. While in storage, endogenous sugar levels and plantlet dry weight remained unchanged for all treatments. Following storage, plants originally cultured in 5% and 7% media sucrose had higher dry weight and less leaf chlorosis than those cultured in 1% and 3% media. Differences in endogenous soluble sugar levels at the end of stage III rooting, and after storage were related to the sucrose concentration of the initial stage II multiplication medium. Increased media sucrose levels during the multiplication cycle has a positive, long-term effect on plant morphology and quality.

Introduction

Hostas are shade-tolerant, hardy, perennial plants native to East Asia. Valued for variations in leaf-size, texture and variegation pattern, hostas have been rediscovered in the modern landscape (Armtiage, 1997). Breeding programs have led to the introduction of numerous new cultivars with micropropagation effectively decreasing the time to market. Successful commercial micropropagation techniques have increased availability and decreased prices for selected, superior cultivars.

Maintenance of micropropagules in active growth necessitates frequent subculture making labor the most expensive component of commercial micropropagation (Chu, 1995). Demand for plants is seldom distributed evenly through the year, either from climatic considerations for the crop, or due to seasonal markets for the customer. It is impractical for a commercial laboratory to train seasonal labor for production peaks, and discarding unsold products off-season would cause heavy financial loss. Storage of *in vitro* plants through slower seasons is a desired option to manage seasonal cycles.

Low temperature storage has been the most popular method of plant storage (Bajaj, 1991). Low temperatures reduce active growth and subsequently delay the necessity for subculture. *In vitro* plants of chrysanthemums (*Chrysanthemum morifolium* Ramat.) and Petunia (*Petunia hybrida* Vilm.) were stored at 4–5 °C for up to 6 years (with occasional exposure to light in the culture room) and flowered after being trans-

planted to pots (Bajaj, 1986). Storage in darkness reduces growth rate of cultures and subculture frequency. However, low light during short-term storage was shown to preserve the photosynthetic potential and increase re-growth capacity in broccoli (*Brassica oleracea* L. Botrytis group 'Ryokurei') (Kubota et al., 1994). Hosta plantlets stored in a medium without sucrose did not recover following 4-week dark storage at 5 °C, but plantlets recovered following 12 weeks at 5 °C under 5–7 μmol $m^{-2}s^{-1}$ of light. Also, Hosta plantlets cultured in media containing 2% sucrose survived after 12 weeks of dark storage at 5 °C (Wilson et al., 1998). These results show that providing a carbohydrate source during storage, either in media or through photosynthesis, is critical for survival of plantlets during low temperature storage.

Tissue cultures are grown most often on agar medium with 3% sucrose as the carbohydrate source. Liquid culture micropropagation systems have been reported to increase plant quality and lower production costs in many different crops (Smith and Spomer, 1995; Etienne and Berthouly, 2002). Hostas grown in liquid culture had greater dry weight and faster nursery growth than those cultured on agar (Adelberg et al., 2000).

The present research investigated the effects of sucrose at varying concentrations (1–7% sucrose) in liquid cultures of Hosta 'Newberry Gold' in the presence or absence of BAP, a commonly used growth regulator for micropropagation. Plant quality was measured in terms of dry weight, soluble carbohydrate concentration, and ability to withstand 5 weeks of storage in dim light at 10 °C. Long-term effect on plant quality and soluble carbohydrate status subsequent to storage was observed.

Methods and materials

Plant material and culture conditions

Uniform, stage II *Hosta tokudama* Tratt 'Newberry Gold' buds were obtained from a commercial laboratory (Southern Sun Propagation Systems, Norris, SC) and were transferred to 180 ml glass jars with Magenta B-cap enclosures (Magenta Corp., Chicago IL). Each jar contained 35 ml of liquid culture medium. Murashige and Skoog (1962) medium was modified by addition of 4.5 mM NaH_2PO_4 and 0.5 mM adenine hemisulfate. The culture treatments were comprised of 8 factorial combinations of sucrose (1%, 3%, 5%, or 7% w/v) and BAP (0 or 3 μM). Seven jars with 6

Table 1. Effects of media sucrose concentration during stage II culture on shoot and root glucose, fructose, sucrose, and total soluble sugars (TSS)

Sucrose in stage II medium (%)	Glucose (mg g^{-1})	Fructose (mg g^{-1})	Sucrose (mg g^{-1})	TSS (mg g^{-1})
		Shoot		
1	26.1	18.6	5.9	50.6
3	47.8	42.9	8.0	98.8
5	80.4	70.4	15.0	165.8
7	103.7	97.1	17.0	217.2
Contrasts[1]				
Linear	***	***	***	***
Quadratic	ns	ns	ns	ns
		Root		
1	–[2]	–	–	–
3	36.2	36.5	10.3	83.0
5	54.5	51.0	11.0	116.6
7	63.0	56.1	13.1	132.3
Contrasts[1]				
Linear	***	***	ns	***
Quadratic	*	*	ns	*

[1]ns, * or *** indicates non-significant, significant at $p = 0.05$ or 0.001.
[2]Root sample was not sufficient for sugar analysis.

Table 2. Comparison of total soluble sugar content in shoots at the end of stage II, end of stage III (prior to storage) and end of storage

Sucrose in stage II medium	Total soluble sugars (mg g^{-1})		
	End of stage II	End of stage III	End of storage
1%	55.2 d B[1]	87.7 b A	71.8 b AB
3%	89.7 c A	96.0 b A	92.5 ab A
5%	172.4 b A	102.7 b B	94.9 ab B
7%	217.0 a A	141.9 a B	109.1 a B
Contrasts[2]			
Linear	***	**	*
Quadratic	ns	*	ns

Plants were grown in 1%, 3%, 5%, or 7% media sucrose during stage II for 5 weeks and transferred for rooting (stage III) in media with 3% sucrose for 4 weeks and subsequently stored at 10 °C under 5 μmol m^{-2} s^{-1} of PPF for 5 weeks. Each number represents the mean of 3 culture vessels with 9 plantlets.
[1]Mean separation within columns or rows by LSD at $p = 0.05$. Means with the same letter are not significantly different. Lower case letters indicate the mean separation within a column and upper case letters indicate the mean separation a within row.
[2]ns, *, **, *** = non-significant or significant at $p = 0.05$, 0.01, 0.001 respectively.

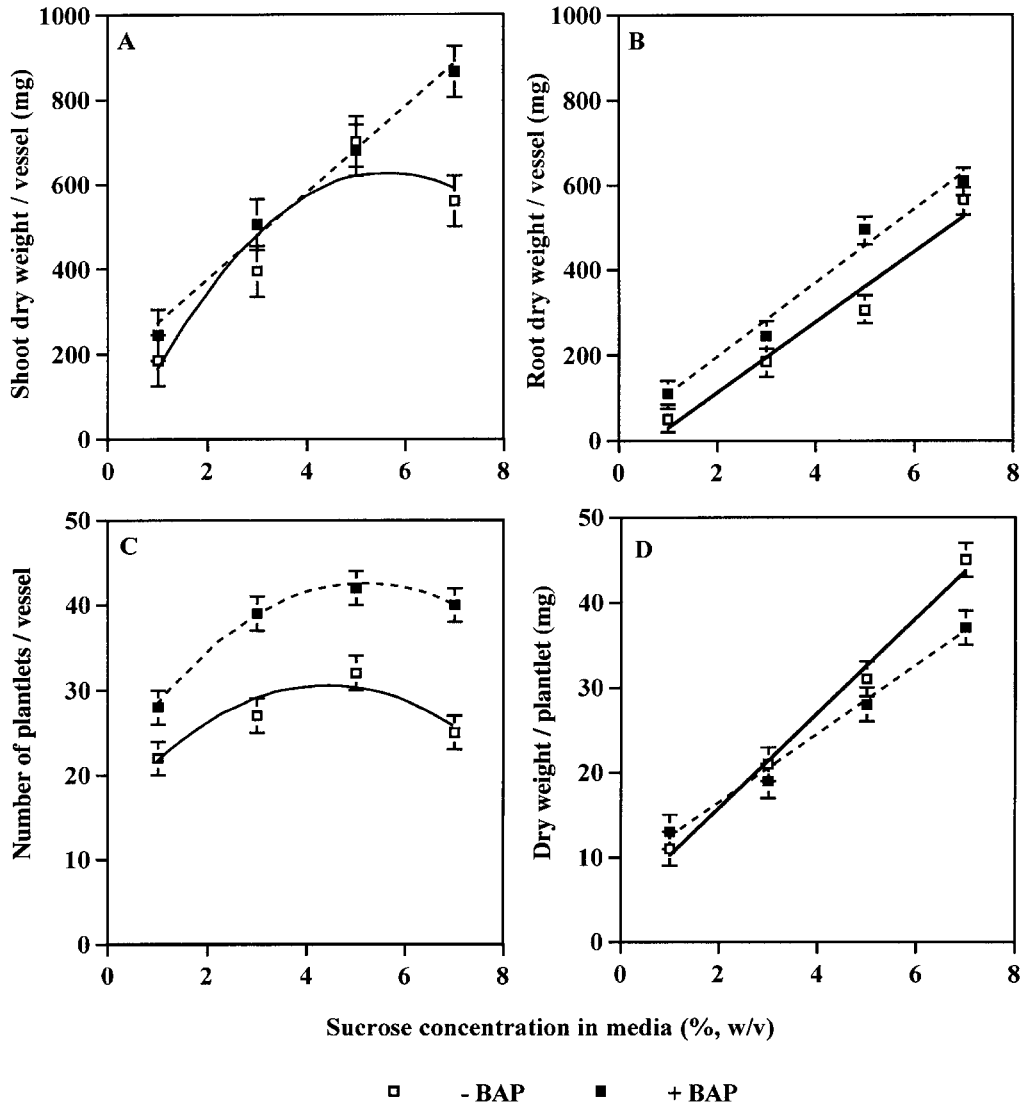

Figure 1. Effects of media sucrose concentration and presence or absence of BAP during stage II on shoot biomass (A), root biomass (B), number of plantlets (C), and average dry weight per plantlet (D) at harvest. Stage II cultures were grown in 1%, 3%, 5%, or 7% media sucrose with or without BAP for 5 weeks. Vertical bars indicate S.E.

buds in each were initiated for each of the 8 treatment combinations. The cultures were placed on an orbital shaker (90 rpm) and maintained for 35 days at 25 ± 2 °C, under photosynthetic photon flux (PPF) of about 20 μmol $m^{-2}s^{-1}$ (from cool white fluorescent light) with a 16-h photoperiod. At the end of stage II, samples from each treatment combination were harvested, frozen and stored at −80 °C for soluble carbohydrate analysis. Sufficient root samples were not available from the 1% sucrose treatment at the end of stage II for carbohydrate analysis. Therefore, this treatment was considered only for dry weight determination. Dry weights of the sampled micropropagules were recorded after freeze-drying.

Remaining plantlets were transferred to stage III where they were cultured in liquid medium on Sorbarod plugs (Ilacon Industries, UK) as supporting material, in Magenta boxes GA7 (Magenta Corp., Chicago, IL). Each box contained 9 plugs and 40 ml of MS basal medium with 3% sucrose, without BAP. Six magenta boxes were prepared from each of the 8 stage II culture combinations. Stage III cultures were maintained for 4 weeks under the same conditions of

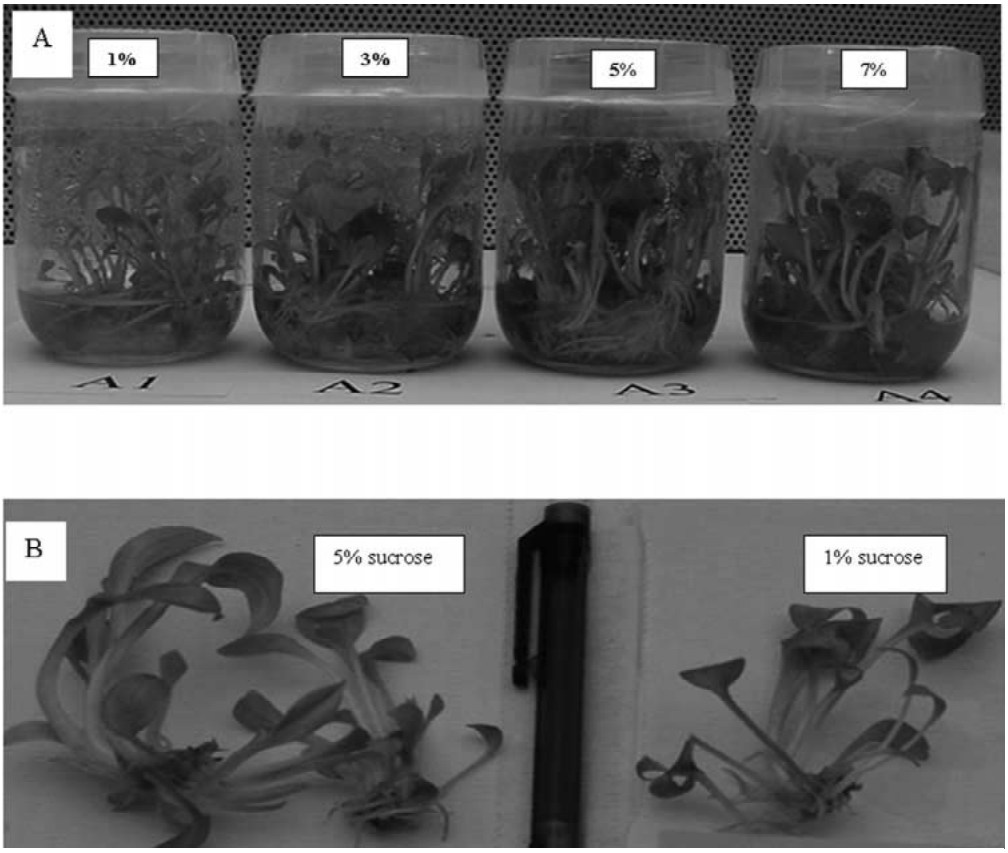

Figure 2. Influence of media sucrose concentration on biomass increase per vessel (A) and size of individual plantlets (B) at the end of stage II. Stage II cultures shown here were grown in 1%, 3%, 5%, or 7% media sucrose without BAP for 5 weeks.

Table 3. Percentage leaf chlorosis at the end of storage. Plants were grown in 1%, 3%, 5%, or 7% media sucrose during stage II for 5 weeks and transferred for rooting (stage III) in media with 3% sucrose for 4 weeks and subsequently stored at 10 °C under 5 μmol m^{-2} s^{-1} of PPF for 5 weeks. Each number represents the mean of 3 culture vessels with 9 plantlets

Sucrose in stage II medium	Leaf chlorosis (%)
1%	50
3%	48
5%	21
7%	24
Contrasts[1]	
Linear	**
Quadratic	ns

[1]ns or **indicates non-significant or significant at $p = 0.01$, respectively.

light and temperature previously described. At the end of stage III, three culture vessels from each treatment were harvested, plant material frozen and stored at −80 °C for dry matter and carbohydrate determination. The remaining three vessels were taken to storage in the same culture vessel with the partially depleted medium from stage III. The cultures were stored at 10 °C under continuous light from cool fluorescent lamps with a PPF of 5 μmol $m^{-2}s^{-1}$. At the end of 5 weeks of storage, the percentage of chlorotic leaves was recorded and the plants were harvested for dry matter and carbohydrate content determination.

Soluble carbohydrate analysis

Following visual quality assessment, shoots and roots were separated, frozen in liquid N_2, freeze dried and dry weight was recorded. Shoots and roots were separately ground and soluble sugars from 25 mg of plant tissue were extracted overnight with methanol: chloroform: water (12:5:3, by volume) and prepared as described by Miller and Langhans (1989). Sucrose, glucose, and fructose were separated using a Dionex DX-300 High Performance Liquid Chromatography

system with a 4 × 250 mm CarboPac^{TM} column and detected with an electrochemical detector (Dionex, Sunnyvale, CA). Quantification of above sugars was based on the calibration curves obtained from their respective standards. Starch content was determined by enzymatic hydrolysis of starch (using amyloglucosidase, Sigma Chemicals, St. Louis, MO) into glucose (Haissig and Dickson, 1979).

Experimental design and statistical analysis

The experiment was conducted as a factorial arrangement in a completely randomized design. The two factors were sucrose (1%, 3%, 5%, or 7%) and BAP (present or absent). Culture vessels were placed randomly on the shaker during stage II and on the culture racks during stage III. Five culture vessels of each treatment combination were used for the determination of dry weight and number of buds at the end of stage II. One culture vessel from each of the 8 treatment combinations was used to obtain five samples for extraction and subsequent carbohydrate analysis after stage II. To determine the dry matter and carbohydrate status of the pre-storage and post-storage micropropagules, three culture vessels of each treatment were used at the end of stage III and storage, respectively. Data were analyzed using analysis of variance (general linear modes procedure of SAS software, SAS Inst. Inc., Cary, NC). Linear contrasts were used to determine the order of polynomials to fit the data. Means for measurements showing significant differences in the analysis of variances were tested using Fisher's least significant difference (LSD).

Results

The presence or the absence of BAP did not significantly affect shoot or root soluble sugar accumulation. Therefore, the data were pooled for the presentation. Shoot and root soluble sugar concentrations (glucose, fructose, and sucrose) increased linearly across the media sucrose concentrations tested (Table 1). Shoots from plants grown in 7% sucrose medium had approximately 4× more soluble sugars than those cultured in 1% sucrose medium. In all treatments, most of the endogenous sugars were glucose and fructose, with sucrose concentration being much smaller. Soluble sugars were less in roots than shoots. In plants cultured in medium containing 3% sucrose, shoots had ∼20% more total soluble sugars than roots. The mean difference increased linearly to ∼60% when shoots and roots from 7% sucrose medium were compared ($p = 0.0045$, $R^2 = 0.89$). Inadequate root growth in 1% sucrose did not allow root sugar determination to be made. During multiplication, the shoots were more active carbohydrate sinks than the roots, based on greater accumulation of soluble sugars in shoots. Starch concentrations were relatively small and sucrose concentrations in medium did not affect starch concentration in the shoots or in the roots (data not shown).

Shoot biomass (dry weight per vessel) increased linearly as the sucrose concentration increased in medium from 1% to 7% when BAP was present. In the absence of BAP, shoot biomass followed a quadratic relation and did not increase with sucrose concentration in medium beyond 5% (Figure 1A). Root biomass increased linearly with sucrose concentrations in the medium, both in the presence and absence of BAP (Figure 1B). BAP had increased the shoots physiological sink strength allowing plants in the 7% sucrose medium to be most effective in converting sugars to shoot dry matter. BAP is known to increase cell division, break apical dominance and effect source/sink relations for carbohydrates (D'Agostino and Kieber, 1999). The average number of buds produced per vessel was greater in the presence of BAP. The number of buds produced per vessel followed a quadratic relationship with greatest rate of multiplication occurring at about 5% sucrose in the presence or absence of BAP (Figure 1C). Individual plantlets were larger with greater dry weights in the cultures grown with greater levels of sucrose (Figures 1D, 2A, B).

Plantlets cultured in 1% sucrose media during stage II had an increase (59%) in total sugars at the end of stage III (before storage) due to the higher concentration of sucrose in the medium (3%) during stage III (Table 2). In plantlets cultured in 3% sucrose medium during stage II, the total shoot soluble sugar concentration remained unchanged during stage III. In plantlets cultured in 5% or 7% sucrose, shoot soluble sugar levels decreased by about 40% during stage III. The differences in endogenous total soluble sugar concentration was somewhat diminished during the three weeks on 3% sucrose rooting medium (e.g. ratios of total soluble sugars between the 1% and 7% treatments being reduced from 4× to 1.6×). However, the total soluble sugar concentrations following stage III rooting were still related to the sucrose concentration from prior stage II multiplication treatments. Soluble

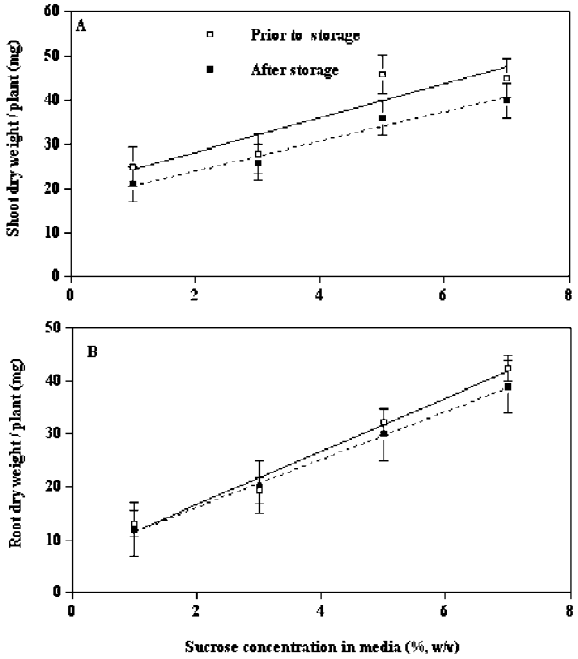

Figure 3. Shoot and root dry weight prior to and after storage. Plants were grown in 1%, 3%, 5%, or 7% media sucrose during stage II for 5 weeks and transferred for rooting (stage III) in media with 3% sucrose for 4 weeks and subsequently stored at 10 °C under 5 μmol m^{-2} s^{-1} of PPF for 5 weeks. Each number is the average of 3 culture vessels with 9 plantlets. Vertical bars indicate S.E.

sugar levels did not change during 5 weeks storage at 10 °C (Table 2). A linear relationship between sucrose in the media at stage II and endogenous soluble sugars following storage was maintained for eight weeks (3 weeks rooting and 5 weeks storage).

During storage, shoot and root biomass did not change significantly (Figure 3). There was a linear relationship between sucrose concentration in medium during multiplication phase and shoot, and root biomass subsequent to storage (Figure 3). The difference in shoot dry weight between plants cultured in 1% and 7% sucrose was 1.6× and the difference in root dry weight was 3×. Plantlets cultured in 1% or 3% had ∼50% leaf chlorosis compared to ∼20% leaf chlorosis in the plantlets cultured in 5% or 7% media sucrose (Table 3). Overall post-storage visual quality was poor in plantlets cultured in media containing lower sucrose concentrations (1% and 3%), which displayed wilted leaves and petioles (data not shown). Sucrose uptake during *in vitro* multiplication had a long-term benefit on plant size and quality after storage.

Discussion

Most often micropropagation of hosta uses agar medium with 3% media sucrose. Growth (dry weight), multiplication (plantlets per vessel) and increased concentrations of soluble sugars at the end of stage II were observed in the cultures with greater sugar concentration (e.g. 5% and 7%). A positive correlation between sucrose in the medium and dry matter content in *in vitro* plants has been reported previously (Capellades et al., 1990). In our current work at the rate of 170 explants per liter of medium, 5% sucrose was optimal for shoot bud multiplication. Although optimal multiplication occurred at approximately 5% sucrose, dry matter continued to increase over all ranges of sucrose tested in the presence of BAP suggesting that growth was not retarded due to osmotic stress.

During stage III rooting, endogenous soluble sugar concentration decreased in plantlets previously grown in 5% and 7% sucrose media when transferred to lower sugar concentration (3%) in the culture medium. In plantlets cultured in 1% sucrose media, an increase in internal sugar levels took place during stage III growth. The cultures from 5% and 7% sucrose treatments also had greater shoot and root dry weight per plant at the end of stage III compared to cultures from 1% and 3% sucrose treatments. Increased availability of sugars in heterotrophic systems has been shown to increase cellulose synthesis, which was correlated to increase in dry weight (Amor et al., 1995; Babb and Candice, 2001). Similarly, a considerable part of the increased dry weight gain in our work with elevated sucrose concentrations may be associated with structural carbohydrates in the cell wall, the largest component of a plants' dry weight.

Culturing buds in 5% and 7% sucrose medium during stage II pre-disposed plantlets for superior post-storage plant quality while plantlets from 1% and 3% sucrose medium had greater foliar chlorosis following storage. Soluble carbohydrate content of the shoots during storage was somewhat related to enhanced post-storage plant quality.

Culturing buds in high concentration media sucrose (i.e. 5%) during stage II led to an increased concentration of endogenous soluble sugars, greater plant multiplication and dry weight, and overall quality of *in vitro* grown hosta plants. The presence of BAP did not change the concentration of total soluble sugars, however it led to enhanced growth and proliferation. Subjecting the plants to greater concentrations of sucrose in medium contributed to plantlet quality

during stage II and enhanced dry weight (per plantlet) during stage III leading to post-storage plantlets with greater biomass and less chlorosis. We conclude that increased sucrose concentration in stage II media has a lasting impact on plant quality following storage. This should help laboratory managers to better handle seasonal demand.

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