

# MORPHO-PHYSIOLOGICAL CHANGES IN PINEAPPLE PLANTLETS [*Ananas comosus* (L.) Merr.] DURING ACCLIMATIZATION

## Mudanças morfofisiológicas em plântulas de abacaxi [*Ananas comosus* (L.) Merr.] durante a aclimatização

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### ABSTRACT

Pineapple producing countries lack good quality propagation material to expand cultivars into new areas. Previously, research protocols may increase the offer of high quality plantlets and speed the introduction of new pineapple cultivars. The present work is to evaluate the morpho-physiological changes in plantlets of pineapple [*Ananas comosus* (L.) Merr. 'MD-2'] during the acclimatization phase. Plantlets were acclimatized under 80% relative humidity, 25.5 °C temperature and photosynthetic photon flux of 400-500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  as average for 45 d under natural photoperiods. All measurements (plant length, number of leaves and roots, fresh weight, width and length of leaf 'D', net photosynthesis and total transpiration rate) were carried out at the end of *in vitro* rooting phase coincident with 0 d of acclimatization and at 15, 30 and 45 d thereafter. Photosynthetic activity of *in vitro* plantlets did not increase during the first 30 d of the acclimatization phase. After 30 d, photosynthetic activity ranged from 5.72 to 9.36  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  while total transpiration ranged from 6.0 to 1.42  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ . During the first 30 days there were no significant differences in number of leaves, length or width of the longest ('D') leaf (cm) or plant length (cm). However, after 45 days plant fresh weight (g), length and width of the 'D' leaf (cm) and root number all increased significantly, while transpiration ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) declined. There were small but significant decreases in chlorophyll a and b ( $\mu\text{g g}^{-1} \text{mf}$ ). Increased photosynthetic activity after 30 d shows that the increase in light intensity and the reduction of relative humidity during acclimatization did not constitute inhibitory factors.

**Index terms:** Chlorophyll, *ex vitro* conditions, photosynthesis, transpiration.

### RESUMO

Os países produtores do abacaxi precisam de mudas de boa qualidade para a introdução de novas culturas nas áreas de cultivo. Os protocolos de pesquisa podem aumentar a oferta de mudas de alta qualidade e acelerar a introdução de novas culturas do abacaxi. O estudo apresentado teve como objetivo avaliar as alterações morfofisiológicas de mudas de abacaxi [*Ananas comosus* (L.) Merr. 'MD-2'], durante a fase de aclimação. As plantas foram aclimatizadas baseadas em um regime de 80% de umidade relativa, uma temperatura de 25°C e um fluxo médio de fótons fotossinteticamente ativos de 400-500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , durante 45 dias em condições naturais de iluminação. As medições todas (comprimento da planta, número de folhas, comprimento, largura da folha maior 'D', taxa fotossintética e taxa transpiratória total) foram feitas no final da fase do enraizamento *in vitro* a coincidir com a aclimatização no período de 0, 15, 30 e 45 dias após aclimatização. Depois de 30 dias, a atividade fotossintética variou entre 5,72 e 9,36  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , enquanto a transpiração total variou de 6,0 a 1,42  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ . Durante os primeiros 30 dias, não foram registradas diferenças significativas entre o e comprimento da planta, número de folhas e comprimento e largura da folha maior 'D'. No entanto, após 45 dias a massa fresca da planta (g), comprimento e largura da lâmina 'D' (cm) e o número de raízes aumentaram significativamente, enquanto a transpiração ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) foi reduzida. As clorofilas a e b ( $\mu\text{g g}^{-1} \text{mf}$ ) diminuíram e mostraram diferenças pequenas, mas significativas. O aumento da atividade fotossintética após 30 dias mostrou que o aumento da intensidade luminosa e redução da umidade relativa não são fatores inibidores desta atividade durante a aclimatização.

**Termos para indexação:** Clorofila, condições *ex vitro*, fotossíntese, transpiração.

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### INTRODUCTION

Pineapple [*Ananas comosus* (L.) Merr.] is vegetatively propagated so expanding the available supply of planting material required to expand production in new areas or introduce a new cultivar is an expensive and time consuming process. Based on typical planting densities for various cultivars, one hectare of the crop requires from

35 000 to 75 000 propagules (BARTHOLOMEW; PAULL; ROHRBACK, 2003).

The MD-2 cultivar, developed by Del Monte Fresh Produce International Inc., has almost completely taken over the international fresh fruit market (LOEILLET; DAWSON; PAQUI, 2011). In order for the pineapple industries to remain competitive, it is imperative that they expand plantings of 'MD-2' pineapple on a commercial

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scale (BARTHOLOMEW et al., 2009). Rapid expansion of planting material requires the implementation of a meristem bank to establish plantlets production schemes with the use of biotechnology techniques (LEAL; COPPENS D'EECKENBRUGGE, 1996).

The key means of regeneration is the use of meristematic tips (BOTELLA; FAIRBAIN, 2005). All these protocols have disadvantages related with the low coefficients of multiplication and the high cost and the low possibility of automation (WATT, 2012). That is why the explosive expansion of this industry depends on the existence of new technologies to facilitate automation and improved protocols to optimize the acclimatization of plants (KITTO, 1999).

However, the potential use of these procedures is limited by the low survival rates and plant growth during the acclimatization stage. Plants cultured *in vitro* are exposed to high levels of organic and inorganic nutrients, high relative humidity, low light intensity, and limited exchange of CO<sub>2</sub> and O<sub>2</sub>. As a result, *in vitro* cultivated plants have morphological and physiological abnormalities (BARBOZA et al., 2006). Those modifications make them very sensitive to the abrupt environmental changes experienced when they are removed from culture containers and placed under *ex vitro* conditions (KELLER; LUTTGE, 2005; BRAGA et al., 2011). As a result, a gradual acclimatization period is normally required after *ex vitro* transplantation to reduce stress as *in vitro* plants progressively acclimatize to the new growing condition (YANES; GONZÁLEZ; RODRÍGUEZ, 2000).

Bioplant Center developed a novel protocol for the production of *in vitro* cultivated plants based on the use of liquid medium and temporary immersion technology coupled with the implementation of a semiautomatic system. The system reduces the time required to generate sufficient quantities of plantlets for the creation of high quality pineapple plantlets banks. Using this technology, a pilot plant produced about one million 'MD-2' plantlets in a small growth chamber (ESCALONA et al., 1999). However, little is known about the morpho-physiological changes undergone by 'MD-2' plantlets during the acclimatization phase of the process. The objectives of this research were to characterize the morphological and physiological changes that occurred in plantlets during the acclimatization process. It is expected that the information would guide further research on improvements in acclimatization that would speed the process or improve rates of establishment under field conditions.

## MATERIALS AND METHODS

### Plant material and growth conditions.

Pineapple plantlets (*Ananas comosus* 'MD-2') were micropropagated according to the protocol proposed by Daquinta and Benegas (1997) using the crown buds as explants.

For Phase I (*in vitro* establishment), the culture medium contained 10 mg L<sup>-1</sup> Murashige and Skoog (1962) salts (MS), 1.0 mg L<sup>-1</sup> thiamine, 0.1 g L<sup>-1</sup> myo-inositol, 1.0 mg L<sup>-1</sup> naphthaleneacetic acid (NAA), 30 g L<sup>-1</sup> sucrose, 1.0 mg L<sup>-1</sup> 6-benzylamino purine (BAP) and 6.5 mg L<sup>-1</sup> agar. The culture medium for Phase II (multiplication) contained 10 mg L<sup>-1</sup> MS salts, 1.0 mg L<sup>-1</sup> thiamine, 0.1 mg L<sup>-1</sup> myo-inositol, 0.3 mg L<sup>-1</sup> NAA, 30 g L<sup>-1</sup> sucrose and 2.1 mg L<sup>-1</sup> BAP. For Phase III (pre-elongation) culture medium was prepared with 10 mg L<sup>-1</sup> MS salts, 1.0 mg L<sup>-1</sup> thiamine, 0.1 mg L<sup>-1</sup> myo-inositol, 0.5 mg L<sup>-1</sup> NAA and 30 g L<sup>-1</sup> sucrose. For the phase IV (rooting), the culture medium contained 10 mg L<sup>-1</sup> MS salts, 1.0 mg L<sup>-1</sup> thiamine, 10 mg L<sup>-1</sup> myo-inositol, 0.5 mg L<sup>-1</sup> NAA and 30 g L<sup>-1</sup> sucrose. Prior to sterilization, the medium pH was adjusted to 5.7 for all phases of the *in vitro* culture. Each *in vitro* phase lasted 45 days.

### *In vitro* culture conditions.

All *in vitro* manipulations were performed in aseptic conditions. The culture media were sterilized by autoclaving at 121° C at 118 kPa pressure. The plantlets were grown in transparent magenta plastic containers (7.2 x 8.6 x 9.2 (H) cm) with 40 mL of liquid medium. The photoperiod was of 16 h at a photosynthetic photon flux (PPF) of 75 μmol m<sup>-2</sup>s<sup>-1</sup> and 26±2° C of temperature during all phases.

### Environmental conditions in acclimatization phase.

Uniform micropropagated plantlets of ≥ 4.0 cm height, 7.0-8.0 g fresh weight and containing 5-6 true leaves were selected from the plastic culture vessels (ESCALONA et al., 1999), dipped in 3.0 mL L<sup>-1</sup> Previcur Energy® (Bayer Cropscience) for 5 minutes and planted in 1:1 (v:v) red ferralytic soil and filter cake (derived from sugarcane bagasse) in polyethylene bags (396.26 cm<sup>3</sup>) as containers for acclimatization (VILLALOBOS et al., 2012). Plantlets were acclimatized under 80% relative humidity (RH), 25.5° C temperature and photosynthetic photon flux (PPF) of 400-500 μmol m<sup>-2</sup> s<sup>-1</sup> as average for 45 d under natural photoperiods. During the first week, plants were misted for 15 seconds every 45 minutes beginning at 9:00 am each

day. A foliar fertilizer mix containing 16.0 g of crystalline N-P-K and 1.0 g of Multimicro Combi (Haifa Chemicals Ltd., Haifa Bay 26120, Israel) in 16 L of water was applied every ten days.

Morpho-physiological measurements were carried out at the end of *in vitro* rooting phase coincident with 0 d of acclimatization and at 15, 30 and 45 d thereafter.

### Morphological and physiological analysis.

Forty plants were collected at each time interval and data were taken on plant length (cm), number of leaves and roots; fresh weight (g), width and length of leaf 'D', defined as a leaf forming a 45 degrees with the vertical axis.

Chlorophyll was extracted from five leaf disks (0.28 cm<sup>2</sup>) taken from the 'D' leaves of five plantlets.

Disks were incubated in 3 mL DMSO at 65° C for 1 hour, and absorbance was measured at 645 and 663 nm. The chlorophyll concentration of the extracts was calculated using the equations described by Porra et al. (2002).

### Photosynthesis measurements.

Net photosynthesis ( $A$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) was measured at the ambient relative humidity and air temperature. The analyzer and cuvette (PP Systems CIRAS-2 Portable Photosynthesis System and PLC6 2.5 cm<sup>2</sup> cuvette, <http://www.ppsystems.com/>) were automatically calibrated before every measurement. Light was fixed at 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for the determination of the  $A/C_i$  curve, while the  $\text{CO}_2$  was fixed at 400  $\mu\text{mol mol}^{-1}$ , these values were obtained from saturation curve of light and  $\text{CO}_2$  in pineapple plants.

Measurement of  $A$  and total transpiration rate ( $T$ ,  $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$ ) were always made on the youngest fully expanded leaves and between 9:00 am and 10:00 am.

### Statistical analysis.

At each sampling day, six plants were randomly selected to measure photosynthesis and transpiration, 40 for counting leaves, width and length of leaf 'D' (cm), plant length, root number, 20 for measuring fresh weight and 10 for measuring chlorophyll content.

Analysis of variance was conducted using SPSS Program. Duncan's multiple range tests and Student's  $t$ -distribution were used for mean separation at  $p < 0.05$  level.

## RESULTS AND DISCUSSION

Total transpiration of *in vitro* leaves was highest at 0 d and declined significantly by 15 d after transfer to *ex vitro* conditions, after which it remained constant until the end of the experiment (Figure 1 A). Leaves initially became shorter and wider when plantlets were subjected to the higher light levels of the acclimatizing environment (Table 1). The low light levels during *in vitro* culture likely caused some etiolation of the leaves.

It appears that in only 15 d, the stomata become functional to control water loss since transpiration was stable from that point on. This observation is consistent with the fact that during acclimatization, the transpiration is gradually reduced as stomata become functional and cuticular water loss declines (HERRERA, 2009). When stomatal and cuticular transpiration rates in *in vitro* leaves are very high it is difficult to maintain plant turgidity after transplanting *ex vitro* (SEON et al., 2000).

Photosynthesis at 0 d was low at the end of *in vitro* phase and remained for the first 30 days after transfer to *ex vitro* conditions (Figure 1 B). The significantly higher level

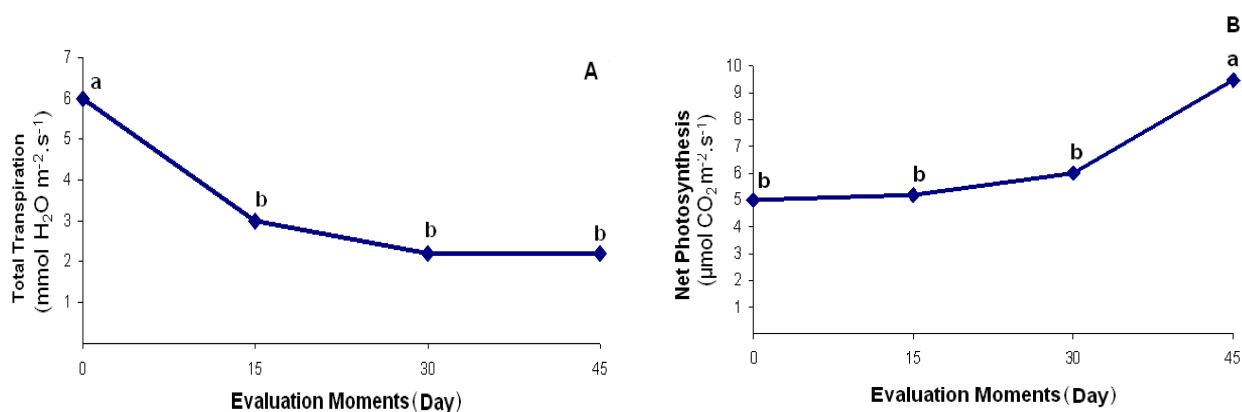


Figure 1 – (A) Behavior of total transpiration rate ( $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$ ) and (B) net photosynthesis ( $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ) in pineapple plantlets (*Ananas comosus* 'MD-2') during acclimatization. Means followed by different letters in rows are significantly different using ANOVA, Duncan's test,  $p < 0.05$ .

of photosynthesis at 45 d demonstrated that pineapple plantlets had adapted to increment of light intensity in the acclimatizing environment. The decline in transpiration and increase in CO<sub>2</sub> fixation by day 45 might indicate the plant was adapted to the new conditions during acclimatization phase and maybe of plants having Crassulacean acid metabolism (ZHU; BARTHOLOMEW; GOLDSTEIN, 2005). Ceusters et al., (2010) found in bromeliad, a photosynthetic plasticity in response to a range of environmental factors that include [CO<sub>2</sub>], water availability, light intensity and temperature.

Cote, Folliot and Andre (1993) found that *in vitro* pineapple plants required a few months and a plant fresh weight of about 30 g to complete the transition from C3-dependent photosynthesis to predominantly CAM assimilation. Thus, it seems unlikely that plants in this study, which weighed only about 11 g at 45 days after transfer to *ex vitro* conditions (Table 2), would demonstrate significant CAM activity.

In the evaluation at 'D' leaf width, it was unchanged at 15 d after plant transference to acclimatization after which width increased progressively with time to day 45 (Table 1). Plant length was significantly less after 15 and 30 d than it was at day 0, but by day 45 had regained the height at day 0.

Aragon et al. (2012) found that stress imposed by water deficit and higher photosynthetic photon flux

switched pineapple plants from C3 to CAM behavior. In our results we can find more number of roots, higher fresh weight and photosynthetic activity at 45 d in acclimatization period to report for this author at 56 d in CAM induced pineapple. Maybe, the environmental conditions used in acclimatización process (increase in light intensity and quality of natural light) were decisive factors for it results.

The effects of increment in light intensity and the gradual reduction of relative humidity at early stage were demonstrated in pineapple plantlets (*Ananas comosus*) by Yanes, González and Rodríguez (2000) and Silva et al. (2008).

Batagin et al. (2009) found an increase on cuticle thickness, on wavy contours of epidermal cells, and on the distribution and quantity of mesophyll fibers were observed during acclimatization process, evidencing the light conditions interference in morphological characteristics of the pineapple plantlets. *In vitro* propagation of pineapple has demonstrated that light is the factor with more influence on plant quality (GONZALEZ et al., 2005) and provides better agronomic and anatomical change during the acclimatization phase (DAMASCENO; SOUZA; GOMES, 2008).

During the acclimatization phase, plantlet increased 1.41 the root number, while stem diameter was 0.32 cm and the plant fresh weight reached 3.25 g. The main cause of fresh weight increase was found in the new emission of root, stem diameter, width of leaves and length of plants

Table 1 – Change of morphological variables of pineapple plantlets (*Ananas comosus* 'MD-2') during acclimatization.

Variables	Time of evaluation (days)				SE
	0	15	30	45	
Number of Leaves	9.80 a	8.76 c	9.01 c	9.36 b	0.090
Width of leave 'D'(cm)	1.40 c	1.54 c	1.73 b	2.02 a	0.024
Length of leave 'D' (cm)	10.17 a	8.73 c	9.24 b	9.56 b	0.140
Length of Plant (cm)	10.24 a	9.01 c	9.66 b	10.37 a	0.110

Means followed by different letters in rows are significantly different using ANOVA, Duncan's test,  $p < 0.05$ .

<sup>z</sup>Number of days after transplantation.

Table 2 – Change in morphological variables of pineapple plantlets (*Ananas comosus* 'MD-2') at the initial and final evaluation during acclimatization.

Variables	0	45 days <sup>z</sup>	SE
Root number	5.90 b	7.31 a	0.040
Stem diameter (cm)	0.73 b	1.05 a	0.014
Plant fresh weight (g)	8.10 b	11.35 a	0.060

Means with different letters in rows indicate significant (Student's t-  $p < 0.05$ ).

<sup>z</sup>Number of days after transplantation.

leaves during the experiment. In pineapple, the major dry-matter accumulation is associated with a high leaf area index and the ability to maintain their photosynthetic capacity for long periods of time (BARTHOLOMEW; MALÉZIEUX, 1994). However, in pineapple plantlets the fresh weight increased significant during 45 days, presumably due the new environmental conditions and the photoautotrophic behavior of the plantlets.

Generally, it is recognized that environmental change during *in vitro* to *ex vitro* conditions cause stress in plants, expressed in terms of accumulation of abscisic acid (ABA), proline and reactive oxygen species (CARVALHO et al., 2006; BATROVA; POSPOSILOVA; SYNKOVA, 2008). However, results on *in vitro* pineapple have shown that it is possible to affect the carbon metabolism by changing the environmental temperature during the light and the dark period (NIEVOLA et al., 2005). Temperature and plant heights also had influences notably in the morphological variable in plants cultivated in a greenhouse (DAMASCENO; SOUZA; GOMES, 2008). In our results, apparently, no prolonged stress was observed during acclimatization phase, demonstrated by the growth of the evaluated variables in all process.

After transfer of plantlets to acclimatization condition a sharp and not significant decrease in chlorophyll a and b was observed during the first 15 days (Table 3) and was constant until 45 d. The chlorophyll a and b content continued to decrease until 45 d (8.1 and 3.8  $\mu\text{g g}^{-1}$  FW respectively). This higher content in chlorophyll in young *in vitro* leaves is indicative of adaptation to shade. Under these conditions, the light intercepting units are extensive, and higher chlorophyll are associated with higher investment of the plant into light harvesting complex of photosystem II (LHC II), and antenna pigments. This might be explained by a rapid dismantling of the light harvesting antenna to avoid over-reduction of the photosynthetic electron chain, which may lead to photoinhibition (BATROVA; POSPOSILOVA; SYNKOVA, 2008).

Chlorophyll a/b ratio showed no significant reduction (0.02  $\mu\text{g g}^{-1}$  FW) at the times of transfer to different environmental conditions during the first 30 d. This is not in contradiction with the result found by Fila et al. (2006) that recorded high chlorophyll contents in plantlets growth under *in vitro* condition compared with *ex vitro* acclimatized.

Table 3 – Change in chlorophyll a, chlorophyll b, and total chlorophyll content in pineapple plantlets during acclimatization phase.

Chlorophyll b ( $\mu\text{g g}^{-1}$ FW)	0	15	30	45 days <sup>z</sup>
Chl a	90.1 a	87.0 ab	85.3 ab	82.0 b
Chl b	70.0 a	68.2 ab	67.4 b	66.2 b
Chl a+b	160.1 a	155.2 ab	152.7 b	148.2 b
Chl a/b	1.28 a	1.27 a	1.26 ab	1.23 b

Means followed by different letters in rows are significantly different using ANOVA, Duncan's test,  $p < 0.05$ .

<sup>z</sup>Number of days after transplantation.

### CONCLUSIONS

At 30 days, pineapple plantlets showed photoautotrophic conduct, due to the new formation of roots and new emission of photoautotrophic leaves.

Photosynthetic activity was tolerant of relatively high environmental stress. Thus, there was significant acclimation of photosynthesis until a maximum was reached at 45 d in the *ex vitro* environment, which suggests that high photosynthetic capacity was acquired slowly, and thus impacted strongly on subsequent growth and physiological development of the plants while chlorophyll content showed no significant reduction during the first 30 d.

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