Valeria Paloma Ferrario Bazalar

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Orientador: Prof. Dr. Gilberto Barbante Kerbauy

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“Science, my boy, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.”

— *Jules Verne, Journey to the Center of the Earth.*

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ABSTRACT

Epiphytic orchids are important components of the tropical and subtropical forests. In the epiphytic environment these are subjected to many stressful conditions, among which stand out intermittent rainfall or even drought. The strategies these orchids employ to survive and develop in such conditions are of great interest between researchers. In the present study we investigated some of the effects of water deficit on the physiology, biochemistry and morphology of protocorms of the epiphytic orchid *Laelia lobata* Lindl. The protocorm is a post-embryonic stage of the orchids, considered a simple and unique structure which is particularly unprotected but with a high ability to survive in a highly constrained environment. To mimic water shortage conditions, we used three different osmotic treatments adding polyethylene glycol (PEG) 6000 to a previously selected culture medium. It was observed that the decrease in water potential increased the survival of the protocorms, their photosynthetic activity, and induced both a large accumulation of cuticle waxes as well as deep changes in the waxes composition. Moreover, it was also observed that *L.lobata* protocorms have stomata on the whole surface and present values of δ^{13} (-15‰ ~ -14‰) which indicated that they putatively display crassulacean acid metabolism (CAM). On the other hand, the osmotic treatments provoked a marked reduction in the protocorms growth, in total soluble sugars content, cell wall monosaccharides, polyols and other carbohydrates, which are thought to be accumulated to act as osmoprotectans. The data contributes to a better understanding of the effects of water shortage on the protocorms of *L.lobata* and some of the tolerance mechanisms they display to cope with such stressful condition, highlighting that some responses differed from what was expected according to literature.

RESUMO

As orquídeas epífitas são componentes importantes das florestas tropicais e subtropicais. No ambiente epifítico estas estão sujeitas a várias condições estressantes, dentre as quais destacam-se as chuvas intermitentes ou até a seca. As estratégias empregadas por estas orquídeas para sua sobrevivência e desenvolvimento nessas condições são de grande interesse entre os pesquisadores. No presente estudo, investigamos os efeitos do déficit hídrico na fisiologia, bioquímica e morfologia de protocormos da orquídea epífita *Laelia lobata* Lindl. O protocormo trata-se de um estágio pós-embrionário do desenvolvimento das orquídeas, considerado como estrutura peculiar e que a despeito da sua simplicidade estrutural apresenta uma elevada capacidade de sobrevivência em um ambiente altamente restritivo. Para imitar as condições experimentais de restrição hídrica, foram empregados três tratamentos osmóticos diferentes, obtidos por meio da adição de polietilenoglicol (PEG) 6000 a um meio de cultura previamente selecionado. Foi observada que a diminuição do potencial hídrico aumentou a sobrevivência dos protocormos, sua atividade fotossintética, matéria seca e induziu um grande acúmulo de ceras de cutícula, além de profundas alterações nos componentes destas. Também foi observado que os protocormos de *L. lobata* possuem estômatos em toda a superfície e apresentam valores de δ13 (-15 ‰ ~ -14 ‰) o que putativamente indicaria um metabolismo ácido das crassuláceas (CAM). No entanto, foi observado que os tratamentos osmóticos causaram decréscimos no crescimento dos protocormos, assim como nos teores de açúcares solúveis totais, monossacarídeos da parede celular, polióis e outros carboidratos, sendo que estes normalmente são acumulados para atuarem como osmoprotetores. Os dados obtidos contribuem para uma melhor compreensão dos efeitos da escassez de água sobre os protocormos de *L.lobata* e alguns dos mecanismos de tolerância que eles exibem para lidar com essa condição estressante, destacando que algumas das respostas diferem das esperadas segundo a literatura.

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1. GENERAL INTRODUCTION

The importance of water is undeniable; it is one of the most important substances on earth and indispensable for the development of life. The variety and amount of vegetation throughout the planet depends on water availability more than any other single environmental factor (Kramer & Boyer, 1995). The water shortage limits the growth and normal development of plants as a consequence of internal imbalances. However, the severity of the impact on the plant depends on the stress degree and time, the species and the developmental stage (Demirevska *et al.*, 2009).

Water scarcity triggers a cascade of responses which affect both the physiological and biochemical processes of plants development, such as the plasmatic membrane, water potential, enzymatic activities, stomata closure, chlorophyll synthesis and photosynthetic activity, hormone levels, respiration ratio, etc. (Wang *et al.*, 2002; Silva *et al.*, 2009; Samarah *et al.*, 2009; Fujita *et al.*, 2011; Yang *et al.*, 2016).

Epiphytic plants inhabit an environment subjected to multiple abiotic stressful conditions, in particular the intermittence of water availability. The declining of epiphytes diversity along geographic rainfall gradients compared to other plants is an evident proof of that limitation (Gentry & Dodson, 1987).These plants have evolved, adapted and survived under such circumstances and among all of them the Orchidaceae family stands out by their prevalence, features and beauty.

The scope of this investigation is to find out which are the physiological, biochemical and morphological effects of water deficit in the protocorms (pro-embryos) of the epiphytic orchid *Laelia lobata* (Lindl.) and the presumable tolerance mechanisms; starting from those who have been seen in previous research in other plants. Since our study model, the protocorm is a tiny nude pro-embryo without neither cotyledon nor endosperm that manages to survive in nature under stressful conditions emphasizing the intermittence of water availability.

1.1. Epiphytes main characteristics

An epiphyte is a plant that grows on other plants, using them as mechanical support and not parasitizing them. Their botanical hosts are call phorophytes and lacking of a ground to access to nutrients and water, they draw these with less conventional alternatives (Benzing, 2004; Zotz, 2016).

Their existence, according to fossils, date from the Carboniferous and many modern species yet present some ancient patterns. Overtime, these plants evolved into a peculiar habitat growing far from the ground and instead living above the trees and other plants under a constrained environment, adapting to adverse conditions which make them a great group for studies related to water balance, nutrition, reproduction and evolution (Benzing, 2004).

They are responsible for much of the biodiversity that makes humid tropical forests the most complex of all the world's terrestrial ecosystems (Gentry & Dodson, 1987). As Lüttge (2012) said, environmental factors imposing stress are not only considered as the forces driving evolution but also as parameters underlying ecophysiological diversity.

There are few vascular types found in mid or higher latitudes (Hsu *et al.*, 2002), some of them occur in dry sites such as the cactus/shrub forests in Mexico and Peru. Temperatures below freezing limit the geographical distribution of most epiphytes; therefore, with the exception of lichens and bryophytes, relatively few species are found outside the tropics (Benzing, 2008).

To cope with the stressful conditions, epiphytes exhibit well-developed structures and physiological mechanisms to tolerate drought and avoid photo-injury that are rather distinctive or more common among vascular epiphytes when compared to soil-rooted flora (Benzing, 1998). For example, the foliage of the epiphytes has some expected traits, Orchidaceae (Dressler, 1981), Bromeliaceae (Benzing & Bennett, 2000) and Melastomataceae (Reginato *et al.*, 2009) exhibit leaf succulence because of their water-rich parenchyma cells.

Among the 28,000 species of vascular epiphytes (Zotz, 2013), there are two distinct groups especially dominant among epiphytes, Bromeliaceae and Orchidaceae (Benzing, 1987).

Bromeliads have impounding tanks and leaf scales (Zotz, 2016). The leaf scales also known as trichomes reflect infrared radiation reducing the heat load of leaves exposed to sunlight. Among atmospherics (a peculiar bromeliad stage of development), has been observed a correlation between the amount and dimension of leaf scales that and the moisture of the environment. It is thought that they serve to reduce transpiration by increasing boundary layer thickness (Benzing, 1976).

Epiphytic orchids form pseudobulbs unlike terrestrial, what matches the idea of the pseudobulbs as a representation of the shift from a terrestrial to epiphytic habit (Freudenstein & Chase, 2015). They are utterly associated to water and mineral nutrients storage (Hew, 1996; Ng & Hew., 2000)**.** They also have huge tangle aerial roots, as in *Catasetum* species (Oliveira, 2019) in addition to the presence of the velamen radicum which are adaptations of the roots in orchids (Zotz & Winkler, 2013).

Another so called adaption of epiphytes is the seeds size. Approximately 75% of the genera have seeds that are less than 1 mm in length, favouring them to be dispersed by wind or transported by rain drops, besides they can easily accommodate in tiny spaces like cracks on barks to germinate and grow (Baskin & Baskin, 1998).

1.2. Adaptations and mechanisms for tolerance to water deficit

Tolerance water limitation involves numerous changes, including a reduction in growth, the activation/increased expression of tolerance related genes, the increasing of abscisic acid levels, the accumulation of osmolytes and antioxidants among others (Bartels & Sunkar, 2005). Certainly, the type of response depends on the species, the intensity and length of the stressful condition, the developmental stage and the presence/absence of reserve organs.

As the flow of water into the plants is a matter of great significance, plants look forward to preserve the internal water and minimize its loss. Therefore, their initial response is to accumulate organic and inorganic solutes to maintain cell turgor. This is known as osmotic adjustment or osmoregulation and consists of an accumulation of compatible solutes; they accumulate in the cytoplasm without prejudicing the cells even in great concentrations and neither interact with other compounds (Yancey *et al.*, 1982). The most common osmoprotectants are the aminoacid proline, nonstructural carbohydrates as sugar alcohols, raffinose series oligosaccharides, trehalose, sucrose and hexoses (Stancato *et al.*, 2001; Bartels & Sunkar, 2005; Gupta & Kaur, 2005; Anjum, 2011).

While the stress is prolonged may cause oxidative stress which gives rise to the appearance of reactive oxygen species (ROS). The accumulation of ROS can provoke both negative consequences in the plant but also triggers tolerance mechanisms to neutralize the negative effects (Bailey-Serres & Mittler, 2006).

The accumulation of the phytohormone abscisic acid (ABA) is one of the tolerance responses induced by ROS, and this in turn controls downstream stress responses (Wang *et al.,* 2002; Bernacchia & Furini, 2004; Vishwakarma *et al.,* 2017). ABA promotes stomatal closure hence reducing internal water loss. However, this affects carbon dioxide (CO_2) uptake promoting a reduction in the photosynthesis rate (Cornic, 2000; Seki *et al.,* 2007; Osakabe *et al.,* 2014).

To counter osmotic stress some plants accumulate polysaccharides in the form of mucilages inside some particular cells called idioblasts. They are usually much larger than the surrounding cells and are present in many genera including orchids, where the mucilage is composed by glucomannans associated to oxalate raphides (Meier & Reid, 1982). These glucomannans are water-soluble polysaccharides that represent a major reserve of carbohydrates in epiphytic orchids (Stancato *et al.*, 2001; Sezik, 2002; Sailo *et al.*, 2014

Structural carbohydrates related to cell wall composition may also play a role in drought tolerance. This is in behalf of the cell wall elasticity (CWE); the increase of CWE has been correlated to plant drought tolerance, contributing to the maintenance of cell turgor.

Martínez *et al.* (2007) studied six cultivars of common bean under drought stress and observed a higher CWE in the most drought resistant cultivars which may reflect differences in the wall structure that could contribute to a higher resistance to water stress.

A physiological adaptation largely found in vascular epiphytes (Zotz, 2004) is the Crassulacean acid metabolism (CAM); this pathway permits some plants to survive in semiarid habitats or habitats with low or infrequent water availability.

CAM is one of the three modes of photosynthesis and implies a temporal separation where the CO_2 uptake occurs at night and its fixation at daytime (Cushman, 2001). As the carbon dioxide is taken during the night, water loss by transpiration is reduced (Zotz, 2004). In some bromeliad the photosynthesis can be facultative under drought conditions (Maxwell *et al.*, 1994; Mioto & Mercier, 2013; Rodrigues *et al.*, 2013) however this changeable photosynthetic pathway has not been seen in epiphytic orchid species so far.

Water loss in plants as a consequence of water scarcity in the environment, not only occurs through stomata but also across the cuticle. The development of a water-resistant cuticle was fundamental since the colonization of plants in land; it constituted the first barrier among the hostile environment and the plant (Edwards *et al.*, 1996).

Plants possess waxy cuticles, whose nature is hydrophobic and are composite by biopolymers, cutin and cuticular lipids. These vary in composition and structure depending on the species (Bargel *et al.*, 2006; Shepherd & Griffiths, 2006).

The cuticular waxes are accumulated rapidly under water stress (Premachandra *et al.*, 1991).Kim *et al.* (2007) observed in sesame plants leaves submitted to drought, an increased deposition of waxes (especially in the amount of alkanes). Cominelli *et al.* (2008) studied the gene AtMYB41 in *Arabidopsis* which encodes the transcription factor R2R3-MYB. This gene turned out to be greatly expressed in response to drought, salt high concentrations or ABA treatments. Transgenic lines overexpressing MYB41 demonstrated to induce wax accumulation and cell expansion (a role related to cell wall modification), cuticle synthesis and deposition. Oliveira *et al.* (2003) observed differences between the constitution and thickness of waxes of caatinga species and cerrado species wherein the components promoted a reduction in water permeability resulting in a greater resistance to water evaporation.

1.3. Epiphytic orchids

Among many plant species, orchids have evolved exceptional adaptations due to adverse environmental conditions, particularly because of a limited access and conservation of water (Pridgeon *et al.*, 2001). They were originated as terrestrial forest understory herbs approximately 76-85 million years ago (Ramírez *et al.*, 2007) and were widely extended in many different habitats (Zotz, 2013, 2016) by adapting and modifying their stems, leaves, reproductive structures, seeds and roots (Arditti, 1980; Benzing, 2004).

There is a great prevalence of CAM in epiphytic orchids (Hansen, 2016) which provides important water economy by stomata closure during the day (Lüttge, 2004) and of which the Epidendroideae subfamily is the richest in epiphyte CAM species (Kerbauy *et al.,* 2012).

Some of their most important features are the roots. In addition to serve as anchor to trees or rocks and absorb water and nutrients, they are photosynthetic and act as a link between the orchid and mycorrhizal fungus. These roots have a spongy layer of cells consisting of multiple epidermises known as velamen radicum. It is assumed to rapidly uptake water and nutrients and retaining them (Zotz & Winkler, 2013). Moreover, according to Pridgeon (1987) the fact that the velamen is whitey makes it reflect solar radiation in exposed roots protecting them from overheating and slowing the root transpiration (Benzing *et al.*, 1982).

Another interesting peculiarity is the modification of their stems, which have enlarged portions that form the so-called pseudobulbs whose function is the storage of water, nutrients and carbohydrates and also have photosynthetic activity. The pseudobulbs as well as the leaves have thick cuticles to diminish moisture loss (Benzing, 2004).

1.3.1. *Laelia lobata* Lindl.

Orchidaceae is one of the two largest families of flowering plants. Chase *et al.* (2015) recognized 736 genera in Orchidaceae. The epiphytic orchid *Laelia lobata* (Lindl.)

belongs to the subfamily Epidendroideae which is one of the five subfamilies that constitute Orchidaceae which comprises the largest number of orchids, approximately 21.160 species (Freudenstein & Chase, 2015).

L.lobata is endemic to Rio de Janeiro (Brazil) and known to occur on inselbergs, a peculiar habitat that consists on rock formations that arise within a humid tropical forest (Constantino & Fraga, 2005). They are considered epiphytes but also lithophytes, this plasticity is fairly common between orchid species, increasing their options to colonize adjacent microenvironment (Barros, 1990).

This species has a recognized ornamental value because of the size and color of their flowers, which has led to its indiscriminate extraction from nature , and furthermore these localities are greatly affected by tourism (rock climbers) and fires during the dry season (Gomes *et al.*, 2017); for that reason, currently CITES considers *L. lobata* as an endangered species.

1.3.2. **Characteristics of a protocorm**

Epiphytic orchid as the majority of epiphytic plants, have very peculiar seeds.

An orchid seed pod contains millions of tiny seeds inside (Gregg, 1991; Arditti & Pridgeon ,2013).These are so small that are called “dust-like seeds”, their size allow them to be transported by the wind. Their length goes from 0.05 mm to 6 mm, and have a loose thin-textured seed coat that make them very light (Arditti & Ghani, 2000; Benzing, 2004; Arditti & Pridgeon, 2013).

Orchids need a mandatory symbiotic association with mycorrhizal fungi to germinate. However, Knudson (1946) demonstrated that the majority or epiphytic orchids can germinate asymbiotically in suitable culture medium supplemented with a carbon source (sugar).

The orchid embryos lack of endosperm and cotyledon, so the nutrient reserves are stored directly in their cells (Baskin & Baskin, 1998). These reserves are mostly lipids (Richardson *et al.*, 1992) but also protein bodies (Arditti, 1980).

The endosperm is absent because it fails to develop during the embryo development. In other plants this normally results in the embryo abortion (Lester & Kang, 1998) and even though the orchid embryos manage to survive, this restricts them further histodifferentiation beyond the globular stage. In other words, the orchid embryo doesn't pass through all the development stages; it passes from the globular stage to becoming a protocorm (Yeung, 2017).

Unfortunately there is still no clear and outright definition of the term "protocorm". In the midst of the definition controversy some authors have describe it as a group of parenchymatic cells delimited by a unistratified epidermis without cotyledon nor endosperm, a stage of development right before the appearance of the first adventitious root which takes place after the differentiation of the first leaves (Alvarez and Sagawa, 1965; Kraus *et al.*, 2006; Pereira *et al.*, 2015). On that basis, the present study was carried out.

2. HYPOTHESIS AND OBJECTIVES

From the literature and previous research, we have come to know how water deficit affects the plants development and some of their tolerance mechanisms. Since epiphytic orchids are highly exposed to water shortage and they arise as a peculiar and sensitive "nude" pro- embryo in the nature, we wondered how this tiny protocorm manages to survive in such adverse conditions. For this reason, we choose the protocorms of *Laelia lobata* Lindl. as biological model, being the main goal of the present investigation to determine how water deficit affects them physiologically, biochemically and morphologically, and identify some putative tolerance mechanisms using as a baseline the results seen in other vascular plants.

For this purpose, we outline the following specific objectives:

- Determine the changes that occur in the protocorms due to different levels the osmotic stress, such as:
    - **a.** Water content.
    - **b.** Osmotic potential.
    - **c.** Survival.
    - **d.** Growth.
    - **e.** Non-structural and structural carbohydrates.
    - **f.** Cuticle wax.
    - **g.** Photosynthetic pigments and chlorophyll fluorescence.
- Determine the *L. lobata* photosynthetic pathway through the carbon isotope discrimination and describe its external morphology through the observation under a scanning electron microscope.

3. MATERIAL AND METHODS

3.1. Plant material and growth conditions

Laelia lobata Lindl. seeds were germinated in Vacin and Went (1949) modified medium with Phytagel 0.23% as gelling agent. The capsules of the orchid were disinfected with commercial sodium hypochlorite 30% and some drops of commercial liquid detergent for 30 minutes. Subsequently, they were rinsed three times with sterilized distillated water and then transferred to the culture medium.

The seeds were then incubated at 20 μmol $m^{-2}s^{-1}$ of light intensity emitted LED lamps, temperature 25°C ±2, and 12 hours of photoperiod. They were kept under the described conditions for four months (time needed to germinate and turn into a protocorm in this species).

Figure 1. Protocorm of *Laelia lobata* after four months in growth culture medium, displaying the presence of the first two leaves and no roots.

3.2. Osmotic stress treatments

The protocorms were transferred into a liquid culture medium consisting of Vacin & Went solution (without Phytagel) and three different concentrations of polyethylene glycol-6000(PEG). PEG was used as osmopriming agent; in other words, it was used to imitate water stress conditions in plants, decreasing the water potential of the medium without penetrating plant cells.

The treatments used the next PEG concentrations: 10%PEG (T1), 15%PEG (T2), 30%PEG (T3); and our control group was kept in Vacin & Went liquid medium without PEG (T0).

Each treatment consisted of five biological replicas adding a total of twenty flasks. We used 15 mL of medium per flask, volume necessary to cover the mass of protocorms. These flasks were daily hand agitated and under the same incubation conditions as previously described for seeds germination and. The osmotic treatments were imposed for forty days.

It is important to emphasize that due to the small size of the protocorms each biological replica was represented by the total mass of protocorms of each flask, which was composed by several protocorms, not individuals.

In view of their size it was imperative to germinate a large amount of seeds to reach the amount of mass required for all the schemed experiments.

After the treatments, the protocorms were harvested and stored at -20 °C unless they were needed to be used fresh for any of the experiments.

3.3. Water content determination

Representative aliquots were weighed before and after being lyophilized, and then the following formula was used to determine the percentages of water content:

$$\%\text{Water content} = \frac{(Fresh\ weight - Dry\ weight)}{Fresh\ weight} \times 100\%$$

3.4. Medium osmotic potential and plant tissue osmotic potential

Samples of liquid Vacin &Went medium with the different PEG concentrations were analyzed in an osmometer as well as the plant extracts obtained from freshly collected protocorms submitted to the treatments.

To obtain the plant extracts, the protocorms were crushed and centrifuged for five minutes at 2400 rpm. The supernatants were extracted and filtered with Millipore filter (0.22 µm) and then stored at -40°C.

The measurement of the osmotic potential was made with the vapor pressure Osmometer 5520 (Wescor Vapro ®, USA) facilitated by the Ecophysiology Laboratory of the Botany Institute of São Paulo.

3.5. Survival

To determine the percentage of survival of the *Laelia lobata* Lindl. protocorms, we picked one hundred individuals in good conditions, i.e. turgid and green, and submitted them into the osmotic stress treatment as described beforehand.

After the treatment period, the dead and surviving protocorms of each sample population were counted meticulously. We considered as dead those who turned brown and flaccid, and alive those who remained turgid and green.

3.6. Size measurement

To determine how the treatments affect the growth of the protocorms we measured the surface area of the protocorms. Ad hoc, fresh protocorms were harvested and placed into the Chlorophyll Fluorescence Imager CF0040 (Technologica Ltd., UK) to be photographed. These photos were seized for this experiment since the equipment was mainly used to determine chlorophyll fluorescence.

The ImageJ 1.50i software was used for the analysis of the photos and the measurement of the surface area of each protocorm.

3.7. Carbohydrates analysis

For the following experiments we used lyophilized and grounded plant material.

Total soluble sugars

The extraction of soluble sugars was made according to the Colorimetric method using phenol- sulfuric acid by Dubois *et al.* (1956). It starts with a sequence of extractions with ethanol 80% at 80 °C until the total extraction of the soluble sugars. The extracts were collected and read in a cuvette UV Vis spectrophotometer Ultrospec 3000 (Pharmacia Biotech, UK) to obtain the absorbance values. With these data and a glucose standard curve we determined the concentration of total soluble sugars in each sample.

Sugars from the ethanolic extract

One ml of each ethanolic extract was dried in a CentriVap Concentrator Vacuum (LABCONCO©, USA). The dried samples were resuspended in 100µL of methanol and derivatized with 50µL of pyridine and 50 µL of N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) in dry bath at 75°C for one hour. The samples were analyzed with a gas chromatograph-mass spectrometer (GC-MS) GCMS-QP2010 SE (SHIMADZU©, Japan). The chromatograph was equipped with a fused-silica capillary column (30m, ID 0.25 mm, 0.25 µm thick internal film) DB-5 MS (Agilent Technologies, USA) stationary phase using helium as the carrier gas at a flow rate of 24 mL min^{-1}. The injection volume was 1µL and the heating ramp of the column followed the next programming: initial temperature of 100 °C to 300 °C in at a rate of 6°C · min^{-1}, and final temperature maintained for ten minutes.

The identification and quantification of the sugars was made by corresponding peaks of standards and calibration curves.

Starch dosage

After the extraction of the soluble sugars, the leftover pellets were processed according to Amaral *et al.* (2007). Then, aliquots were taken and Glucose PAP Liquiform reagent was added. This gives a reddish coloration to the solution allowing the determination of the concentration of glucose by colorimetry. The absorbance values were determined in a Multiskan FC® Microplate Photometer (Thermo Fisher Scientific, USA) at 490nm. The concentrations were determined with a standard curve.

Glucomannans

The leftover pellets from the starch extraction were rinsed with ethanol 80% and then dried and grounded. From these pellets 1mg of each was aliquoted and solubilized with 47.9µL Ammonium Acetate buffer solution (pH 5.0). They were left overnight in gentle continuous agitation at 30°C.

When totally solubilized, 1.25µL of mannanase was added and the samples incubated in agitation at 40 °C for 24 hours. After the enzymatic digestion, they were boiled for 5 minutes to finish up the enzymatic reaction. Once the samples were cooled down, were centrifuged and filtered with Millipore filters (PVDF 0, 22 µm).

The quantitative analysis was performed by high-performance anion exchange chromatography (HPAEC-PAD) equipped with a CarbonPac PA-100 (ICS-3000 system, Dionex- Thermo®) eluted with 88mM sodium hydroxide and 200mM sodium acetate (0.9 ml · min^{-1}) for 45 min.

This experiment was made in collaboration with the Laboratory of Ecological Physiology of Plants (LAFIECO) in the Institute of Biosciences of the University of São Paulo.

Cell wall monosaccharides

Aliquots consisting of 2 mg from the leftover pellets of the starch extraction were taken and 1ml of Trifluoroacetic acid (TFA) 2M was added, incubated at 100°C for 1 hour at 1250 rpm of agitation and totally dried in an Acid Resistant CentriVap Concentrator Vacuum (LABCONCO©, USA).

The samples were resuspended by adding 1 ml of MilliQ water and filtered with Millipore filters (PVDF 0, 22 µm). The quantitative and qualitative analysis was made with the HPAEC- PAD of the LAFIECO in the Institute of Biosciences of the University of São Paulo. The volume of injection was 10µL and the analysis was made through the injection of hydrolysate into a CarboPac SA10 column (ICS 5.000, Dionex-Thermo®). The column was eluted isocratically with 99.2% of water and 0.8% sodium hydroxide (1mL $\cdot$ min^{-1}). To identify the monosaccharides we used a post-column containing 500 mM of NaOH (0.5 mL $\cdot$ min^{-1}) and standard solutions of fucose, arabinose, galactose, rhamnose, glucose, xylose, and mannose.

Histological analysis for the determination of mucilaginous idioblasts

The protocorms were fixed with Karnovsky's fixative under vacuum for 24 hours and washed with phosphate buffer (0.1M). Then dehydrated with an ethanol and embedded into a mixture of 95% ethanol and pure resin 1:1 (Historesin Leica, prepared according to manufacturer's instructions) for eight hours. For the infiltration we used pure resin and placed the samples into a desiccator under vacuum, this resin was renewed every 24 hours for three days. Later, the protocorms were embedded with a mixture of resin and a polymer.

The blocks were sectioned with a rotary Microtome HM 340E (Thermo Scientific™, USA) and the sections stained with toluidine blue (O'Brien *et al.*, 1964). For the analysis and photographic documentation was used a Leica DM LB light microscope coupled to a digital Leica DFC320 camera (Leica Microsystems, Germany).

This experiment was made in collaboration with the Laboratory of Plant Anatomy of the Institute of Biosciences of the University of São Paulo.

3.8. Extraction, quantification and identification of epicuticular waxes

For the extraction of cuticular waxes we used dichloromethane (CH_2Cl_2) as solvent. To ensure no contamination, all the glassware used was previously rinsed with the very same solvent.

The protocorms were weighed and then the extraction was made by successive immersions in dichloromethane three times during 30 seconds each (modified from Fernandes *et al.*, 1964). The solvent was evaporated, and the concentrated wax resuspended in dichloromethane and transferred to new vials with known weight. The extracts were taken to evaporation and maintained within a desiccator until reaching a constant mass. The total wax was calculated (µg) and the values expressed on dry mass basis.

The waxes were resuspended in dichloromethane and derivatized with a solution of 50:50 µL of N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine at 80°C for 40 minutes in dry bath (modified from Jetter *et al.*, 2000). The analysis was made by GC-MS with a System Agilent 6850/Agilent 5975C equipped with a capillary column Agilent HP5-MS (30m x 250µm x 0.25µm). The injection volume was 1µL and helium was used as carrier gas with a flux of $1mL \cdot min^{-1}$. The injector temperature was adjusted to 300°C and the heating ramp of the column followed the next setting up: initial temperature of 100 °C for 5 minutes, and 5°C per minute until reaching 320 °C (final temperature) held for 8 minutes. The total time employed for the analysis was of 57 minutes. Temperatures of MS source and quadrupole were adjusted to 230°C and 150°C, respectively. The electron impact ionization was 70 eV. MS spectra obtained in full scan, with mass range of 50 to 800 with 1.99 scans per second and threshold of 50. The wax compounds were identified by comparison of MS fragmentation patterns using NIST digital library (v2.0, 2008), retention time of authentication standards.

All these procedures were made in collaboration with the Laboratory of Phytochemistry of the Institute of Biosciences in the University of São Paulo.

3.9. Photosynthetic pigments

Chlorophylls *a* and *b*, and carotenoids were determined by the method established by Porra *et al.* (1989) with modifications according to Wellburn (1994) and Minocha *et al.* (2009).

Stored samples (at 4°C) were grounded in liquid nitrogen and aliquots of 10 mg were transferred to 1.5 ml microtubes. For the extraction, it was used 1mL of N, N-Dimetilformamida (DMF) and the samples were kept in darkness and constant agitation for 24 h.

The extracts were centrifuged at °4C for 10 minutes at 13000 rpm and the absorbance was determined using the UV-Vis spectrophotometer Ultrospec 3000 (Pharmacia Biotech, UK) at the following wavelengths: 664nm, 647nm, and 480 nm. To calculate the concentration of the pigments the following formulas were used:

$$\text{Chlorophyll } a\ (\mu g.\ g\ FW^{-1}) = (12 \times A664) - (3.11 \times A647)$$

$$\text{Chlorophyll } b\ (\mu g.\ g\ FW^{-1}) = (20.78 \times A647) - (4.88 \times A664)$$

$$\text{Total Chlorophylls } (\mu g.\ g\ FW^{-1}) = \text{Chlorophyll } a + \text{Chlorophyll } b$$

$$\text{Carotenoids } (\mu g.\ g\ FW^{-1}) = \frac{(1000 \times A480) - (1.12 \times Chlorophyll\ a) - (34.07 \times Chlorophyll\ b)}{245}$$

3.10. Chlorophyll Fluorescence analysis: Operating Efficiency of the photosystem II (PSII)

Chlorophyll fluorescence imaging is a rapid and noninvasive technique that permits to monitor the fluorescence parameters of numerous plants at the same time under equal conditions. It is a sensitive method for assessing the PSII fluorescence activity in response to abiotic factors (Murchie & Lawson, 2013).

The operating efficiency (Fq'/F_m' or ϕPSII) gives the proportion of absorbed light (quantum efficiency) that is actually used in PSII photochemistry in a light –adapted state (Genty *et al.*, 1989; Maxwell & Jhonson, 2000; Baker, 2008).

For this experiment we used 30 protocorms as representatives of each biological replica and measured the operating PSII efficiency with the Chlorophyll Fluorescence Imager CF0040 (Technologica Ltd., UK) following the next programming: (i) 2s of actinic light PPFD 20 µmol $m^{-2}s^{-1}$, (ii) 2 minutes of pulse (1 cycle) PPFD 6120 µmol $m^{-2}s^{-1}$.

3.11. Scanning Electron Microscopy (SEM)

The protocorms were fixed in FAA (18:1:1 of 50% ethanol, acetic acid and formalin), for 24 hours, dehydrated in a grades ethanol series and dried by critical point using Balzers critical point dryer 030 (Balzers, Germany). Subsequently, the material was mounted on aluminum stubs and sputter coated with gold with a Balzers SCD-050 sputter coater (ONLINK Technologies GmbH, Germany).

The samples were observed and photographed with a Zeiss DSM 940 scanning electron microscope (Carl Zeiss, Germany).

3.12. Carbon isotope discrimination ($\delta^{13}C$)

Crassulacean acid metabolism (CAM) is one of three metabolic pathways found in vascular plants. This photosynthetic via is considered an adaptation to environments with low water supply. In this experiment it was pretended to investigate whether the epiphytic orchid *Laelia lobata* Lindl. was a CAM plant, based in the analysis of its $\delta^{13}C$.

Several studies have demonstrated that plant $\delta^{13}C$ is related to different photosynthetic pathways and to leaf gas exchange characteristics. Because of differential enzyme-mediated discrimination against $^{13}CO_2$ during photosynthetic carbon assimilation between CAM and other photosynthetic pathways (Bender, 1968) it is possible to determine whether a plant is CAM, C_4 or C_3 (Silvera *et al.*, 2005).

For the experiment we used two groups of samples: one composed of three replicas of an only leaf of an adult *Laelia lobata* Lindl. grown and raised in the green house of Department of Botany of the Biosciences Institute under normal conditions (no stress imposed); the second group was constituted by three replicas of the control group of protocorms grown in vitro.

All samples were weighed, macerated and grounded in liquid nitrogen as soon as they were collected. The analyses were performed in the UC Davis Stable Isotope facility of the Department of Plant Sciences of the University of California (USA).

3.13. Statistical analysis

All the data were analyzed with the STATSOFT STATISTICA software. Results with $p < 0.05$ were considered significantly different.

The number of replicas varies in each analysis and is detailed afterwards in the results of this document as well as the tests used.

The graphics were generated with the software GraphPad Prism 5.00.

4. RESULTS

4.1. Water content and osmotic potential

Table 1 shows the values of osmotic potential of the Vacin & Went (1949) culture medium with PEG added and of the tissue osmotic potential of *L. lobata* protocorms submitted to the osmotic treatments. To the extent that the concentrations of PEG were higher, it was observed statistical differences in the osmotic potential among all the treatments. As a whole, these results appear to follow the largely known rule, according to which the tissue osmotic potential is more negative than osmotic potential of the substrate solution, which is a crucial factor for water uptake by plants. However, in T3 the osmotic potential of the plant extracts was less negative than the media culture.

Table 1. Mean values of osmotic potentials. Data averaged with three biological replicas ± SD. Different superscript letters denote significant difference between the control and osmotic stress treatments, according to Tukey's test.

Treatment	Osmotic potential (MPa) of media culture	Osmotic potential (MPa) of plant extracts
Control(T0)	-0.882 ± 0.019^{a}	-1.046 ± 0.011^{a}
10%PEG(T1)	-1.024 ± 0.012^{b}	-1.193 ± 0.014^{b}
15%PEG(T2)	-1.175 ± 0.016^{c}	-1.265 ± 0.013^{c}
30%PEG(T3)	-2.050 ± 0.020^{d}	-1.759 ± 0.039^{d}

As the protocorms were submitted to the treatments they lost water as observable in Figure 2. All of the treatments clearly provoked a decrease in water content, but was in T3 where the loss was nearly of 14% compared to the control.

On the other hand, the treatments T1 and T2 showed similar responses to the treatments, displaying not statistical differences between them.

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Figure 2 showcases T0 with a great percentage of water, and due to the treatments the percentages decrease slightly in T1 and T2 displaying a greater decrease (-14%) in the most stressful treatment.

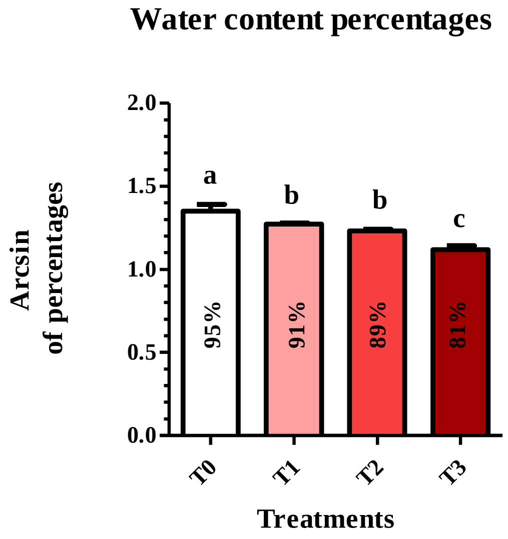

Figure 2. Percentages of water content**.** Means obtained with 5 biological replicas. Data analyzed with ANOVA one way and Tukey's test. Bars represent standard deviation and letters represent whether the treatments are or are not statistically different.

4.2. Survival and size analysis

Water restriction causes a limitation on growth and development of plants and when that condition becomes critical may conduce to death. Figure 3 shows photographically the effect of the treatments on the size of the protocorms of *Laelia lobata* Lindl. Table 2 displays the mean values of the surface area (mm^2) of the protocorms. The decrease in each treatment is clear, highlighting that the average of the protocorm size in the T3 treatment (4.20±1.307) is approximately one third of the respective control (12.96±1.427).

Regarding to the survival experiment, it was observed, surprisingly, that all the osmotic treatments enhanced the survival of the protocorms; in such way that we found that the T3 treatment has almost 100% of survival (Table 2).

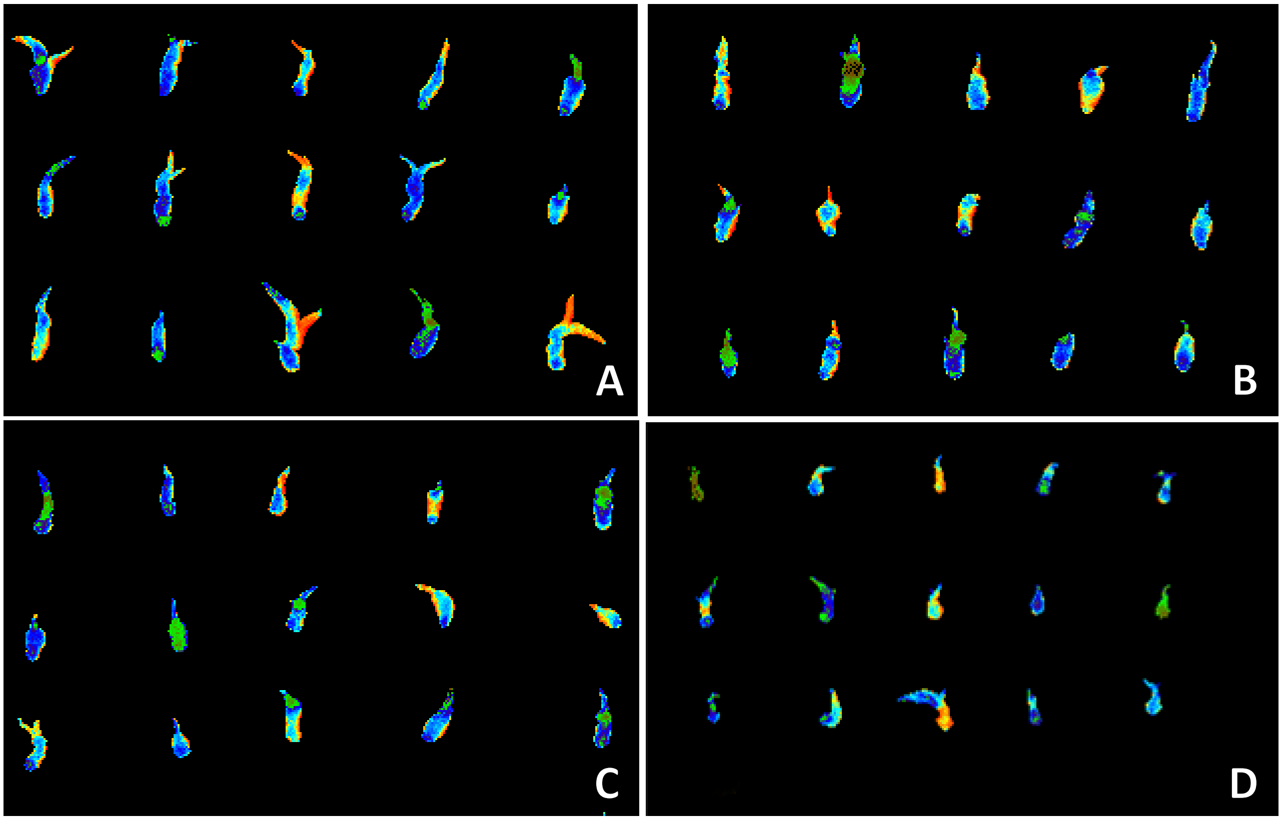

Figure 3. Protocorms size variations of protocorms after treatments. **A**. Control group; **B**, T1; **C**, T2; and **D**, T3.

Table 2. Surface area measurements and percentages of survival of protocorms in each treatment. Mean values obtained from 5 biological replicas ± SD. Different superscript letters denote significant difference according to Tukey's test.

Treatment	Size (mm^2)	% of surviving protocorms
Control (T0)	12.96±1.427^a	62.8±8.729^c
10%PEG (T1)	7.59±1.030^b	87.2±4.970^b
15%PEG (T2)	6.35±1.568^{bc}	97.8±1.304^a
30%PEG (T3)	4.20±1.307^c	99.2±0.837^a

4.3. Carbohydrates analysis

In order to determine the content of endogenous carbohydrates, both structural and non-structural, a series of extractions and dosages were performed.

In respect of the total soluble sugars they gradually decreased in the protocorms submitted to the treatments but declined abruptly in T3 having less than a quarter the concentration found in control; in case of the starch content, there were no statistical differences between the treatments (Table 3).

The ethanolic extract was used to determine the total concentration of soluble sugars, but also to quantify some polyols as mannitol and sorbitol, a disaccharide (trehalose) and a trisaccharide (raffinose), which have been frequently related to plant responses to water deficit. Despite of what was expected, none of these sugars appeared to increase with the treatments as shown in Figure 4.

Mannitol and sorbitol are represented together in the same graph because of limitations of the equipment. It can be seen from Figure 4 that their concentrations decline with the treatments, remarkably in T2 and T3. Trehalose content is apparently unaffected by the treatments and concerning to raffinose it decreases from T0 to T2 but slightly increases in T3 where the response is statistically similar to T1. The variations are so reduced that seems difficult to outline a trend in these results.

Considering the role of glucomannans in orchids as mentioned previously, we extracted this polysaccharide and determined its approximate concentration using the mannose: glucose ratio expressed in µg per milligram of dry weight. Regardless of what was expected, glucomannans content did not increase under water deficit conditions, being the control group the one with the higher value and significantly decreasing in T1, with no statistical differences between any of the treatments.

Table 3. Weight of total soluble sugars, starch and glucomannans. Means averaged with 5 biological replicas ± SD. Different superscript letters denote significant difference according to Tukey's test.

Treatment	**Total soluble sugars (µg · mg^{-1}DW**	**Starch (µg · mg^{-1}DW)**	**Glucomannans (µg · mg^{-1}DW)**
Control (T0)	592.62±81.858^{a}	215.98±58.459	0.37±0.136^{a}
10%PEG (T1)	351.98±90.303^{b}	313.63±76.633	0.11±0.036^{b}
15%PEG (T2)	348.43±85.583^{b}	311.04±85.005	0.17±0.030^{b}
30%PEG (T3)	141.07±20.415^{c}	243.52±79.794	0.16±0.059^{b}

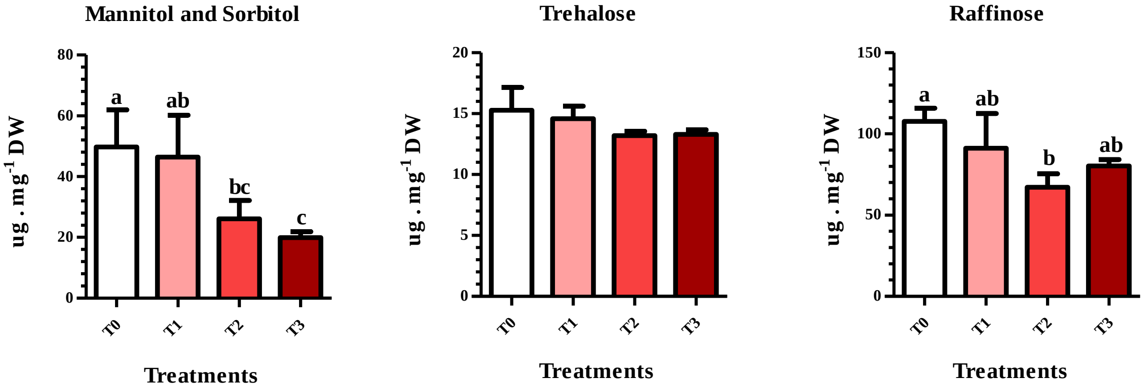

Figure 4. Content of mannitol, sorbitol, trehalose and raffinose in the ethanolic extract of protocorms (µg · mg^{-1} DW). Means averaged with 4 biological replicas. Bars represent standard deviation and different letters represent significant differences among treatments according to Tukey's test.

Structural monosaccharides from cell walls were also studied in order to elucidate if any of them varied as a result of the water deficit. The monosaccharides analyzed were fucose, arabinose, galactose, rhamnose, glucose, xylose and mannose and the concentration of each one was calculated in microgram per milligram of dry weight.

As Table 4 shows none of these carbohydrates increased with the treatments, in fact all of them have a slight decrease because of the treatments; yet some of them are not statistically different from the mean concentration of the monosaccharide in the control group like in arabinose, rhamnose, glucose and mannose. These results show that the cell walls appear to have very little variations in the structural carbohydrates that compose them owing to the treatments.

Table 4. Quantification of monosaccharides of the cell walls**.** Mean values averaged with 5 biological replicas ± SD. Different superscript letters denote significant difference between treatments according to Tukey's test.

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Treatments **Sugars ($\mu g \cdot mg^{-1}DW$)**	**T0**	**T1**	**T2**	**T3**
Fucose	2.37 ± 0.48^{a}	1.92 ± 0.30^{ab}	1.69 ± 0.27^{b}	2.06 ± 0.19^{ab}
Arabinose	17.74±3.49	14.45±2.84	13.39±2.17	14.88±1.07
Galactose	23.26 ± 3.87^{ab}	23.70 ± 3.19^{a}	19.871 ± 3.33^{ab}	17.73 ± 1.86^{b}
Rhamnose	2.00±0.34	1.86±0.37	1.79±0.34	1.86±0.27
Glucose	6.89±0.79	7.06±1.28	7.07±1.59	5.42±1.11
Xylose	10.62 ± 2.02^{a}	8.11 ± 1.84^{ab}	7.56 ± 1.37^{b}	8.83 ± 0.88^{ab}
Mannose	7.40±1.72	7.98±2.07	8.89±2.27	5.98±1.64

Aiming to evaluate if the protocorms of *L. lobata* accumulated mucilages (another storage product to counter water deficit), a histological analysis was performed. This was made instead of a quantitative analysis because would help us to determine whether their presence in the protocorms was relevant or not since it was needed large amounts of fresh mass for their quantification.

The presence of the mucilage idioblasts was corroborated. They appeared like big cells with a yellowish or slimy substance (mucilage) associated with raphides (Figures 5 and 6). We did not find any relationship between idioblasts incidence and the PEG treatments as can be observed in the next figures.

The cells of the protocorms of T1 appear as turgid as the control protocorm cells; however Figures 6B and D show undulations in their cell walls which mean plasmolysis.

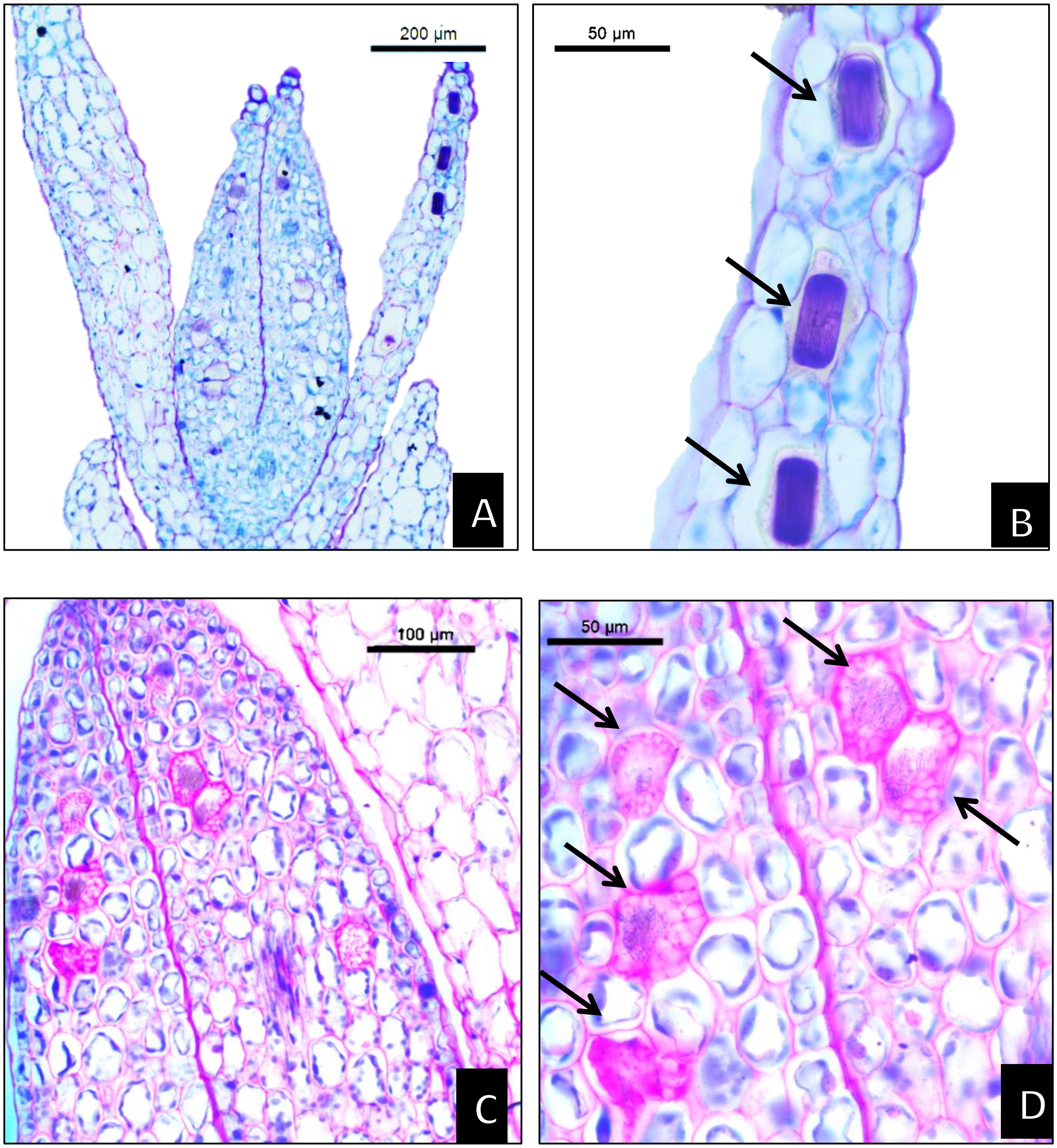


Figure 5. Histological slides of the *L.lobata* protocorms highlighting the presence of the mucilage idioblasts with black arrows. A and B (control group), C and D (T1).

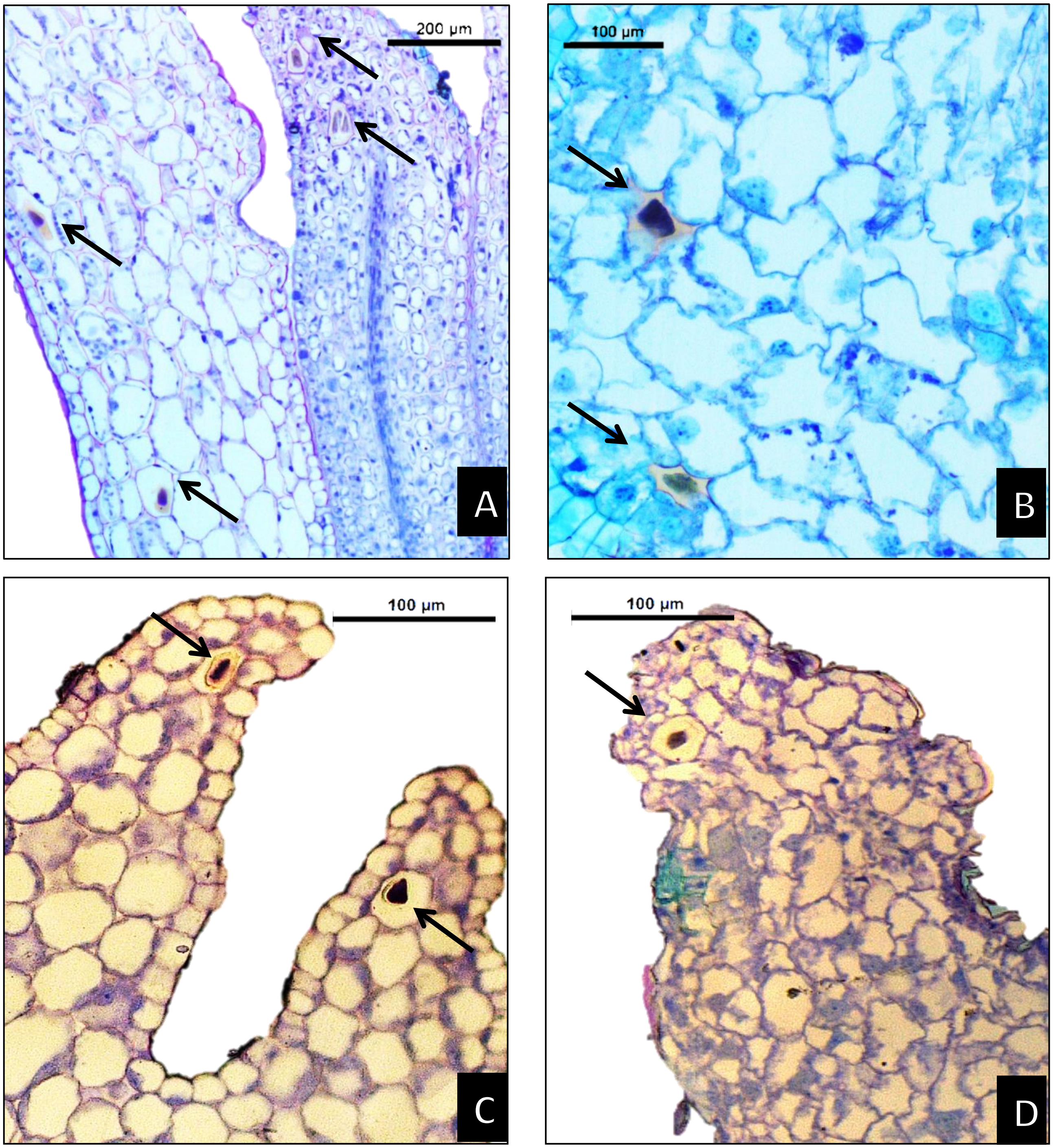


Figure 6. Histological slides of the *L.lobata* protocorms highlighting the presence of the mucilage idioblasts with black arrows. A and B (T2), C and D (T3).

The color difference in the figures (5A-B, 5C-D, 6 A-B and 6 C-D) is due to small differences during the staining of the slides.

4.4. Cuticular waxes

The analysis of the cuticular waxes of the protocorms of *L.lobata* was made in three levels. As a general view, the total load of waxes in each group protocorms under osmotic stress is graphically depicted in Figure 7. The treatments enhanced gradual and conspicuously the cuticle wax content as the water deficit got more intense, reaching in T3 more than 70 fold the concentration of the control group. These results seem to point out a significant relationship between the production of cuticular waxes and water deficit.

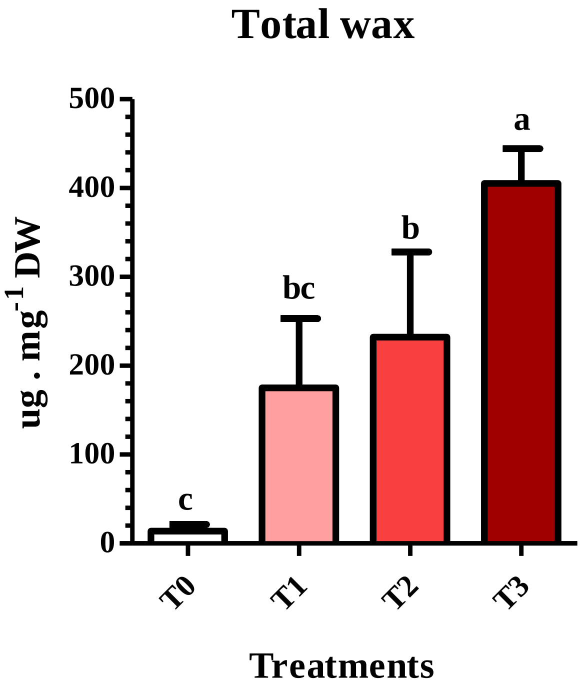


Figure 7. Total load of cuticular waxes of the protocorms submitted to water deficit. Mean values determined with 3 biological replicas. Bars represent standard deviation and letters denote significant differences among treatments according to Tukey's test.

As cuticular waxes are composed of a complex mixture of different chemical compounds such as cyclic and long-chain aliphatic components, we identified and grouped them by classes (Figure 8).

Broadly speaking, the alkanes and the steroids declined from the first osmotic treatment (T1); on the other hand, fatty acids seemed to increase; the primary alcohols, just as much as the triterpenes and the esters decreased gradually in each treatment until appear unquantifiable in T3. It is important to elucidate that some compounds are in such low quantities that are considered traces and that a great percentage of the accumulated substances could not be identified (shown as not id., brown portion of the bars in Figure 8).

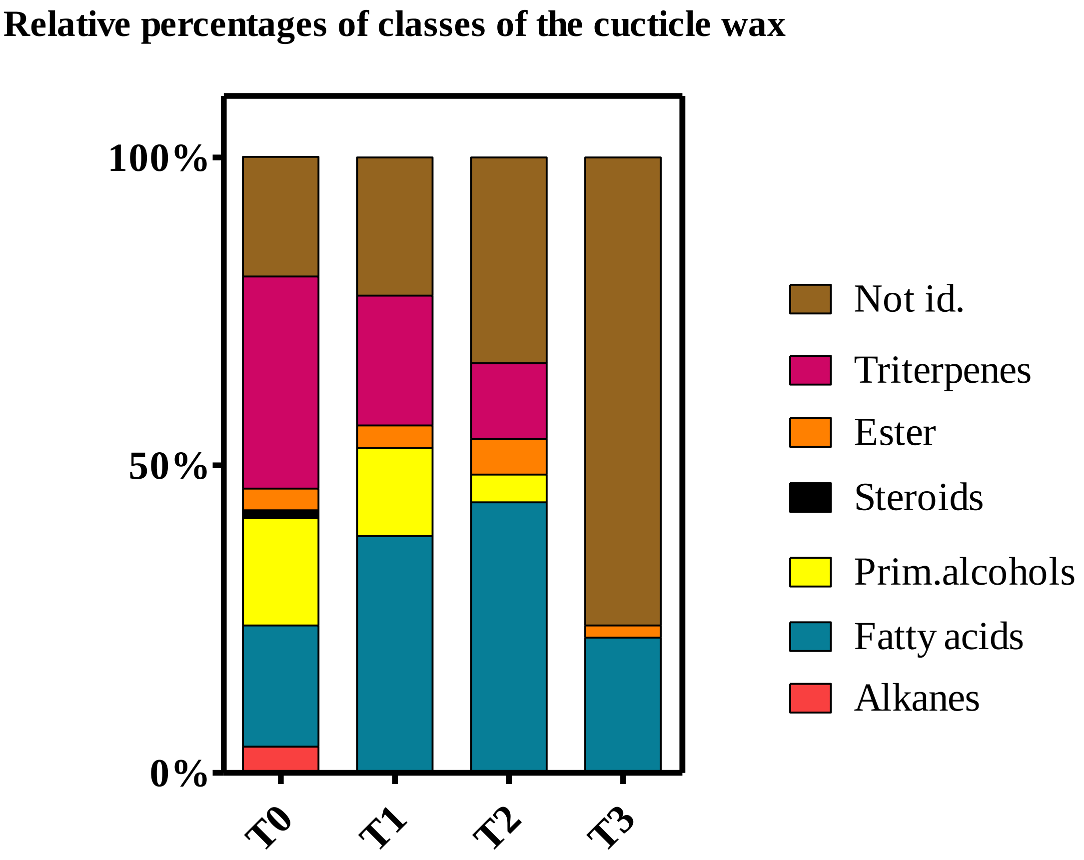
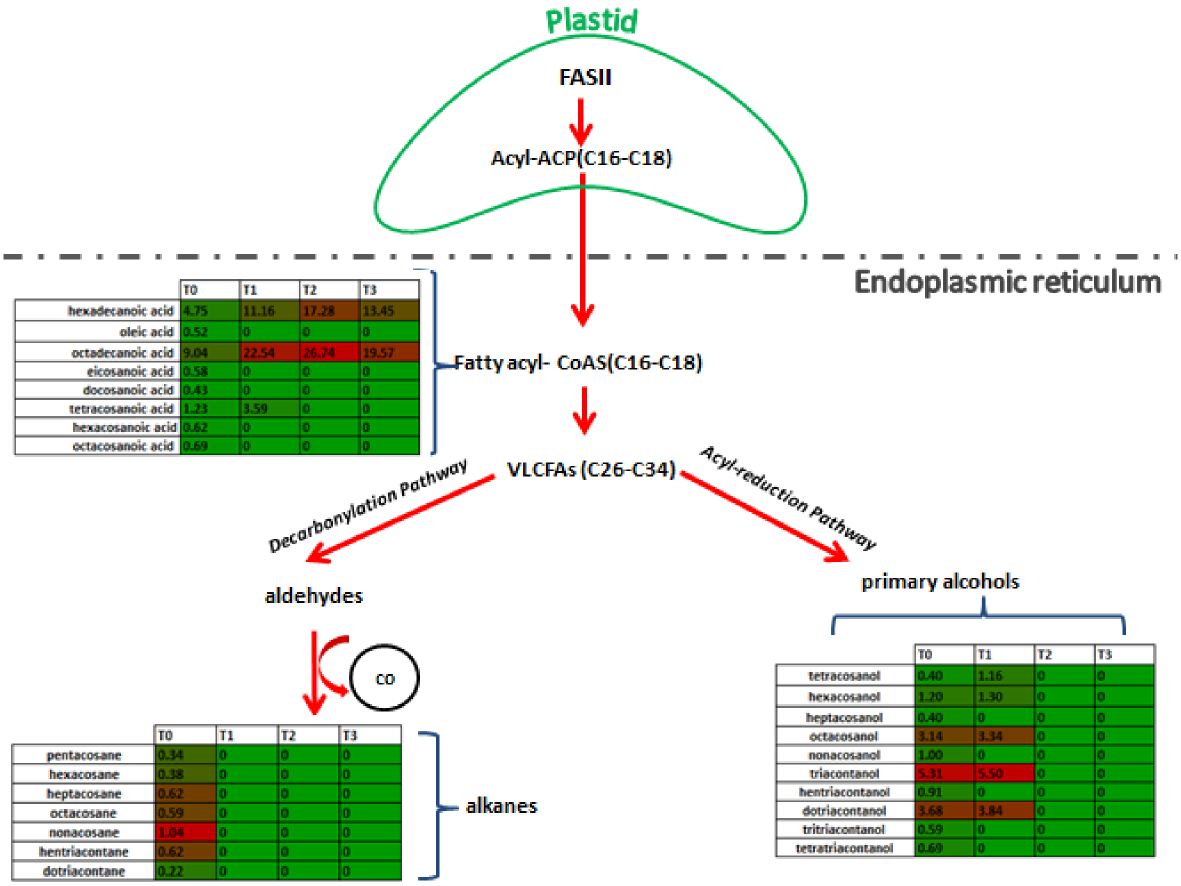


Figure 8. Stacked column chart representing the relative percentages of the classes constituting the wax cuticle of the protocorms of *Laelia lobata* Lindl. after the treatments.

Figure 9 displays more an in-depth view of the cuticle waxes. It represents briefly the biosynthesis of the cuticular wax in plants but also, in the form of heatmaps, the variations of some of the homologues identified.

The biosynthesis of the cuticular waxes initiates in the plastids where short-carbon chain molecules (C_{16}, C_{18}) continue to be elongated and modified ($C_{>20}$) in the endoplasmic reticulum constituting different components (primary alcohols, esters, etc.)

The interesting aspect of these results is that apparently the Acyl-ACP elongation is interrupted at some point during the treatments causing the decline in the production of alkanes and primary alcohols; moreover, the variety of fatty acids is lost and only few homologues are highly produced: the hexadecanoic and octadecanoic acids.



	T0	T1	T2	T3
hexadecanoic acid	4.75	11.16	17.28	13.45
oleic acid	0.52	0	0	0
octadecanoic acid	9.04	22.54	26.74	19.57
eicosanoic acid	0.58	0	0	0
docosanoic acid	0.43	0	0	0
tetracosanoic acid	1.23	3.59	0	0
hexacosanoic acid	0.62	0	0	0
octacosanoic acid	0.69	0	0	0

	T0	T1	T2	T3
pentacosane	0.34	0	0	0
hexacosane	0.38	0	0	0
heptacosane	0.62	0	0	0
octacosane	0.59	0	0	0
nonacosane	1.04	0	0	0
hentriacontane	0.62	0	0	0
dotriacontane	0.22	0	0	0

	T0	T1	T2	T3
tetracosanol	0.40	1.16	0	0
hexacosanol	1.20	1.30	0	0
heptacosanol	0.40	0	0	0
octacosanol	3.14	3.34	0	0
nonacosanol	1.00	0	0	0
triacontanol	5.31	5.50	0	0
hentriacontanol	0.91	0	0	0
dotriacontanol	3.68	3.84	0	0
tritriacontanol	0.59	0	0	0
tetratriacontanol	0.69	0	0	0

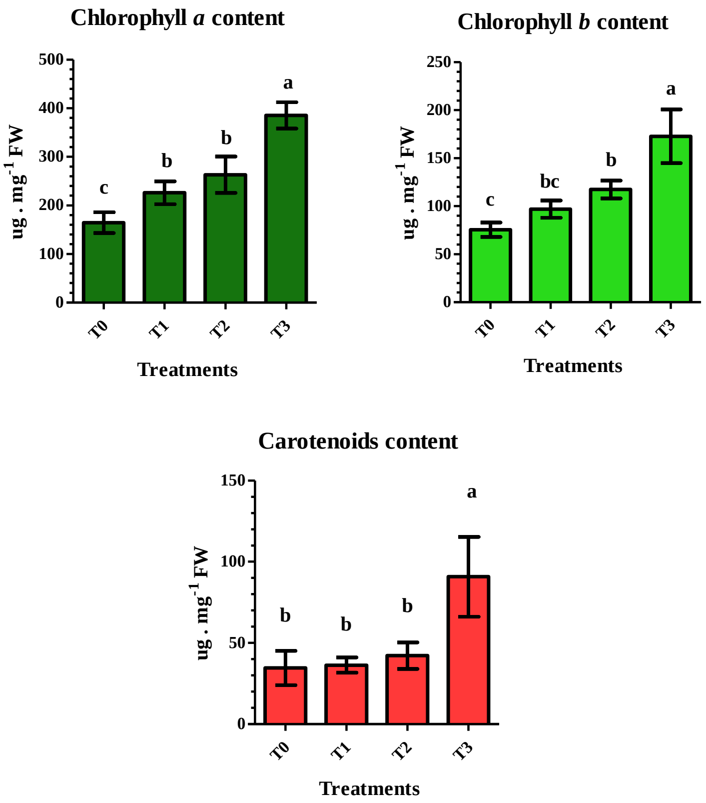
Figure 9. Cuticle wax biosynthesis: elongation of fatty acids under the effects of the osmotic treatments over some of their components. FAS: fatty acid synthase II; VLCFAs: very long chain fatty acid.

The reason why some compounds, as the primary alcohols in T2, appear in Figure 8 but not in Figure 9 is because in order to elaborate Figure 8 we considered all the relative percentages obtained by the equipment during the processing of the samples (even the traces); although when filtering the data, some of them had to be dismissed to avoid a great standard deviation (more than 35% from the mean value).

4.5. **Photosynthetic pigments**

The photosynthetic pigments of the protocorms displayed significant differences when submitted to water deficit treatments (Figure 10). All the pigments quantified increased markedly in all the treatments, registering their higher concentrations in T3.

Both chlorophyll *a* and *b* registered different statistical responses, increasingly having higher values since the T1. Regarding to the carotenoids, they remained very similar to control in T1 and T2 but increased almost threefold the control in T3.


Chlorophyll *a* content

Chlorophyll *b* content

Carotenoids content

Figure 10. Pigments content represented on fresh weight basis (µg · g FW^{-1}). Means obtained with 5 replicates. Bars represent standard deviation, and different letters represent significant differences among treatments according to Tukey's test.

4.6. Operating Efficiency (OE)

These results are shown below (Figure 11), representing the values obtained directly from the fluorometer

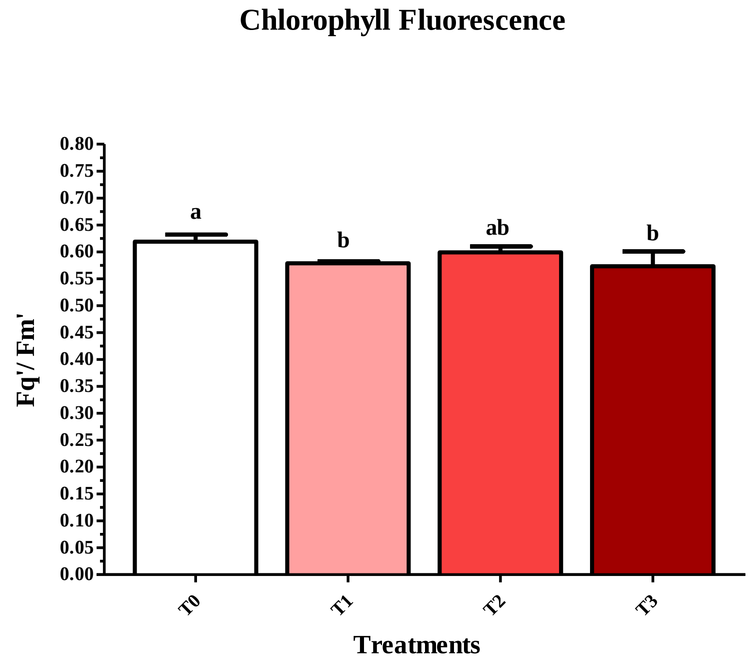


Figure 11. Operating efficiency (Fq’ / Fm’) of the PSII of the protocorms. Bars represent deviation and different letters represent significant differences among treatments according to Tukey’s test.

The net means of operating efficiency in each treatment are: 0.62(control), 0.58 (T1), 0.60 (T2), and 0.57 (T3), these values fluctuate slightly among them and the treatments T1 and T3 had the lowest OE.

4.7. SEM

The SEM photos of the surface of the protocorms of *Laelia lobata* Lindl. highlighted very interesting details about some of their anatomical features.

Several rhizoids were observed all over the basal part of the protocorm (Fig 12); we also noticed the presence of stomata through all the epidermis of the protocorm (Fig 13A, B, and C).

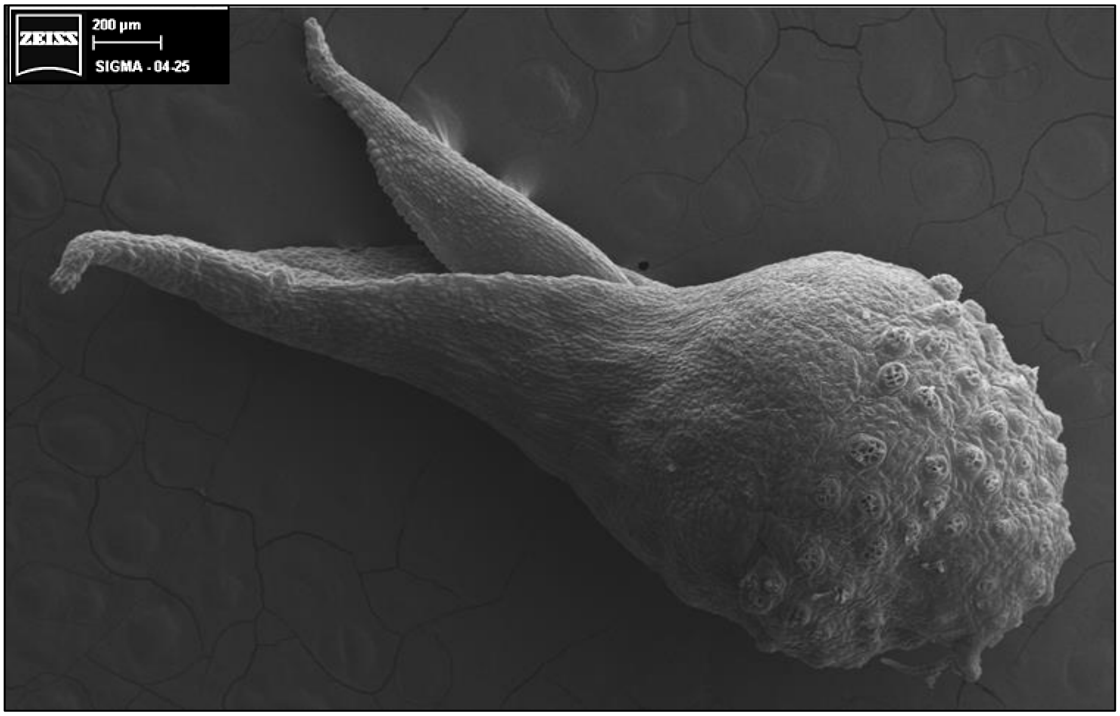


Figure 12. Scanning electron microscopy of an entire protocorm.

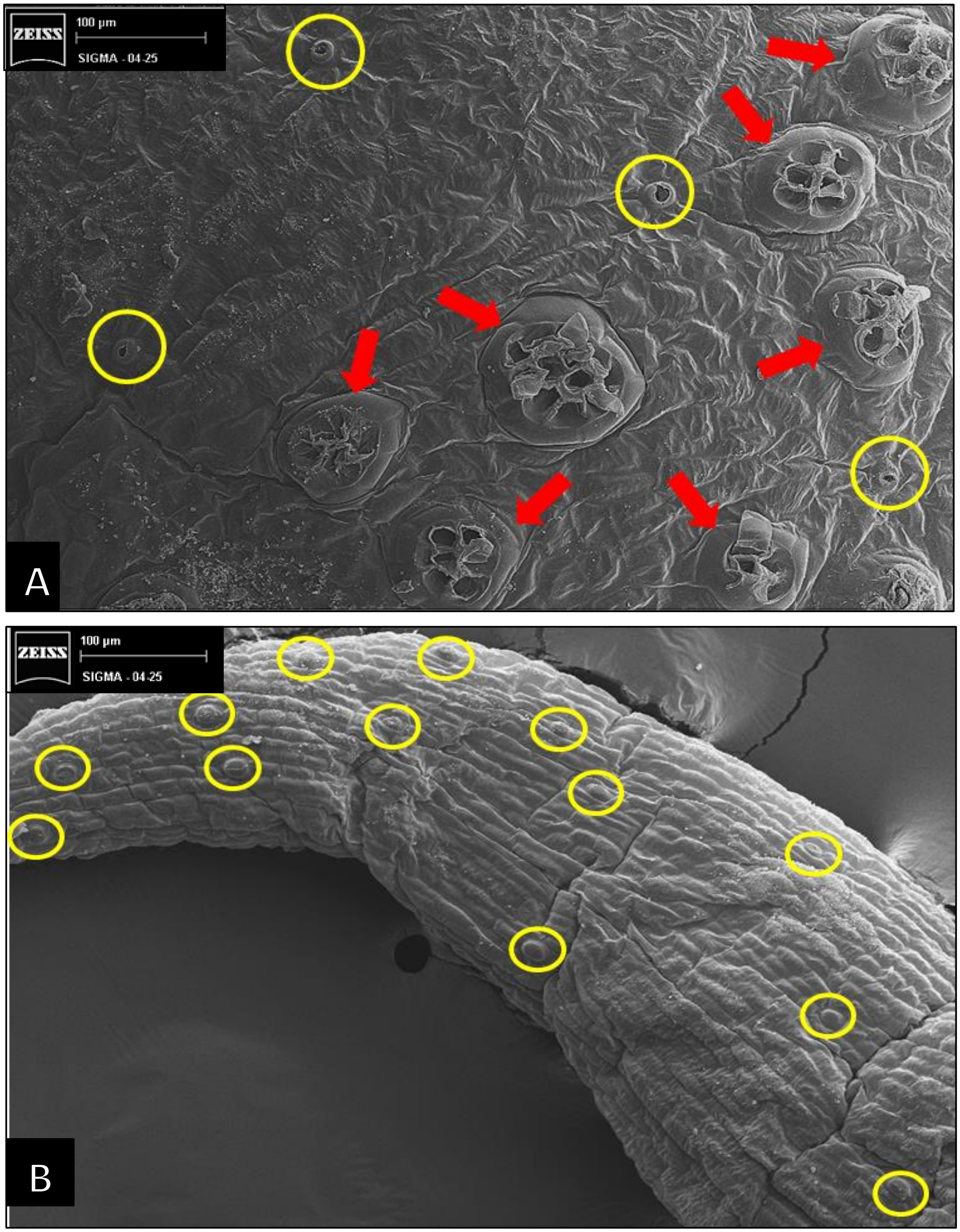


Figure 13A. Basal part of the protocorm emphasizing the basal cells of the rhizoids (signaled with red arrows) and stomata (circled in yellow circles). Only the basal cells of the rhizoids are present because they were lost during the preparation of the plant material for the SEM. **B.** Abaxial part of young leaf.

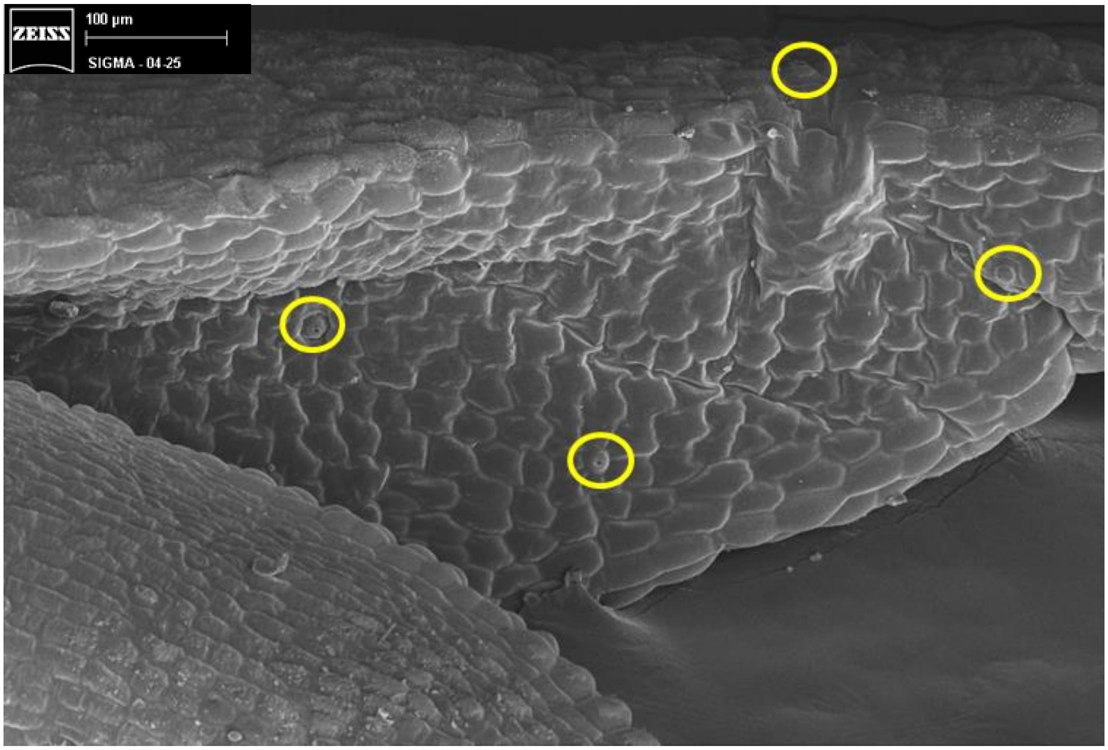


Figure 14. Adaxial part young leaf. Stomata circled in yellow.

4.8. Carbon isotope discrimination

This experiment was made in order to determine which photosynthetic pathway is performed by *Laelia lobata* Lindl. both for the adult plant as much as the protocorms. Figure 15 exhibits the δ^{13}values of each sample, where the δ^{13} of the adult plant leaves is more negative (-15.79‰) than the δ^{13} of the protocorms (-14.36‰). These values indicate that they could be whether CAM or C4; this will be discussed afterwards.

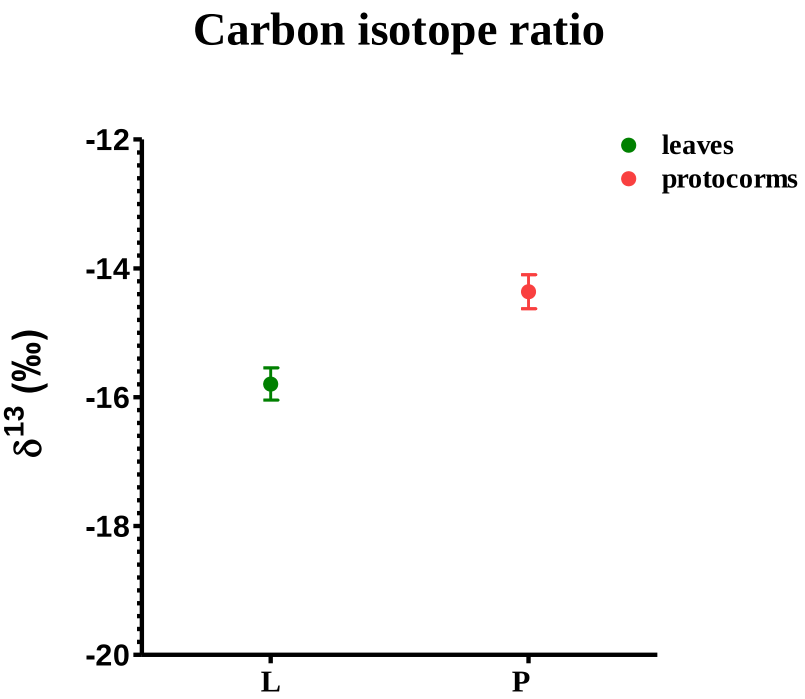


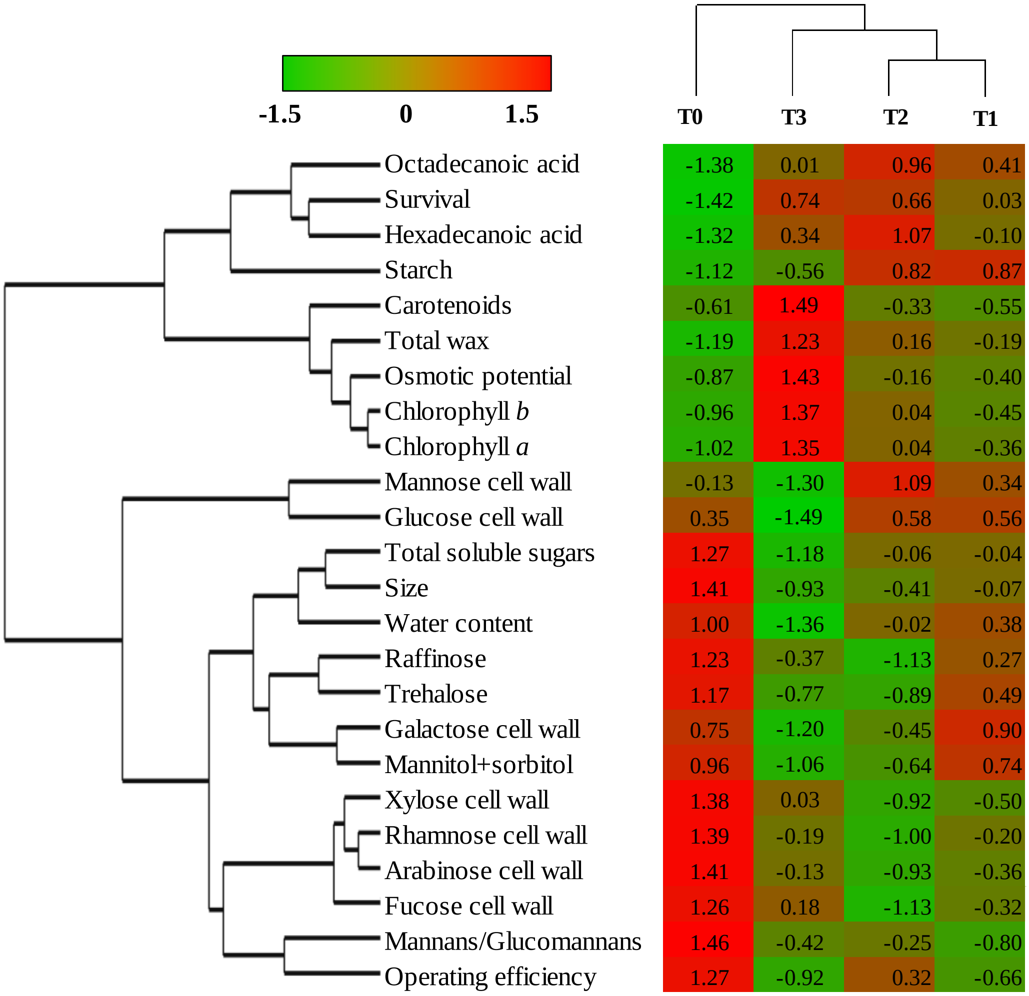
Figure 15. Carbon isotope discrimination of leaves of an adult plant and the protocorms of *L.lobata*. Means averaged with four replicas (represented by dots) ± standard deviation (represented by bars). T-test (p=0.002).

4.9. Data set analysis

In order visualize simultaneously all the data, a double dendrogram also known as clustered heatmap was made. The data is displayed in clusters organized in columns and rows.

The clusters in the columns are in respect of the treatments, and it is clear (as seen beforehand) that T3 and T0 have very contrasting responses while T1 and T2 are in the same cluster due to the similarities in their responses throughout the majority of experiments.

Meanwhile, the clusters in the rows are in respect of the experiments, and distinctly shape two big categories; the first great branch (from the octadecanoic acid to chlorophyll *a*), groups the data where the values increased due to the treatments, with the lowest value in T0; the second branch (from mannose of the cell wall to operating efficiency), groups the data where the values decreased and where T0 had the highest values. There is a small cluster which derives from the second great branch conformed by mannose and glucose from the cell wall, this may have happened because they do not fulfill completely the trend of the rest of the results of the second category but have their lowest values in T3 and not in T0 as to be included in the first category.



	T0	T3	T2	T1
Octadecanoic acid	-1.38	0.01	0.96	0.41
Survival	-1.42	0.74	0.66	0.03
Hexadecanoic acid	-1.32	0.34	1.07	-0.10
Starch	-1.12	-0.56	0.82	0.87
Carotenoids	-0.61	1.49	-0.33	-0.55
Total wax	-1.19	1.23	0.16	-0.19
Osmotic potential	-0.87	1.43	-0.16	-0.40
Chlorophyll *b*	-0.96	1.37	0.04	-0.45
Chlorophyll *a*	-1.02	1.35	0.04	-0.36
Mannose cell wall	-0.13	-1.30	1.09	0.34
Glucose cell wall	0.35	-1.49	0.58	0.56
Total soluble sugars	1.27	-1.18	-0.06	-0.04
Size	1.41	-0.93	-0.41	-0.07
Water content	1.00	-1.36	-0.02	0.38
Raffinose	1.23	-0.37	-1.13	0.27
Trehalose	1.17	-0.77	-0.89	0.49
Galactose cell wall	0.75	-1.20	-0.45	0.90
Mannitol+sorbitol	0.96	-1.06	-0.64	0.74
Xylose cell wall	1.38	0.03	-0.92	-0.50
Rhamnose cell wall	1.39	-0.19	-1.00	-0.20
Arabinose cell wall	1.41	-0.13	-0.93	-0.36
Fucose cell wall	1.26	0.18	-1.13	-0.32
Mannans/Glucomannans	1.46	-0.42	-0.25	-0.80
Operating efficiency	1.27	-0.92	0.32	-0.66

Figure 16. Clustered Heatmap. the double dendrogram displays hierarchical aggrupation (Euclidean analysis) of the data obtained in each experiment and treatment. The color scale of the heatmap represents higher values in red and lower values in green.

5. DISCUSSION

Although the Atlantic forest is generally humid, the epiphytes that inhabit it are subjected to an environment with intermittence of water (Madison, 1977; Benzing, 1990; Zotz, 2016). Therefore, it is not surprising that many epiphytes are thought to be considerably tolerant to drought stress (Coutinho 1964, 1969; Lambers *et al.*, 1998).

Similarly to other plant groups, the water shortage even in epiphytes leads to higher mortality rates in seedlings and juvenile phases (Ackerman *et al.*, 1996; Zotz & Hietz, 2001). The protocorms of *Laelia lobata*, as described above, are at an early stage of development and quite unprotected. However, curiously the results showed a rise in the survival percentage of the protocorms submitted to the water shortage treatments. Probably, the PEG treatments were not intense enough to provoke their mortality but to enhance their survival as reported by Skirycz *et al.* (2011) who analyzed *Arabidopsis* responses to osmotic stress using mannitol and found that severe drought conditions enhanced the survival of the resistant lines was because of the activation of water-saving mechanisms.

According to Martin *et al.* (2004) there are two types of responses that epiphytes may display under drought conditions that are closely interrelated. First, to possess a highly negative tissue osmotic potential allows the plant to uptake water from the substrate. Second, having a less negative tissue osmotic potential would help the plant to avoid plasmolysis in case of a longer period of drought.

As the protocorms were exposed to an increasing osmotic stress, the osmotic potential of control, T1 and T2 plants were more negative than the medium culture, thereby inducing the entrance of water into the protocorms; however, plants under T3 treatment, with twice as much the stress of T2, registered an opposite response having a higher tissue osmotic potential. Thus, as suggested by Martin *et al.* (2004), this process may take place to avoid the cell lysis since the external osmotic potential was too intense.

The maintenance of the cell turgor not only depends on the fluctuations of water potential and osmotic adjustments by the accumulation of osmoprotectans (Murakeözy *et al.*, 2003; Seki *et al.*, 2007; Lambers *et al.* 2008), but also on the elasticity of the cell walls.

The elasticity depends on the cell-walls components, while thicker or just rigid they have high elastic modulus (Ɛ) which means they are less elastic; in its turn, flexible cell walls have low elastic modulus. This prevents cells from shrinking during long periods of water shortage and allows them storing greater amounts of water when water sources are available (Fan *et al.*, 1994; Lambers *et al.*, 1998; Saito & Terashima, 2004; Martinez *et al.*, 2007; Moore *et al.*, 2008).

CAM plants typically have elastic cell walls (Rygol *et al.*, 1989; Ogburn & Edwards, 2010) and according to the obtained carbon isotope discrimination results, *Laelia lobata* is most likely a CAM plant. On that basis we could propose that the cell walls of this orchid are elastic too, and when submitted to water deficit their components may vary to avoid damage in the cytoplasm. For instance, Balsamo *et al.* (2006) found a correlation between the cell wall biochemical composition and drought resistance in resurrection grasses (*Eragostris*) which are largely known to be extremely tolerant to desiccation.

The plant cell walls are made up of cellulose, hemicellulose, proteins and carbohydrate polymers. Hemicelluloses constitute a large part of the polysaccharides of the cell walls and are composed mainly by xylans, arabinoxylans, mannans, galactomannans, glucomannans and arabinogalactan II; and pectic substances such as rhamnogalacturonan I, rhamnogalacturonan II, arabinan, galactan, arabinogalactan I and D-galacturonan (Heredia *et al.*, 1995).

Several of the polysaccharides aforementioned are constituted by the monosaccharides found in the protocorms of *Laelia lobata*. The decrease of these saccharides, as a result of the osmotic stress, may have resulted in a reduction of the cell wall thickness, then less rigidity. This elasticity of the cell wall is only an assumption since we did not estimate the Ɛ module and according to Cosgrove (2015) the assembly of the components of the cell walls to make them extensible still remains an enigma.

Also regarding to polysaccharides, many taxa (*e.g.* cacti, orchids, etc.) have shown to accumulate mucilages to enhance their water storage capacity (Meier & Reid, 1982; Nobel *et al.*, 1992; Ogburn & Edwards, 2010). However, the quantification of glucomannans (main component in orchid's mucilages), as well as the presence of mucilaginous idioblasts of the protocorms of *Laelia lobata* did not showed any clear effect of the treatments on their prevalence.

It is well reported in the literature the occurrence of shifts in the concentration of non-structural carbohydrates in plants under drought stress. For instance, Muller *et al.* (2011) reviewed many cases of carbohydrates accumulation; Mohammadkhhani & Heidari (2008), induced osmotic stress using different concentrations of PEG in two maize varieties and observed an accumulation of soluble sugars and a decline in starch content; Griffin *et al.* (2004) obtained similar responses in two ecotypes of the leguminous Eastern redbud *Cercis canadensis*, being D-pinitol the most accumulated carbohydrate. Nevertheless, other authors have found divergent responses, where soluble sugar content decreased or remained invariable (Morgan, 1992; Stancato *et al.*, 2001) such as our own results.

It is known that carbohydrates metabolism is deeply associated with photosynthesis and both are affected by water shortage. Decreased chlorophylls, carotenoids and chlorophyll fluorescence of the PSII under water stress conditions have been reported in many species (Munné-Bosch & Alegre, 2000; Colom & Vazzana, 2003; Manivannan, 2007) and considered as a symptom of oxidative stress and a putative result of pigment photo-oxidation and degradation (Anjum *et al.,* 2011) and disturbances in the photosynthetic apparatus.

Sapeta *et al.* (2013) observed an abrupt reduction of the PSII operating efficiency in *Jatropha curcas* under drought conditions. Same responses had Iqbal *et al.* (2019) with *Glycine max* in water deficit conditions using PEG-6000 in concentrations less harsh than the ones we used (6% as its highest concentration).
According to the results of chlorophyll fluorescence in the protocorms of *L.lobata*, there is a slight decrease of OE in the treatments (±6%) which remains stable with no abrupt reductions not even in T3.

The non- accumulation of carbohydrates in the protocorms may not be a consequence of a disruption in the photosynthetic activity. In fact, the values of OE such as the increase in photosynthetic pigments indicate that the photosynthetic apparatus could be keeping its integrity and functionality, probably because the intensity and duration of the osmotic treatments were not enough to provoke a destabilization of the photosystem, or because it constitutes as such a tolerance mechanism of the *Laelia lobata* protocorms.

One other important factor that affects photosynthetic activity is the reduction in the stomatal conductance in water deficit conditions to avoid water transpiration. In this study we did not measure the stomatal conductance but was confirmed the presence of stomata in the protocorms which sheds the possibility of conducting this experiment further on.

The number of stomata found in the protocorms of *Laelia lobata* was limited but still a nice feature to find out considering that the protocorms are in such an early stage of development; however, their existence per se does not appear to be a sine qua non condition for CAM orchids. According to Winter *et al.* (1985) who studied CAM metabolism in the leafless orchid *Campylocentrum tyrridion* and Cockburn *et al.* (1985) who investigated the photosynthetic carbon assimilation in shootless orchid *Chiloschista usneoides*, the absence of stomata does not affect the CO_2 uptake in some epiphytic orchids.

Some of the main components that could affect the overall isotope discrimination during photosynthesis are the CO_2 diffusion, interconversion (CO_2 to HCO^-_3), the assimilation by PEP carboxylase and RuBP carboxylase, and respiration (O'Leary, 1980). As the isotopic values seem to have a linear relation to the carbon dioxide uptake by the plants, e.g. the CO_2 incorporation in C_3 plants is limited and have more negative a $\delta_{13}C$ values, we presume that the higher values of $\delta_{13}C$ of the protocorms compared to the adult leaves might be associated to a higher efficiency of CO_2 fixation.

The carbon (C) supply, which accomplishes many roles in plants, is obtained by photosynthesis. If the photosynthetic activity is affected by water restriction, accordingly, the plant growth would be also affected. However, according to Muller *et al.* (2011) the limitation of plants growth not necessarily has to do with limitations of C source but more with the employment of these C molecules to generate tolerance responses. As seen in our results, there was a significant limitation in the protocorm growth while the biomass increased greatly. On that basis, we could hypothesize that the C molecules and energy obtained from the high photosynthetic activity could be being invested in the formation of complex molecules in order to increase the water deficit tolerance of the protocorms.

The cuticle waxes of the protocorms were specially affected by the osmotic treatments. This was reflected through a significant wax accumulation and large variations in their components. Indeed, the accumulation of cuticular waxes during water deficit has been largely reported, considering alkanes as the most accumulated and most effective barrier against water loss (Oliveira *et al.* 2003; Kim *et al.*, 2007; Bi *et al.*, 2017).

Yet in our results, the most accumulated substances were the fatty acids. We ponder that this might be because of the reduction/interruption of the fatty acids elongation through their pathway to conform alkanes and primary alcohols since these two classes decreased to became traces from the very first treatment; instead, the short chain fatty acids were repeatedly synthetized particularly the hexadecanoic and octadecanoic acids.

Unfortunately, the most accumulated substance could not be identified; a profound investigation of this is still pending since it represents a great percentage of the total load of cuticle waxes of the treated protocorms.

We got to analyze the cuticle waxes of leaves of non-stressed adult plants of *L. lobata* (data not included in this work), and the composition of the waxes turned out to be very similar to the results of the control group of protocorms.

Although there is no correlation between the chemical composition of the cuticular wax deposition and phylogeny, we found some interesting similarities among our findings and the results obtained by Pansarin *et al.* (2008). They analyzed the cuticular waxes of leaves of adult plants of 13 species of *Cleistes* (Orchidaceae) and found a predominance of either fatty acids or primary alcohols highlighting that most of the species are characterized by having as main fatty acids the hexadecanoic and octadecanoic acids.

6. FINAL CONSIDERATIONS

The present study rose up some interesting aspects regarding to the effects of water deficit in the protocorms of *Laelia lobata*. Despite their small size and fragile appearance, they proved otherwise, showing to be extremely tolerant to the osmotic treatments due to nothing more than internal mechanisms.

Some of the results obtained differed in several grades of what was expected. The enhancement of survival, the non-increased polyols known as osmoprotectans, the stability (and even increased activity) of the photosynthetic apparatus and augmentation of the photosynthetic pigments, the decrease of alkanes and increased fatty acids and unknown substances in the cuticle waxes and so.

One of the remained questions was in regard to the osmoprotectans; from all the putative substances analyzed, none of them resulted to be accumulated in the treatments so we can conclude that they are not acting as such. Probably, the nature of their osmoprotectants is very different from the ones we analyzed (e.g. amino acids as proline, glycinebetaine).

By the succulence of their leaves and the analysis of the carbon isotopes we could determine that *Laelia lobata* most likely a CAM orchid; an analysis of titratable acidity would be needed to confirm this result.

Further studies are needed to elucidate which other biochemical and physiological mechanisms these protocorms display to cope with water deficit. We propose the analysis of proteins (even in a molecular level), phytohormones and a deeper analysis of the wax cuticle.

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