RESEARCH ARTICLE

Monitoring the end of the *in vitro* phase of *Anthurium andreanum* Lindl. plantlets

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Received: 01 September 2009; Accepted: 14 May 2010

ABSTRACT

Estimulation of autotrophy in *in vitro* plantlets could be achieved through changes in the culture medium, or by changing the traditional hermetic caps by one that could allow gas exchanges between the culture and the environment. Besides that, the use of lamps with distinct emission spectrum irradiaction has propitiated successful results. This work was carried out aiming to evaluate the either the combined or the single action of some factors that can induce autotrophy on *in vitro A. andraeanum* cv. Eidibel plantlets. 3 sucrose concentrations were used: 0, 15 and 60 mM and for each one, to kinds of flasks according to the cap ventilation: under (0.038 $L.h^{-1}$) and without ventilation. Flasks were kept under cold light fluorescent lamps or under gro-lux lamps. At the end of the experiment showing the highest shoot dry mass treatment was 60 mM, under ventilation and gro-lux, and the treatment which accumulate root dry mass to a lesser extent were 0 mM with ventilation and cold light and 15 mM without ventilation and cold light. In average, treatments with higher sucrose content in the culture medium, that is, 60 mM, under gro-lux lamps, presented the highest chlorophyll *a*, *b* and *total* contents, than those under cold lamp. Steps of carbohydrates metabolism could be associated with the total soluble sugars (sucrose and reducing sugars) levels, highlighting the steps where nutrient requirements were higher, showing the role of the plantlets sink.

Key words: acclimatization, Araceae, plantlets metabolism

RESUMO

Monitoramento do final da fase *in vitro* de plântulas de *Anthurium andraeanum*. O estímulo à autotrofia em plântulas *in vitro* pode ser alcançado através de mudanças no meio de cultura, ou pela troca de tampas herméticas por tampas que permitam a troca de gases entre a cultura e o ambiente. Além disso, o uso de lâmpadas com distintos espectros de irradição tem propiciado bons resultados. Este trabalho foi conduzido com o objetivo de avaliar a ação isolada ou em conjunto de fatores que podem induzir a autotrofia em plântulas de *A. andraeanum* vc. Eidibel *in vitro*. 3 concentrações de sacarose foram usadas: 0, 15 e 60 mM e para cada concentração foram empregados frascos com ventilação (0,038 $L.h^{-1}$) ou sem ventilação. Os frascos foram mantidos sob lâmpada fluorescente fria ou gro-lux. O final dos experimentos mostrou que o maior acumulo de massa seca da parte aérea ocorreu a 60 mM, sob ventilação e gro-lux, e os tratamento que acumularam menor massa seca de raízes foram 0 mM com ventilação e sob luz fria e 15 mM sem ventilação e luz fria. Em média, os tratamentos com o maior teor de sacarose no meio, 60 mM, sob lâmpada gro-lux, apresentaram as maiores concentrações de clorofila *a*, *b* e total, do que sob luz fria. Os níveis de açúcares solúveis totais (sacarose e açúcares redutores) mostraram passos do metabolismo de carboidratos nessas plantas, realçando os momentos em que a exigência por nutrientes foi maior, destacando-se o papel de dreno das plântulas.

Palavras-chave: aclimatização, Araceae, metabolismo de plântulas

INTRODUCTION

Anthurium andreanum has been of great significance in the Brazilian flowers production either for the cut flowers market or for the pot plants one, and the development of cultivars suitable to the tropical conditions (Tombolato, 2004) has contributed to the increasing growing area as well as to the high quality of flowers.

The *Anthurium* market has shown interest in plants uniformity, high floral quality as well as high yield, and the method of production through tissue culture *in vitro*, has been an important tool for the consecution of those purposes.

It is known that plantlets grown *in vitro* show feeble systems of protection against water loss, and don't have yet developed photosynthetic machinery for CO_2 assimilation. Therefore is essential to provide suitable conditions to plantlets development from the beginning to transplantation. According to literature (Grattapaglia and Machado, 1998) besides low irradiance around and appropriate mineral nutrition (N:P:K), plantlets *in vitro* need high relative humidity, between 70 and 80% and air temperature never below 15ºC.

The success of a *in vitro* culture depends mainly on the physiological and anatomical adjustments that plantlets undergo at the last stage *in vitro* and throughout the *ex vitro* one, and the dry mass accumulation will be a consequence of the interaction of the environmental conditions and carbohydrates metabolism.

In general, the control of the environment and the medium culture changes are part of a serie of estrategies performed throughout the pre-acclimatization of plantlets, being of great importance in its growth, development, and proper morphological changes promotion, *in vitro* (Kozai et al., 1987; Kozai et al., 1991). Estimulation of autotrophy could be achieved through changes in the culture medium, or by changing the traditional hermetic caps by one that could allow gas exchanges between the culture and the environment (Kozai, 1991). Besides that, the use of lamps with distinct light emission spectrum has propitiated successful results, but mostly restrict to few plant species.

This work was carried out aiming to evaluate the either the combined or the single action of some factors that can induce autotrophy on *in vitro A. andraeanum* cv. Eidibel plantlets.

MATERIAL AND METHODS

A. *andraeanum*, cv. Eidibel plantlets kept in Murashige and Skoog (1962) medium were selected, being subsequently submitted to the same medium but under half salt concentration, in 300 mL capacity flasks with 50 mL of culture medium, with 5 plantlets per flask, for forty five days. Three sucrose concentrations were used: 0, 15 and 60 mM and for each one, to kinds of flasks according to the cap ventilation: under (0.038 $L.h^{-1}$) and without ventilation, u/v and w/v, respectively. Flasks were kept under cold light fluorescent lamps or under gro-lux lamps.

The experimental design was a random one, with three sucrose concentrations, and the use or not of ventilation and 2 kinds of illumination, that is twelve treatments and 10 replicates. Analyses were performed at 0, fifteen, thirty and forty five days of growing *in vitro*, and for each treatment all the 10 replicates were analyzed.

Plantlets dry weight was performed by submitting them to 70°C temperature, until constant weight. At the end of the experiment chlorophyll *a*, *b* and total contents were evaluated, according to the Arnon (1949) method and the results were expressed in mass units. Total soluble sugars contents were determined according to Dubois et al. (1956), reducing sugars contents were determined according to Somogyi (1952), and sucrose contents, according to Händel (1968).

RESULTS

There was no significant difference in shoot dry weight among treatments at the 0 day experiment (Table 1). From the onset of dry weight accumulation on, high values have been observed for the treatments 15 mM u/v and cold lamps (CL) corresponding to 5.2 mg and the treatment 60mM, under the same conditions, corresponding to 5.3 mg. At the 30 th days the higher dry mass accumulation was for the treatments 0 mM u/v and gro-lux (GL), corresponding to 5.3 mg; 15 mM u/v and CL, corresponding to 5.5 mg, and 60 mM u/v and CL, 5.6 mg. At the end of the experiment showing the highest shoot dry mass treatment was 60 mM u/v and GL, corresponding to 7.2 mg.

Treatments that accumulated shoot dry mass to a lesser extent were: at the 15 th day, 0 mM w/v and CL, and 60 mM w/v and GL; at the 30 th day treatments 0 mM w/v and CL, 15 mM w/v and GL, and 60 mM w/v and GL; at 45 th day the 0 mM w/v and CL.

Regarding to the root dry weight accumulation (Table 2), at the 30 th day better performance was observed on treatments 0 mM, under and without ventilation and GL, corresponding to

1.4 mg, 15 mM u/v and CL, corresponding to 1.6 mg, 15 mM u/v and GL, corresponding to 1.4 mg and 60 mM u/v and GL, corresponding to 1.5 mg. At the end of the experiment the treatment which accumulated root dry mass to a lesser extent were 0 mM u/v and CL, and 15 mM w/v and CL, both presenting 1.5 mg root dry weight.

Tables 1 and 2 show that treatment with the highest sucrose content, under flasks ventilation and GL lamps happened to be the one that accumulated both shoot and root dry weight, that is plantlets under these conditions would be able to survive under the *ex vitro* environment.

Table 1. Average shoot dry mass accumulation (mg) in *Anthurium andraeanum* cv. Eidibel plantlets at the 0, 15 th, 30 th and 45 th days of acclimatization *in vitro*, in media culture with 0, 15 and 60 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

Treatment	Shoot dry mass			
	0 days	15 days	30 days	45 days
0mM w/v CL	3.16ns[1,2]	3.44h	4.08f	4.6g
0mM u/v CL	3.16ns	4.06f	4.44e	4.98f
0mM w/v GL	3.16ns	4.7cde	5.16c	5.51de
0mM u/v GL	3.16ns	5bc	5.3abc	5.72cd
15mM w/v CL	3.16ns	4.4e	4.78d	5.3e
15mM u/v CL	3.16ns	5.16ab	5.54ab	5.96c
15mM w/v GL	3.16ns	3.96fg	4.18ef	5.76c
15mM u/v GL	3.16ns	4.04fg	4.4e	5.94c
60mM w/v CL	3.16ns	4.54de	4.84d	5.36e
60mM u/v CL	3.16ns	5.34a	5.58a	6.94b
60mM w/v GL	3.16ns	3.72gh	4.08f	5.82c
60mM u/v GL	3.16ns	4.8cd	5.26bc	7.22a

[1] According to Tukey test at 5%.
[2] Values followed by same letters on columns do not differ significantly.

Table 2. Average root dry mass accumulation (mg) in *Anthurium andraeanum* cv. Eidibel plantlets at the 0, 15 th, 30 th and 45 th days of acclimatization *in vitro*, in media culture with 0, 15 and 60 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

Treatment	Root dry mass			
	0 days	15 days	30 days	45 days
0mM w/v CL	0.9ns[1,2]	1.04ns	1.22c	1.56g
0mM u/v CL	0.9ns	1.2ns	1.38bc	1.52g
0mM w/v GL	0.9ns	1.08ns	1.4abc	1.86ef
0mM u/v GL	0.9ns	1.16ns	1.4abc	1.74fg
15mM w/v CL	0.9ns	1.3ns	1.46ab	1.52g
15mM u/v CL	0.9ns	1.34ns	1.62a	1.74fg
15mM w/v GL	0.9ns	1.12ns	1.32bc	2.06de
15mM u/v GL	0.9ns	1.14ns	1.42abc	2.34c
60mM w/v CL	0.9ns	1.12ns	1.3bc	1.82f
60mM u/v CL	0.9ns	1.28ns	1.36bc	2.98b
60mM w/v GL	0.9ns	1.12ns	1.24bc	2.2cd
60mM u/v GL	0.9ns	1.28ns	1.46ab	3.36a

[1] According to Tukey test at 5%.
[2] Values followed by same letters on columns do not differ significantly.

Table 3 shows the plantlets leaf area increase throughout the 45 days acclimatization. Significant differences among treatments could be seen after 15 days, and it is worth mentioning treatments 15 mM u/v and CL, 15 mM w/v and GL, 60 mM u/v and GL, and 60 mM w/v and GL, presenting significant larger leaf area until the end of the experiment. It is worth noting that treatments with 15 mM sucrose presented larger leaf areas independently if plantlets were under CL or GL or u/v or w/v, and 60 mM u/v or w/v and GL. Treatments 15 mM w/v and GL, 60 mM u/v and GL, 60 mM w/v and GL, and 15 mM u/v CL, showed leaf areas about 43.1, 41.9, 38.9 and 35.0%, respectively as high as the other ones.

In average, 60 mM sucrose treatments presented higher chlorophyll *a*, *b* and total content (Table 4), while plantlets grown in 15 mM sucrose showed intermediate values and those under in 0 mM sucrose had the lowest chlorophylls content. Chlorophyll *a* content was higher then chlorophyll *b* (Table 4), in every treatment, corresponging to 2.22 on treatments under 0 mM, 2.89 under 15 mM and 2.45 under 60 mM. Total chlorophyll averages were 1.44 and 1.17 higher under 60 and 15 mM sucrose, respectively, when compared with the content observed under 0 mM. The ratio chlorophyll *a*:chlorophyll *b* varied significant among treatments, with average values of 2.2 for the 0 mM, 2.3 for 15 mM and 2.45 for the 60 mM sucrose.

Table 3. Average leaf area (cm^2) in *Anthurium andraeanum* cv. Eidibel, plantlets at the 0, 15 th, 30 th and 45 th days of acclimatization *in vitro*, in media culture with 0, 15 and 60 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

Treatment	Leaf area			
	0 days	15 days	30 days	45 days
0mM w/v CL	0.94ns	1.62d	2.45e	2.94c
0mM u/v CL	0.94ns	2.15cd	2.71de	2.97c
0mM w/v GL	0.94ns	2.29cd	2.92d	3.29bc
0mM u/v GL	0.94ns	2.29cd	2.91d	3.29bc
15mM w/v CL	0.94ns	3.16ab	3.46bc	3.78b
15mM u/v CL	0.94ns	3.58a	4.15a	4.51a
15mM w/v GL	0.94ns	3.17ab	3.82ab	4.78a
15mM u/v GL	0.94ns	2.48bc	2.82de	3.31bc
60mM w/v CL	0.94ns	2.37cd	2.85de	3.27bc
60mM u/v CL	0.94ns	2.49bc	3.12cd	3.86b
60mM w/v GL	0.94ns	3.77a	3.95a	4.64a
60mM u/v GL	0.94ns	3.49a	3.87ab	4.74a

[1] According to Tukey test at 5%. 2 Values followed by same letters on columns do not differ significantly.

Table 4. Chlorophyll content (μg.100 mg^{-1} fresh mass), after 45 days of acclimatization *in vitro*, in media culture with 0, 15 and 60 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

Treatment	Chlorophyll content			Chl *a*/*b* ratio
	Chl *a*	Chl *b*	Total Chl	
0mM w/v CL	2.91e	1.48de	4.39e	1.98c
0mM u/v CL	3.19e	1.46e	4.66e	2.18bc
0mM w/v GL	5.33bcd	2.19abc	7.51bcd	2.43ab
0mM u/v GL	4.48cd	2.02bcd	6.5cd	2.22bc
15mM w/v CL	5.1bcd	2.15abc	7.25bcd	2.37ab
15mM u/v CL	4.09de	1.83cde	5.92de	2.23abc
15mM w/v GL	5.41bc	2.24abc	7.65abcd	2.42ab
15mM u/v GL	4.15de	1.84cde	6.09de	2.26abc
60mM w/v CL	5.59abc	2.31abc	7.9abc	2.42ab
60mM u/v CL	6.67a	2.64a	9.31a	2.53a
60mM w/v GL	4.98cd	2.1abc	7.08cd	2.36ab
60mM u/v GL	6.32ab	2.55ab	8.87ab	2.48ab

[1] According to Tukey test at 5%.
[2] Values followed by same letters on columns do not differ significantly.

Figures 1A, 1B, 1C e 1D show that there were no significant differences among the 0 mM sucrose treatment, independently of flasks being ventilated or not and of the type of illumination. At the 0 day it was observed that plantlets presented low sucrose content while the reducing sugars content was high. At the 15 th day total sugars and reducing sugars content decreased, while sucrose content shows an increase. At the 30 th day the lowest contents of sucrose, reducing sugars and total sugars were observed. Henceforward and then until the end of the experiment at the 45 th day, sucrose content has kept the same or showed a decrease while reducing sugars content showed a sharp increase.

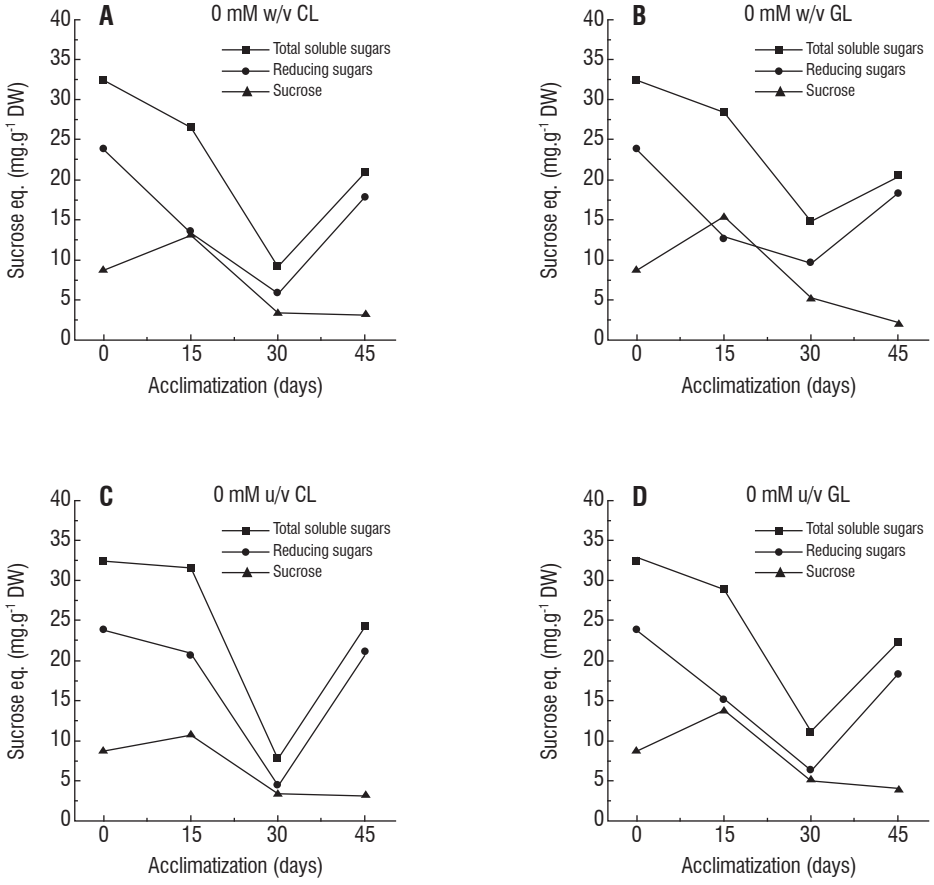

Figure 1. Total soluble sugars, reducing sugars and sucrose content variation, in *Anthurium andraeanum* cv. Eidibel plantlets, throughout 45 days of acclimatization *in vitro*, in media culture with 0 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

The results showed in Figures 2A, 2B, 2C and 2D, under 15 mM sucrose, show that the total soluble sugars, reducing sugars and sucrose content varied significantly throughout the experiment. In general the variations on carbohydrates content seemed to have been similar in all treatments. Under w/v and CL and u/v and GL, the total soluble sugars level showed an increase from the 0 to the 15 th day *in vitro* (Figures 2A and 2C). At the same period, all treatments showed decreases in the reducing sugars, while sucrose content has increased. From the 15 to the 30 th day, a decrease in reducing sugars as well as in sucrose was observed, coupled with a decrease in total soluble sugars content. The lowest level of all the carbohydrates were observed at the 30 th experiment. Figures 2A and 2C, showed that from the 30 to the 45 th day an increase in reducing sugars as well as in sucrose content was observed. At the same period, Figures 2B and 2D, show an increase in reducing sugars level and a decrease in sucrose content, coupled with an increase in total soluble sugars much lower however, than that one observed at the 0 day experiment. It is worth noticing that from the 15 to the 30 th the reducing sugars and sucrose levels were similar.

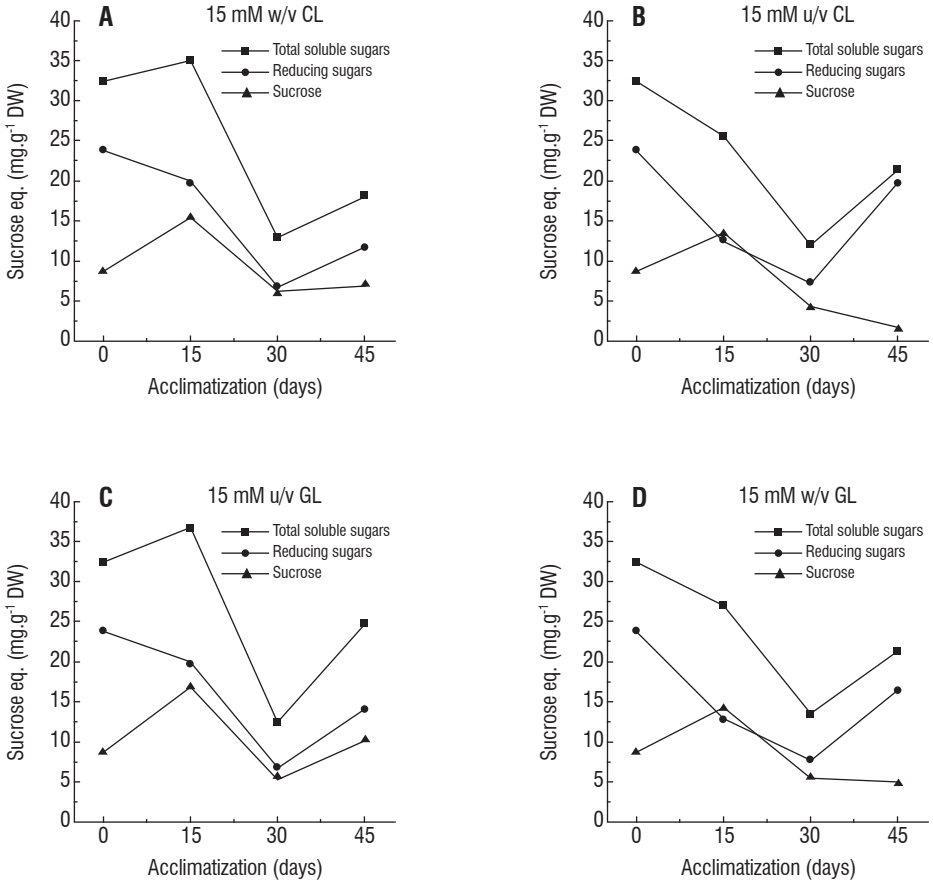

Figure 2. Total soluble sugars, reducing sugars and sucrose content variation, in *Anthurium andraeanum* cv. Eidibel plantlets, throughout 45 days of acclimatization *in vitro*, in media culture with 15 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

Figures 3A, 3B, 3C and 3D show the variations in total soluble sugars, reducing sugars and sucrose contents under 60 mM sucrose. Figures 3A, 3B and 3C, showed that the variations in carbohydrates contents were similar, and that there was no sharp decrease of them after 30 days *in vitro*, as it was observed under 0 and 15 mM. From the 0 to the 15 day, there was a decrease in reducing sugars and in sucrose levels, which were maintained steady from the 15 to the 30 th day. Henceforward until the 45 th day, the sucrose levels increased while those of reducing sugars either unchanged

or showed a slight decrease. Meanwhile, total soluble sugars contents showed an increase, mainly due to the increase in sucrose levels.

Figure 3D shows an atypical performance when compared either with those of Figures 3A, 3B and 3C, or those under the other sucrose concentration in the culture medium (Figures 1 and 2). From the 0 to the 15 th day, the reducing sugars and sucrose contents decreased coupled with the decrease in total soluble sugars content. Henceforth until the end of the experiment at the 45 th day, the reducing sugars as well as the sucrose levels showed a significant increase, coupled with an increase in total soluble sugars levels.

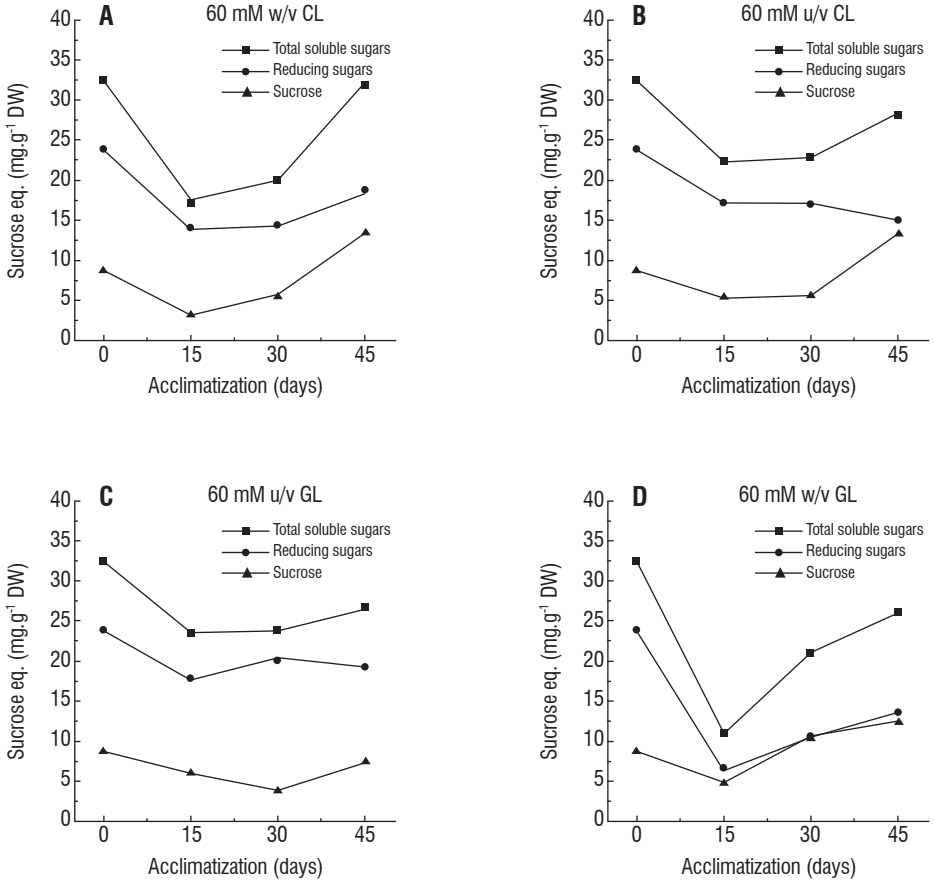

Figure 3. Total soluble sugars, reducing sugars and sucrose content variation, in *Anthurium andraeanum* cv. Eidibel plantlets, throughout 45 days of acclimatization *in vitro*, in media culture with 60 mM sucrose, under (u/v) or without (w/v) flasks ventilation and under cold lamps (CL) or gro-lux (GL).

DISCUSSION

The high total soluble sugars levels, mainly reducing sugars at the beginning of the experiment and the subsequent metabolic changes throughout the 45 days may indicate that previously to the treatments imposition, plantlets had absorbed sucrose from the culture media which enzimatically was broken down to glucose and fructose. Taking into account that the sucrose levels have kept low, the results indicate high catalytic enzymes activity but low sucrose demand, that is the reducing sugars were not consumed highly mainly at the 15 th day. On the 30 th day there was a decrease in the total soluble

sugars, reducing sugars and sucrose level, probably due to a highest demand for photoassimilates at this period, probably due to the maximum sink strength at that moment.

According to Hazarika (2006), growth and respiration *in vitro*, require a steady sucrose exogenous supply as a carbon source. However, high sucrose and salts concentration within the culture medium seem to restrain photosynthetic efficiency. Also, Kovtum and Daie (1995) observed that a exogenous sucrose source speeds leaves growth and development and its transition sink-source in *Beta vulgaris* L. plantlets grown *in vitro*, and concluded that the question was not the source but the sink limitation, until the plantlets themselves developed its capacity of metabolizing carbohydrates. Ticha et al. (1998) have considered that exogenous sucrose would prevent photoinhibition to happen.

The increasing sucrose concentration within the culture medium maximizes the role of the nutrients in persistent leaves (Grout and Millam, 1985; Desjardins et al., 1987), and it is known that high sucrose concentration has propitiate increasing shoot dry weight. Hazarika et al. (2000) observed that pre-conditionement of *Citrus* sp *in vitro* under 3% sucrose has increased subsequent survival and growth *ex vitro*. According to the same authors, a linear increase in the biochemical components with sucrose addition to the culture medium.

From the 45 th day on, there was a sink strength decrease that could be corroborated by the high reducing sugars content, while sucrose content was kept at its previous levels. The results indicate an increase in the sucrose metabolism enzymes which could be due to a decrease of sinks strength or to an imbalance in carbohydrates partitioning (Stancato et al., 2001). Once plantlets from 60 mM treatment presented higher dry weight (Tables 1, 2, and 3) it is possible that a higher demand for reducing sugars has resulted in a higher demand for sucrose.

When the gain of dry mass was related to the leaf area, it seemed that plantlets under 15 and 60 mM sucrose in the culture medium are among those which presented a bigger leaf area, indicating a higher demand for sugars from the culture medium to be allocated to the leaves, resulting a increase in leaf area. By the way, the leaf area does not seem to be a suitable variable for to be used to evaluate the plantlets acclimatization, although Premkumar et al. (2001) have pointed out that the success of acclimatization depends on the sources inside the developing plantlets. According to Ticha et al. (1998), the addition of sucrose to the culture medium influenced positively the increase of biomass and leaf area as well as accumulation of chlorophyll and photosynthetic capacity in tobacco plantlets.

Chlorophyll contents are in agreement with those observed in plantlets of other species *in vitro* (Carvalho et al., 2005). The results show that chlorophyll levels are better related with sucrose concentration in the culture médium than the lamp type. GL lamps have the emission spectrum in the range of blue and red and the CL lamps emit in blue, but the emission spectrum did not interfere on chlorophyll levels, although its capacity of capturing and transforming light energy depends on the simultaneous absortion of blue and red wave lengths. Light intensity is the same for both lamps.

According to Premkumar (2001), while low light inhibits chloroplasts development, sugars content in the culture medium may restrain the photosynthetic enzymes activity, as for instance the Rubisco (EC 4.1.1.39). Watanabe et al. (1990); Tanaka et al. (1991); Rival et al. (1999), observed that *Chrysantemum morifolium* L., *Spathiphyllum wallisi* Regel and coconut plantlets grown *in vitro* with 3% sucrose, presented low Rubisco levels, what is characteristic for *in vitro* plantlets. According to Carvalho et al., (2005) low light intensity inhibit the proper development of chloroplasts, which exhibit low content and activity of Rubisco *in vitro*.

In average, plantlets grown under gro-lux lamps grew faster than those under cold light grown under the same sucrose concentration, that is 60 mM. This may imply that although the lamps emission spectrum has not been involved in the chlorophyll level, there could have been higher Rubisco activity associated to sucrose level in the culture medium, resulting in a better utilization of the light energy. Studies by Kozai et al. (1987) with species of ornamental plantlets showed that the CO_2 level in the flasks, decreased from 3000-9000 ppm, in the dark period to 90 ppm in the light period indicating that plantlets were able to photosynthesize throughout the light period.

Total soluble sugars (sucrose and reducing sugars) showed steps of the carbohydrates metabolism mainly the higher the demand for nutrients by plantlets sinks. Although the carbohydrates level in the culture medium

may have contributed to plantlets heterotrophy, the fact that high sugars concentrations have decreased significantly the medium osmotic potential besides the possible gas exchanges propitiated by the flasks with the holed cap, allowed a degree of autotrophy expressed by the dry weight accumulation in plantlets. Even under low light conditions it is possible to speculate that plantlets grown in 15 and 60 mM sucrose and holed caps have grown not only as a function of nutrients availability in the culture medium, but also via photosynthesis.

CONCLUSIONS

- The plantlets dry mass accumulation were positively correlated with the higher sucrose content in the culture medium;
- The ventilation in the flasks contributed significantly to the plantlets dry mass accumulation and to its acclimatization and consequently to its vigor;
- The emission spectrum of the GL lamps showed a positive influence on the plantlets development.

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