

EFFECTS OF ROOTING PERIOD, CLUMP SIZE, AND GROWTH MEDIUM ON SUGARCANE PLANTLETS IN MICROPROPAGATION DURING AND AFTER TRANSFORMATION TO PHOTOAUTOTROPHY

H. Erturk, P. N. Walker

ABSTRACT. Experiments were conducted to improve the conditions for growth of sugarcane (*Saccharum spp.*) plantlets during and after transformation to photoautotrophy. The experimental treatments included different rooting periods, media, and clump sizes. Photomixotrophic (sugar-containing medium) and photoautotrophic (sugar-free medium) cultures were also compared. The presence of roots was concluded to be essential for plantlet survival during transformation from photomixotrophic to photoautotrophic culture. Six-week rooted plantlets were fully capable of surviving photoautotrophic conditions after transfer whereas three- and four-week rooting periods were insufficient. The test of clump size during transformation to photoautotrophy showed that two plantlets per clump resulted in the highest growth, but insignificantly so, when tested against four plantlets per clump and individual plantlets. Comparison of different sugarless medium types for the photoautotrophic growth of plantlets showed that liquid Murashige and Skoog (MS) medium had the highest growth among other liquid media tested but the differences were not statistically significant. Similarly, gelled MS medium gave insignificantly higher growth rates when compared with liquid MS media. Liquid MS medium was concluded to be suitable for photoautotrophic growth of sugarcane plantlet cultures. The experiments of this study achieved photoautotrophic cultures growing in weight and in number of plantlets, though at a much slower rate than for photomixotrophic cultures. This study is the first time photoautotrophic multiplication of any plant has been demonstrated in micropropagation and further optimization of culture conditions is needed.

Keywords. Shoot culture, Media, Photomixotrophic.

Micropropagation of sugarcane is usually done in three stages. In Stage 1, a small piece of meristematic tissue is taken from a mother plant, sterilized, and cultured aseptically to produce a micropropagule or explant. These propagules are usually multiplied to form a clump of shoots which are separated, placed in fresh multiplication medium and multiplied again over several generations in what is called the multiplication stage (Stage 2). The shoots are then placed in a rooting medium (Stage 3). The rooted plants are transferred to a greenhouse, acclimated to soil growth and outdoor weather conditions (Stage 4), and then transplanted in the field (Walker et al., 1993). Herein medium is used to mean the nutrients and other active ingredients plus water, sometimes combined with a gelling agent.

Photoautotrophic micropropagation, in which plants utilize the energy of light for photosynthesis, has been presented as an alternative for conventional photomixotrophic micropropagation in which plants utilize sugar in the medium as an energy source. Many advantages of growing shoots photoautotrophically were reported by Kozai (1991); most important is that photoautotrophic

micropropagation reduces the loss of cultures due to bacterial, fungal or other biological contamination, which occurs often in conventional micropropagation partly because of the sugar in conventional media.

The general objective of the research presented in this article was to improve the conditions for photoautotrophic micropropagation of sugarcane plantlets, both during and after transformation. The research is part of a longer term effort to develop a septic system for photoautotrophic micropropagation of sugarcane plantlets. Effects of rooting period, clump size, and types of growth media on the growth of plantlets during and after transformation to photoautotrophy were the factors of interest in this study. The term plantlets is used in this study to mean shoots with roots.

The presence of roots is considered to be an important factor in development of photosynthetic ability in plants. Serret et al. (1996) observed that *Gardenia jasmonides* plantlets from the root induction period had more developed photosynthetic characteristics than those from the shoot multiplication period. For both stages (i.e., rooting and multiplication), low sucrose medium stimulated the photoautotrophy of the plantlets *in vitro*.

The clump size and the number of clumps per vessel are important factors for micropropagation and so may be important during transformation to photoautotrophy. The effect of plant or "inoculation" density on shoot and root growth and on protocorm development has been reported for a number of crop species including asparagus (Matsubara, 1973), *Cattleya* (Pierik and Steegmans, 1972), and potato micropropagation (Sarkar et al., 1997).

There are many photoautotrophic growth studies conducted with different medium types. In a study by

Article has been reviewed and approved for publication by the Biological Engineering Division of ASAE. Presented as ASAE Paper No. 98-4160.

The authors are **Handan Erturk**, Assistant Professor, Food Engineering, Izmir Institute of Technology, Izmir 35230, Turkey, and **Paul N. Walker**, *ASAE Member Engineer*, Professor, Agricultural Engineering, Department of Agricultural and Biological Engineering, Pennsylvania State University, University Park, Pennsylvania. **Corresponding author:** Paul N. Walker, Pennsylvania State University, 223 Agricultural Engineering Bldg., University Park, PA 16802, phone: 814.865.4582, fax: 814.863.1031, e-mail: <pnw@psu.edu>.

Kozai et al. (1988a) on the effects of medium types on photoautotrophic growth of carnation plantlets, the growth of the plantlets in the air-leaky vessels was greater in the sugar-free, full-strength Enshi medium than in the media containing sugar with half-strength Murashige and Skoog (MS) or Enshi salts, or in the sugar-free medium with the half strength MS nutrient solutions, all under high photosynthetic photon flux (PPF). They concluded the favorable nutrient solutions for photoautotrophic growth seemed to be different than those developed for photomixotrophic growth of tissue or organs.

MS medium has been used successfully in studies with photoautotrophic cultures by Kozai et al. (1988b) with potato and Kozai et al. (1991) with strawberry plantlets. MS medium was used by other researchers for studies with photomixotrophic sugarcane cultures such as Walker et al. (1991) and Gautz et al. (1991). The optimal sucrose content in MS medium for sugarcane micropropagation was found to be 2% (Walker et al., 1991). The growth of carnation plantlets cultured in vessels with a high ventilation rate under high PPF conditions was better in a sugar-free medium with a typical greenhouse hydroponics solution than in sugar-containing medium with half strength MS salts (Kozai et al., 1990). The findings of Adelberg et al. (1997) showed that greatest fresh weight and number of plantlets of *Cattleya* per vessel were obtained in liquid MS solution at full, half, and quarter strength than in Hoagland's hydroponics medium.

Support material is an important condition for culture. Kirdmanee et al. (1995) compared the growth of *Eucalyptus camaldulensis* cultured *in vitro* photoautotrophically on Phytigel™ with other support materials. They found that the growth *in vitro* was the greatest for vermiculite, followed by the plastic net, Phytigel and agar matrix both with and without CO₂-enrichment of atmosphere.

Although there have been many studies on photoautotrophic micropropagation, only a few such studies involved sugarcane (Walker et al., 1991; Erturk, 1998; Erturk and Walker, 2000). Walker et al. (1991) found that ventilation with CO₂ enriched air did not improve plant growth with or without sucrose in the medium and they were not able to achieve photoautotrophic culture. The effects of light, CO₂ and plant growth regulator levels on the growth of sugarcane shoots during transformation to photoautotrophy were studied in our laboratory (Erturk and Walker, 2000). Light and CO₂ levels were found to have statistically significant and positive effects on the growth of shoots during transformation to photoautotrophy. The following study is an extension of the research on photoautotrophic micropropagation of sugarcane.

MATERIALS AND METHODS

This study contains four distinct parts. The first two address the effects of rooting period and clump size, respectively, on growth of plantlets during transformation from photomixotrophic to photoautotrophic culture. Parts 3 and 4 deal with growing the cultures photoautotrophically in the generations after that transformation. Specifically, Part 3 compares medium types and Part 4 compares the growth rate of photoautotrophic cultures with conventional photomixotrophic cultures. The procedures that apply

generally are discussed here in the materials and methods section. Specific procedures as well as results are discussed in the sections for the individual parts.

GROWTH MEDIA

A stock vitamin solution was prepared with thiamine (0.1 mg l⁻¹), pyridoxine (0.5 mg l⁻¹), nicotinic acid (0.5 mg l⁻¹), glycine (0.2 mg l⁻¹), and myo-inositol (100 mg l⁻¹). Kinetin and benzylaminopurine (BAP) were prepared as stock solutions each with a concentration of 0.1 mg mL⁻¹.

Stock or Gelled MS Medium. Gelled MS medium (Murashige and Skoog, 1964) was used as the stock multiplication medium for the experiments. It was prepared as follows: sugar (20 g l⁻¹) and MS Salts (4.3 g l⁻¹; Sigma Chemicals) were dissolved in distilled water. Then, 2.0 mL vitamin, 1.0 mL kinetin, and 2.0 mL BAP from the stock solutions were put in 1 L of medium. Finally, the gelling agent Phytigel was added to the mixture (1.1-1.2 g l⁻¹).

The medium was autoclaved for 20 min at 121°C, then cooled to about 50°C and aseptically dispensed into previously sterilized 400 mL Magenta (Magenta Corp., Chicago, Ill.) vessels. The medium was left to cool and gel in the vessels. A volume of 90 to 100 mL of multiplication medium per vessel was used for stock cultures and for experiments except where noted otherwise.

Rooting Medium. Rooting medium had the same ingredients as the gelled MS multiplication medium except without the plant growth regulators, i.e., kinetin and BAP. Each vessel used for rooting received 90 to 100 mL of this medium.

Gelled Sugarless MS Medium. The sugarless MS multiplication medium was prepared in the same way as the stock multiplication medium, except no sugar was added.

GROWTH CHAMBER

The growth chamber where the photoautotrophic growth studies were conducted had a PPF of 500 ± 100 μmol m⁻² s⁻¹ (Li-Cor LI-185A quantum sensor) at the location where vessels were placed. The temperature was maintained at 31 ± 2°C.

CULTURE ROOM

The stock culture for the experiments was kept in a separate culture room throughout the study. The temperature averaged 24°C in the culture room. The light level on the shelves, measured as PPF, was provided by cool-white fluorescent lamps with approximately 35 to 85 μmol m⁻² s⁻¹ measured at various locations on the shelves. No effort was made to enrich the atmosphere or increase natural ventilation of the culture room, growth chamber, or the Magenta vessels.

STOCK CULTURE

The stock culture was sugarcane cultivar 85-845 (Crop Genetics, Baton Rouge, Louisiana). Sugarcane shoots were multiplied (Stage 2 of micropropagation), according to the procedures applied by Crop Genetics. The steps in the subculturing procedure were taking the shoot clumps out of their vessels, trimming the tops, gently cutting the clumps with large numbers of shoots into clumps with three to five shoots using a scalpel and transferring these into vessels

with fresh medium. Two to four clumps were placed in each vessel for the stock cultures. The shoots typically were moved to fresh medium within two weeks and subcultured within a month. In moving the shoots to fresh medium, all the shoots from each vessel were moved to a vessel containing fresh medium, working under an aseptic hood and disturbing the shoot clumps as little as possible. Both the stock and the experimental cultures were maintained under aseptic conditions.

PART 1: EFFECTS OF ROOTING PERIOD ON THE GROWTH OF PLANTLETS DURING TRANSFORMATION TO PHOTOAUTOTROPHY

Preliminary observations seemed to indicate that photomixotrophic shoots could be transformed to photoautotrophy only if they had roots. The objective here was to find the appropriate rooting period to enable transformation to photoautotrophy. The treatments for this were three-, four- and six-week rooting periods.

EXPERIMENTAL PROCEDURE

Gelled multiplication and rooting media were used. The plantlets were subcultured into stock multiplication medium about one and one-half months before the experiment and many shoots had some tiny roots on them already. The plantlets were again subcultured at the start of the experiment and divided into three groups, each initially with nine vessels. Each vessel received approximately equal distributions of shoot size and total weight of plantlets and as a result each vessel contained four or five plantlet clumps with three or four plantlets per clump. One group of vessels was transferred immediately into rooting medium and two groups were placed into stock multiplication medium for later rooting.

The second group was transferred to the rooting medium two weeks later and the third group, less one contaminated vessel, was transferred after one more week. The experiment then continued for an additional three weeks. As a result, the first group was in rooting medium for six weeks, the second for four weeks, and the third for three weeks. All plantlets were then transferred to and kept in gelled sugarless MS medium for a period of two weeks.

Rooting and multiplication media were changed each week. The tops of the plantlets were trimmed if the plantlets had grown enough to reach the vessel lids in order to reduce the possibility of contamination caused by the long leaves touching septic surfaces outside the vessels during handling. Fresh weight data were taken at the beginning and end of the experiment. At the end of the experiment photoautotrophy was judged by the healthy green color of the plants. Vessels with pale green or brown plants were judged not to have achieved photoautotrophy.

RESULTS AND DISCUSSION

It was observed from this experiment that all nine of the vessels for the six-week rooting treatment were able to develop photoautotrophy, based on their continued survival and healthy green color in sugarless medium. Four of the nine vessels from the four-week rooting treatment were

Table 1. Comparison of different rooting periods

Treatment	N†	Average Initial Fresh Weight (g)	Average Final Fresh Weight (g)	Average Growth* (%)	Median (%)	S.D. (%)
6-week rooting	9	1.17	2.67	132	111	48
4-week rooting	9	1.02	1.87	78	83	40
3-week rooting	8	1.02	1.29	26	26	25

* Growth p-value: 0.000

† N = number of vessels (samples) at end of experiment.

successful and none of the eight vessels from the three-week rooting treatment developed photoautotrophy.

The fresh weight results from the rooting test are shown in table 1. The growth (percent increase in fresh weight) was found to average 132, 78, and 26% for the six-, four- and three-week rooting treatments, respectively. These results were consistent with the visual photoautotrophy observations. ANOVA (Minitab Software ver. 11) was used to test the treatment effect on the growth of plantlets. The effect of rooting period on the growth of plantlets was found to be statistically significant at the 95% level of significance (i.e., $p < 0.05$), so it was concluded that a six-week rooting period was best.

The finding that longer rooting periods resulted in more successful transformation to photoautotrophy supports the theory that roots are essential for the plantlets to develop photoautotrophy. These results are in agreement with Serret et al. (1996). They observed that plantlets from the root induction period had more developed photosynthetic characteristics than those from the shoot multiplication period. Rooted plantlets in our study apparently developed photosynthetic abilities which helped them survive and grow in sugarless medium.

PART 2: EFFECTS OF CLUMP SIZE ON THE GROWTH OF PLANTLETS DURING TRANSFORMATION TO PHOTOAUTOTROPHY

As a general observation from earlier photoautotrophic growth experiments (Erturk, 1998), it was noted that only a portion of the plantlets in each clump were able to survive when transferred from photomixotrophic (with sugar) to the photoautotrophic (sugarless) medium. The ones that did not develop photoautotrophy died and were discarded eventually. The objective of Part 2 of the study was to find the optimum clump size of the plantlets which will minimize the plant material loss during transformation to photoautotrophy.

EXPERIMENTAL PROCEDURE

This experiment was conducted twice, first with plantlets rooted for eight weeks and again with plantlets rooted for six weeks. The rooting period was changed because previous experiments (table 1) indicated that a six-week rooting period was sufficient to achieve photoautotrophy. Therefore, the difference in rooting time was not used as a treatment and merely represents the status of the plantlet material used for the experiment. The reason for repeating the experiment was that the first

experiment did not give statistically significant differences in treatments. The two sets of stock material were maintained and evaluated separately in the experiments.

There were three treatments to test the effects of clump size, all containing four plantlets per vessel: individual plantlets (4/vessel), 2 plantlets/clump (2 clumps/vessel), 4 plantlets/clump (1 clump/vessel). In subculturing, the plantlets were transferred from conventional sugar-containing medium to sugarless medium. The procedure was performed carefully to transfer only the plantlets that had roots on them. The plantlet clumps that came from each stock culture vessel were cut into smaller clumps or into individual plantlets using a scalpel. Fresh weight data of the plantlet clumps that went into each vessel at the beginning of the experiment were taken aseptically and recorded as were the weights two weeks later at the end of the experiment.

RESULTS AND CONCLUSION

The results for the fresh weight versus clump sizes are shown in tables 2 and 3. The effect of clump size on growth was found to be statistically insignificant (95% level of significance). However, because a clump size of two plantlets gave the highest average growth in terms of fresh weight when compared with both individual plantlets and with a clump size of four plantlets, it was concluded that transferring plantlets in a clump size of two plantlets was suitable for sugarcane plantlets during transformation to photoautotrophy.

Our results did not produce a statistically valid optimum for clump size. However, the results give hope that an optimum can be identified in the future using larger sample sizes. Such a finding would be in agreement with the “inoculation density” or “population” effects as shown by researchers such as Pierik and Steegmans (1972), Matsubara (1973), Start and Cumming (1976), Harris and Stevenson (1982), and Monette (1983).

Table 2. Effects of the clump size using eight-week rooted stock material

Treatment	N†	Average Initial Fresh Weight (g)	Average Final Fresh Weight (g)	Average Growth* (%)	Median (%)	S.D. (%)
Individual plantlets	4	1.19	5.72	380	336	111
2 plantlets/clump	3	1.06	6.80	554	611	134
4 plantlets/clump	2	1.32	4.43	257	257	194

* Growth p-value: 0.120

† N = number of vessels (samples) at end of experiment.

Table 3. Effects of the clump size using six-week rooted stock material

Treatment	N†	Average Initial Fresh Weight (g)	Average Final Fresh Weight (g)	Average Growth* (%)	Median (%)	S.D. (%)
Individual plantlets	2	1.26	5.64	353	353	90
2 plantlets/clump	3	0.89	4.33	389	390	33
4 plantlets/clump	3	1.25	4.64	280	285	81

* Growth p-value: 0.236

† N = number of vessels (samples) at end of experiment.

Sarkar et al. (1997) explained the effect of plant density as related to growth promoting substances diffusing from the explants. They concluded that a favorable change in the microculture atmosphere occurs at higher plant density, which promotes root and plantlet growth during initial culture establishment in potato micropropagation. At some even higher plant density the competition for resources would reduce total production.

PART 3: COMPARISON OF MEDIUM TYPES FOR PHOTOAUTOTROPHIC GROWTH OF PLANTLETS

The medium formulations that were developed for photomixotrophic tissue culture may not be suitable for photoautotrophic growth. Murashige and Skoog (MS) liquid medium is routinely used for photomixotrophic micropropagation of sugarcane and experiments were conducted to find if the MS medium was best for photoautotrophic growth of sugarcane plantlets.

EXPERIMENTAL PROCEDURE

The treatments used four media:

1. MS gel. This is the sugarless MS medium described earlier in the Materials and Methods section.
2. MS liquid. This is the same as above except without Phytigel.
3. Hydro-sol liquid. Hydro-sol (Grace Sierra Horticultural Products Company, Allentown, Pennsylvania) is a commercially used hydroponics medium. Full strength Hydro-sol solution was tried as a multiplication medium and was prepared by dissolving 1.2 g of Hydro-sol and 0.9 g of calcium nitrate in 1.0 l of water.
4. Hoagland’s liquid. One packet of Hoagland’s salts (Sigma Chemicals) was added to 1.0 l of water which gave a concentration of 4.7 g l⁻¹ salt solution.

All media had the same vitamins and plant growth regulators added as for the gelled MS medium. Plantlets used for these experiments were obtained from a photoautotrophic culture grown in sugarless liquid MS medium. Subculturing to vessels with the four different media were performed so that each vessel received two or three individual plantlets. These plantlets had smaller plantlets initiated at their base and so although counted as individual, they were a little bit bigger in size than typical single plantlets. Fresh weight data were collected at the beginning of the experiment and after four weeks.

RESULTS AND DISCUSSION

The average growth of sugarcane plantlets grown photoautotrophically for four weeks in four medium types is shown in table 4. The treatment results were not significant (i.e., p > 0.05). However, the MS liquid gave the highest average growth, compared with the other three liquid medium types. Also, gelled MS medium gave a higher average growth than any of the liquid medium types.

The statistical analysis for the treatment effect (medium type) on the growth showed the treatment effects to be statistically insignificant (95% level of significance). Therefore, it was concluded only that the liquid MS

Table 4. Results from the comparison of medium types test

Treatment	N†	Average Initial Fresh Weight (g)	Average Final Fresh Weight (g)	Average Growth* (%)	S.D. (%)
MS gel	5	2.56	6.88	169	38.4
MS liquid	4	2.52	6.55	160	23.0
Hydro-sol liquid	5	2.57	6.25	143	55.6
Hoagland's liquid	5	2.47	5.68	130	36.1

* Growth p-value: 0.455

† N = number of vessels (samples) at end of experiment.

medium that has been used throughout the photoautotrophic growth studies was at least suitable, though perhaps not optimal, for photoautotrophic multiplication.

Statistically speaking, the higher average growth with the gelled MS medium compared to the liquid media was only a random occurrence. However, higher growth with gelled medium compared to liquid might be expected because the gel acts as support for the plantlets. Plantlets grown on the gel grew vertically while those on liquid medium were more submerged while growing. This vertical, nonsubmerged phenotypic growth may facilitate exchange of gases, especially carbon dioxide and oxygen, with the atmosphere and thereby increase growth rate.

The reason MS medium might be expected to give better growth of sugarcane plantlets than Hydro-sol and Hoagland's medium is because these latter two salts are formulated for general hydroponics culture but MS salts are formulated and widely used for plant tissue culture. This is consistent with the fact that MS medium was used in other studies with sugarcane, such as Walker et al. (1991) and Gautz et al. (1991). MS medium has been used in many studies with photoautotrophic cultures by researchers such as Adelberg (1997) and Kozai et al. (1988b, 1991). So, it was not surprising to conclude that MS salts medium was a suitable growth medium for photoautotrophic micropropagation of sugarcane. Further study with variance reduction through the use of a greater number of vessels is need to determine which of the media tested is really the best.

PART 4: COMPARISON OF THE GROWTH RATES FOR PHOTOAUTOTROPHIC AND PHOTOMIXOTROPHIC MICROPROPAGATION

The growth rates of photoautotrophic cultures seemed to be lower than the growth rates of photomixotrophic cultures. The following experiment was conducted to quantify the differences between the photomixotrophic and photoautotrophic cultures. The growth rates of cultures were measured in terms of weight and number of plantlets.

EXPERIMENTAL PROCEDURE

Plantlets from photomixotrophic cultures were used as stock for the photomixotrophic treatment while plantlets from photoautotrophic cultures were used as stock for the photoautotrophic treatment. Clumps of plantlets from the stock cultures were subcultured (divided) into individual

Table 5. Comparison of growth per vessel of photoautotrophic and photomixotrophic cultures

Culture Type	N*	Average Initial Fresh Weight (g)	Average Fresh Weight after 2 Weeks (g)	Average Fresh Weight after 6 Weeks (g)	Average Growth after 6 Weeks (%)	Avg. No. of Plantlets after 6 Weeks
Photoautotrophic	8	0.67	1.77	4.69	600	9.0
Photomixotrophic	6	0.41	3.44	14.15	3351	33.8

* N = number of vessels (samples) at end of experiment.

plantlets, with four plantlets placed in each treatment vessel. Thirty-five milliliters of liquid MS medium (2% sucrose; same as gelled MS medium described in Materials and Methods section except without Phytigel) and liquid sugarless MS medium (same as previous treatment except no sugar) were placed in each vessel for photomixotrophic and photoautotrophic cultures, respectively. Media in the vessels were changed at the end of each week. Photoautotrophic cultures were grown in the growth chamber while the photomixotrophic cultures were grown in the culture room. The experiment lasted six weeks. The data for the weight and the number of plantlets from this experiment are shown in table 5.

RESULTS AND DISCUSSION

The data from table 5 show that the growth of photoautotrophic cultures was about one-fifth the growth of photomixotrophic cultures after six weeks. The average number of plantlets per vessel obtained in six weeks from photoautotrophic cultures and the photomixotrophic cultures were found to be 9 and 34, respectively, which is consistent with the growth in fresh weight.

The reason the photoautotrophic cultures grew slower than the photomixotrophic cultures is presumably because the plantlets in the sugarless medium are forced to photosynthesize to obtain carbohydrates whereas photomixotrophic cultures utilize the sugar provided by the medium. However, it should be noted that the environmental conditions for photoautotrophic micropropagation of sugarcane have not been optimized, although a start was made in Part 3 of this study (Parts 1 and 2 dealt with transformation to photoautotrophy, not culture after photoautotrophy was achieved). So, the photoautotrophic cultures likely did not have optimum conditions. On the other hand, media and environmental conditions for photomixotrophic culture have been studied and have evolved over decades (e.g., Walker et al., 1991) so that the conditions used for photomixotrophic micropropagation are more nearly optimal.

In our photoautotrophic cultures it is likely that the carbon dioxide levels in the photoautotrophic culture vessels were depressed because photosynthesis used the CO₂ and the vessels were not vented. So, it seems ventilation and/or CO₂ enrichment techniques would increase growth. Also, if the CO₂ levels were indeed depressed the high light levels may have inhibited photosynthesis. However, any ill effects of high light and low CO₂ would likely be less for sugarcane than for C3 plants. Sugarcane and other C4 plants have a higher light saturation point because of their decarboxylation of malate which releases CO₂ in the plant for reuse in photosynthesis.

Nevertheless, it is expected that optimizing medium composition and environmental factors should considerably increase the growth rate for photoautotrophic cultures in the future. Earlier experiments by Erturk and Walker (2000) support this expectation. They showed that higher light levels and CO₂ enrichment stimulated the transformation of shoots from photomixotrophy to photoautotrophy as indicated by final dry weight. However, those experiments deal with transformation to photoautotrophy and not continued photoautotrophic culture.

The Part 4 experiment demonstrated photoautotrophic cultures increasing in weight and in the number of plantlets which was a landmark achievement. Photoautotrophic micropropagation was expressed as increase in the number of plantlets, whereas in earlier work, growth in photoautotrophic micropropagation environments was expressed only as increase in weight and/or the number of leaves. This experiment was the first time photoautotrophic multiplication (increase in the number of shoots under photoautotrophic conditions, starting with shoots from photoautotrophic cultures) of any plant was demonstrated in any micropropagation study. (An increase in number of shoots was also observed for Part 3 of this study, but not recorded.)

CONCLUSIONS

1. It was concluded that roots are necessary for the transformation of sugarcane from photomixotrophic to photoautotrophic micropropagation culture. The best rooting period was found to be six weeks, when compared against three and four weeks. Following six-weeks rooting, 100% of the vessels developed photoautotrophy.
2. Two plantlets per clump (and 2 clumps/vessel) were concluded to be a suitable clump size, although not significantly better than individual plantlets or 4 plantlets/clump.
3. Liquid MS medium gave insignificantly higher growth than liquid Hydro-sol and Hoagland's solutions. MS salts formulation, widely used for general photomixotrophic growth, was concluded to be suitable for photoautotrophic growth.
4. Sugarcane can be photoautotrophically multiplied in micropropagation. This study represents the first time photoautotrophic multiplication in micropropagation has been demonstrated for any plant. However, growth and multiplication for photoautotrophic cultures is only a fraction of that for photomixotrophic culture. It is expected that photoautotrophic culture will improve with further study.

REFERENCES

- Adelberg, J. W., N. V. Desamero, S. A. Hale, and R. Young. 1997. Long term nutrient and water utilization during micropropagation of *Cattleya* on a liquid/membrane system. *Plant Cell Tissue & Organ Culture* 48: 1-7.
- Erturk, H. 1998. Photoautotrophic micropropagation of sugarcane shoots. Ph.D. thesis. University Park, Pa.: Dept. of Agricultural and Biological Engineering, Pennsylvania State University.
- Erturk, H., and P. N. Walker. 2000. Effects of light, CO₂, and hormone levels on the growth of sugarcane shoots during transformation to photoautotrophy. *Transactions of the ASAE* (accepted for publication).
- Gautz, L. D., and J. P. Harris. 1991. Photoperiod effect on *in vitro* cultured sugarcane plantlets. ASAE Paper No. 91-7529. St. Joseph, Mich.: ASAE.
- Harris, R. E., and J. H. Stevenson. 1982. *In vitro* proliferation of *Vitis*. *Vitis* 21: 22-32.
- Huang, L., C. Kohashi, R. Vangundy, and T. Murashige. 1995. Effects of common components of hardness of culture media prepared with Gelrite. *In Vitro Cell Dev. Biol.* 31: 84-89.
- Kirdmanee, C., Y. Kitaya, and T. Kozai. 1995. Effects of CO₂ enrichment and supporting material *in vitro* on photoautotrophic growth of eucalyptus plantlets *in vitro* and *ex vitro*. *In Vitro Cell Dev. Biol.* 31: 144-149.
- Kozai, T., C. Kubota, and I. Watanabe. 1988a. Effects of basal medium composition on the growth of carnation plantlets in auto- and mixo-trophic culture. *Acta Hort.* 230: 159-166.
- Kozai, T., C. Kubota, and I. Watanabe. 1990. The growth of carnation plantlets *in vitro* cultured photoautotrophically on different media. *Environ. Control. Biol.* 28: 21-27.
- Kozai, T., Y. Koyoma, and I. Watanabe. 1988b. Multiplication of potato plantlets *in vitro* with sugar-free medium under high photosynthetic photon flux. *Acta Hort.* 230: 121-127.
- Kozai, T., K. Iwabuchi, K. Watanabe, and I. Watanabe. 1991. Photoautotrophic and photo mixo-trophic growth of strawberry plantlets *in vitro* and changes in nutrient composition of the medium. *Plant Cell, Tissue & Organ Culture* 25:107-115.
- Kozai, T. 1991. Photoautotrophic micropropagation. *In Vitro Cell Dev. Biol.* 27: 47-51.
- Matsubara, S. 1973. Population effects in lateral bud culture of asparagus and promotion of root formation by transplanting. *J. Japanese Hort. Sci.* 42: 142-146.
- Monette, P. L. 1983. Influence of size of culture vessel on *in vitro* proliferation of grape in liquid medium. *Plant Cell Tissue & Organ Culture* 2: 327-332.
- Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant* 15: 473-497.
- Pierik, R. L. M., and H. H. M. Steegmans. 1972. The effect of 6-benzylaminopurine on growth and development of *Cattleya* seedlings grown from unripe seeds. *Z. Pflanzelphysiol.* 68(3): 228-234.
- Sarkar D., R. Chandra, and P. S. Naik. 1997. Effect of inoculation density on potato micropropagation. *Plant Cell Tissue & Organ Culture* 48: 63-66.
- Serret, M. D., M. I. Trillas, J. Matas, and J. L. Araus. 1996. Development of photoautotrophy and photoinhibition of *Gardenia jasmonides* plantlets during micropropagation. *Plant Cell Tissue & Organ Culture* 45: 1-16.
- Start, N. E., and B. G. Cumming. 1976. *In vitro* propagation of *Saintpaulia ionantha* Wendl. *Hort Science* 11: 204-206.
- Walker, P. N., J. P. Harris, and L. D. Gautz. 1991. Optimal environment for sugarcane micropropagation. *Transactions of the ASAE* 34(6): 2609-2614.