Original Article

EFFECTS OF EXOGENOUS PROLINE AND A NATURAL VENTILATION SYSTEM ON THE IN VITRO GROWTH OF ORCHIDS

EFEITOS DA PROLINA EXÓGENA E SISTEMA DE VENTILAÇÃO NATURAL NO CRESCIMENTO IN VITRO DE ORQUÍDEAS

Adriano Bortolotti da Silva[1]; Caroline Oliveira dos Reis[1]; Jairo Osvaldo Cazetta[2]; Samira Domingues Carlin[3]; Paulo Roberto Corrêa Landgraf [1]; MarinaCunha dos Reis[1]

1. Faculdade de Agronomia da Universidade José do Rosário Vellano - UNIFENAS, Alfenas, MG, Brasil. adriano.silva@unifenas.br; 2. Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, Departamento de Tecnologia, Jaboticabal, SP, Brasil; 3. Agência Paulista de Tecnologia dos Agronegócios, Pólo Regional Centro Oeste, UPD, Jaú, SP, Brasil.

ABSTRACT: Micropropagation is an alternative to produce orchid plants in large scale. However, this process presents losses during acclimatization. Exogenous proline use in vitro plant tissue culture can reduce the stress of the plant acclimatization phase. We aimed to verify the growth of orchids in different micropropagation systems with the addition of proline in the culture medium. *Cattleya walkeriana* plants were obtained from the germination of seeds in culture medium. Seeds were germinated in MS medium, added 20 g. L^{-1} of sucrose, solidified with 6 g. L^{-1} of agar and pH adjusted for 5,8. The cultures were incubated in a growth room with temperature of 24 ± 2 ^{0}C, under photoperiod of 16 h. After 5 months, 1-cm long seedlings were placed in a culture vessel according to the treatments, which were composed of two micropropagation systems (conventional and natural ventilation) and three proline concentrations (0, 1, and 2 g·L^{-1}). The experiment was carried out in an entirely randomized design consisting of a 2 × 3 factorial, for a total of 6 treatments, each with 5 replicates. The natural ventilation system with the use of proline (1 g·L^{-1}) promoted higher dry mass accumulation and better control of water loss by plants.

KEYWORDS: *Cattleya walkeriana.* Micropropagation. Abiotic stress.

INTRODUCTION

Species of the *Cattleya* genus and their hybrids are currently the most commercialized orchids in Brazil due to their large, colorful flowers (PRIZÃO et al., 2012). The asymbiotic cultivation of orchids can be used to reduce the effects of their predation in forests, to obtain in vitro germination of a large fraction of their seeds, and to make possible the re-establishment of these species in natural environments. However, plants that originated from this technological process are very expensive and hard to purchase (PAEK et al., 2005), requiring more studies to optimize the micropropagation of this plant.

The in vitro environment of conventional micropropagation is characterized by the lack of gas exchange, high humidity, and low irradiance, which are the main factors responsible for the physiological and morphological disorders of plants in this culture system. This environment, which is entirely different from ex vitro environments, such as greenhouse conditions, often causes the malfunction of stomata (MAJADA et al., 2002), increasing water losses by leaf tissue and potentially leading to a decrease of orchids seedlings survival during acclimatization process (CHA-UM et al., 2010). Photoautotrophic micropropagation is an alternative to overcome conventional micropropagation problems (MOHAMED; ALSADON, 2010).

Photoautotrophic micropropagation is made feasible by using flasks that allow gas exchange between the in vitro culture and ex vitro environment, which is called a natural ventilation system (MOHAMED; ALSADON, 2010). Natural ventilation's benefits are due to the relative reduction of in vitro humidity and to increased aeration, which produce further rustic plants for the ex vitro conditions (MAJADA et al., 2002).

Another way to reduce in vitro culture stress is the use of osmoprotectant substances. The accumulation of osmolytes such as proline and sugars is a well-known adaptive mechanism of plants against stress conditions (MUNNS; TESTER, 2008; BEN AHMED et al., 2010). Proline has a protective action that prevents membrane damage and protein denaturation during severe drought stress (BEN AHMED et al., 2010). There are many studies of tissue culture with abiotic stress and the positive effects of endogenous proline produced by different genotypes in the in vitro culture of plants under saline conditions (ASHRAF; FOOLAD, 2007; EHSANPOUR; FATAHIAN, 2003). However, there is scarce information on the use of exogenous proline in micropropagation. The hypothesis of the present study is that the addition of proline in the culture medium may reduce the

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water loss of plants observed during acclimatization process, producing plants better adapted for transfer from in vitro to ex vitro environments. The aim of this study was to evaluate the in vitro growth of *Cattleya* seedlings in different micropropagation systems (conventional and natural ventilations) and cultivated in the absence or presence of proline in the culture medium.

MATERIAL AND METHODS

Plant material

Seeds of *Cattleya walkeriana* (Orchidaceae) obtained from self-pollination were germinated in MS medium (MURASHIGE; SKOOG, 1962), with 20 $g \cdot L^{-1}$ of sucrose, then solidified with 6 $g \cdot L^{-1}$ of agar, and the pH was adjusted to 5.8. The cultures were incubated in a growth room with a temperature of 24 ± 2 °C, and 16 h photoperiod of 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PPFD). After 5 months, 1 cm long seedlings were placed in the culture vessels for the treatments.

Proline and comparisons among different cultivation methods

In vitro cultivation methods consisted of conventional micropropagation (CM) using 0, 1,or 2 $g \cdot L^{-1}$ of proline, or micropropagation in a natural ventilation system (NV), based on Mills et al. (2004), using 0, 1,or 2 $g \cdot L^{-1}$ of proline, for a total of six treatments. The seedlings were cultivated in MS medium (MURASHIGE; SKOOG, 1962) supplemented with 1.34 $\mu mol \cdot L^{-1}$ of naphthaleneacetic acid (NAA) and 30 $g \cdot L^{-1}$ of sucrose.

For the conventional micropropagation and natural ventilation systems, 500 mL containers were used. Five plants were inoculated in each container with 60 mL of growth medium. The pH was adjusted to 5.8 before autoclaving (121 °C and pressure of 1 $kg \cdot cm^{-1}$) for 20 min.

In the natural ventilation system, filter membranes (0.5µm) were used (Milli Seal, Millipore, Tokyo, Japan) to cover a pair of holes (10 mm diameter) in the culture vessel to allow for gas exchange. Conventional micropropagation used standard culture vessel covers, which do not allow for gas exchange.

Assessments

After 120 days of cultivation, plants were assessed by measuring the following variables: aerial part length (APL), number of leaves (N leaves), number of roots (N roots), root system length (RSL), plant fresh (PFM) and dry masses (PDM), chlorophyll a and b, and water loss. Dry mass was measured after drying for 3 days at 50 °C.

To determine the water loss, plants were removed from the containers and evaluated under laboratory conditions (60% relative air humidity). Every 10 min, 10 mm foliar disks were removed and weighed, for a period of 120 min. At the end of this period, disks were dried at 50 °C for 72 h to measure dry mass (MOREIRA et al., 2013). Water loss values were expressed as the percentage of the foliar tissues that was water.

Chlorophyll extraction was performed with 100 mg of foliar tissue macerated in a mortar and pestle in the presence of 3.0 mL of 80% acetone for 5 min followed by filtration; the procedure was repeated three times with the residue remaining in the filter paper. The absorbances were measured at 470, 647, and 663 nm, and chlorophyll *a* and *b* concentrations were calculated through the equations proposed by Barbieri Junior et al. (2010). Free proline was quantified using 0.1g of fresh leaf tissue assessed by the method developed by Bates et al. (1973).

Data analysis

The experiment was carried out in an entirely randomized design, consisting of a 2 × 3 factorial with two micropropagation systems (conventional and natural ventilation) and three types of culture medium (0, 1, and 2 $g \cdot L^{-1}$ of proline), for a total of 6 treatments with 5 repetitions. The program SISVAR 4.3 was used for the analysis of variances (ANOVA), and the means were compared by a Scott & Knott Test.

RESULTS AND DISCUSSION

Vegetative growth

The orchid in vitro growth was directly affected by the studied factors (micropropagation systems and proline). In addition, a significant interaction was noted between these two factors ($p \leq 0.05$). Plants grown in conventional micropropagation (CM) and in the absence of proline had higher aerial part (APL) and dry (PDM) masses (Table 1). For the root system length (RSL) and for plant fresh masses (PFM), CM in combination with proline (0 or 2 $g \cdot L^{-1}$) and CM in combination with proline (0 or 1 $g \cdot L^{-1}$) produced bigger growth of in vitro plants, respectively (Table 1). The use of proline (1 $g \cdot L^{-1}$) in CM promoted the highest number of leaves (N Leaves) and roots (N Roots). However, these plants had reduced APL and RSL (Table 1) and more nodes.

Table 1. Aerial part length (APL), root system length (RSL), number of leaves (N Leaves), number of roots (N Roots), and plant fresh (PFM) and dry masses (PDM) of in vitro orchid plantlets grown in different micropropagation systems.

MS (*)	Proline (g·L^{-1})								
	0	1	2	0	1	2	0	1	2
	----------APL (cm)---			----------RSL (cm)---------			----------N Leaves---------		
CM(1)	2.5Aa	1.2Bb	1.2Bb	2.4Aa	1.6Bb	2.3Ba	7.0Ab	19.6Aa	8.6Ab
NV(2)	1.7Bc	2.7Aa	2.2Ab	1.6Bc	2.6Ab	3.4Aa	8.3Ab	10.3Ba	7.0Bb
	----------N Roots-------			----------PFM (g)--------			----------PDM (g)----------		
CM	4.0Ab	8.0Aa	5.0Ab	0.448Aa	0.426Ba	0.272Bb	0.035Ba	0.026Bb	0.021Bb
NV	5.6Aa	4.6Ba	4.0Aa	0.461Ab	0.682Aa	0.417Ab	0.054Ab	0.113Aa	0.042Ab

Means followed by the same capital letter in a column or lower case letter in a row did not differ by a Scott–Knott test at 5% probability. (*)Micropropagation system (MS); (1)Conventional Micropropagation (CM), (2)Natural Ventilation (NV).

The natural ventilation system (NV) with proline (1 g·L^{-1}) promoted higher APL growth, but this result did not differ from the best results observed in CM (Table 1). Plants grown under the proline (2 g·L^{-1}) and NV combination had the best results for RSL (Table 1). The number of roots (N roots) in NV was not significantly different among proline doses (Table 1).

Biomass accumulation (PFM) was positively affected by the use of proline (1 g·L^{-1}) in NV, promoting better plant growth when compared with the same treatments in CM (Table 1). Plants grown in NV with proline (1 g·L^{-1}) had the highest PDM, reaching 0.113g (Table 1). As the plant dry mass (PDM) expresses the real growth, due to its relationship to the accumulation of proteins and other substances from photosynthesis, a higher PDM indicates that the treatment yields the best plant performance. The plants grown in vitro in NV conditions had good PDM performance (Table 1). These results are in accordance with the majority of studies of plants grown in natural ventilation systems (MOREIRA et al., 2013; SALDANHA et al., 2012; IVANOVA; VAN STADEN, 2010). Moreira et al. (2013) verified the greater accumulation of PDM in plants grown in NV when compared to conventional micropropagation. Mohamed and Alsadon (2010) reported that plantlets were shorter in ventilated vessels (NV), with fewer nodes and lower fresh mass, but higher dry mass, than plantlets grown in non-ventilated vessels. The same behavior was observed for APL; thus, plants grown in CM (2.5 cm) were taller than those in NV (1.7cm) in the absence of proline (Table 1). However, in the same treatments, higher PDM was observed for plants grown in NV (0.054 g) than in CM (0.035 g) (Table 1).

In the present work, there was higher chlorophyll *b* content (Table 3) in NV (4.01 µg·mg^{-1}leaves) than CM plants (2.87 µg·mg^{-1}leaves) in the absence of proline, which possibly increased the photosynthetic capacity and in vitro growth of plants in NV. Probably, the higher PDM in NV is due to gas exchange and increased CO_2 concentration, which provides a higher photosynthetic rate and biomass accumulation, as observed by Gonçalves et al. (2007) in *Herreria salsaparilha.* These results are supported by the explanation that the membranes in natural ventilation provide higher gas exchange, leading to an increase in plant growth and the content of photosynthetic pigments than in closed systems without a gas-permeable membrane (SALDANHA et al., 2012). The gas exchange in NV has also been reported to increase the photosynthetic rate and biomass accumulation in different species (MOREIRA et al., 2013; MOHAMED; ALSADON, 2010; ZOBAYED et al., 1999).

The addition of proline (1 g·L^{-1}) in the culture medium promoted better in vitro growth of plants in NV (Table 1), including higher APL (2.7 cm), RSL (2.6cm), N leaves (10.3), PFM (0.682 g), and PDM (0.113 g). Probably, proline promoted higher biomass accumulation in plants due to improvements in protein synthesis. Experiments conducted under field conditions have reported the positive effects of the use of exogenous proline on plant growth, photosynthetic rate, and biomass accumulation.

A proline supplement significantly increased the proline content in olive plant tissues

and increased photosynthetic rates, plant water, and shoot elongation (AHMED et al., 2010). Kaya et al. (2007) demonstrated that proline-treated melon plants had more proline in the leaves and produced more biomass than non-treated plants. Similarly, the use of exogenous proline (1 g·L^{-1}) promoted greater in vitro growth of orchid plants in NV (Table 1), as well as higher proline content in foliar tissue (14.69 µg·mg^{-1} leaves) (Table 2). This may explain the performance observed in the growth of plants with the use of this treatment.

Proline content

Endogenous proline content was influenced by the studied factors ($p \leq 0.05$). In the different micropropagation systems, higher proline content was observed for leaves of plants with proline (1g·L^{-1}) in the culture media (Table 2), and it promoted better biomass accumulation compared to other proline levels in NV (Table 1). The same tendency was observed for PDM, although higher concentrations of proline had inhibitory effects (Table 1).

Table 2. Proline content of *in vitro* orchid plantlet leaves in different micropropagation systems, grown with distinct proline concentrations.

MS(*)	Proline (g·L^{-1})		
	0	1	2
	----------Proline (µg·mg^{-1} leaves)----------		
CM(1)	5.2791Ab	15.1958Aa	2.4875Bc
NV(2)	2.1958Bc	14.6958Aa	4.1125Ab

Means followed by the same capital letter in a column or lower case letter in a row do not differ by the Scott–Knott test at 5% of probability. (*)Micropropagation system (MS); (1)Conventional Micropropagation (CM), (2)Natural Ventilation Micropropagation (NV).

The exogenous application of proline has been employed as an osmoprotector substance that improves the performance of plants in saline conditions (ASHARAF; FOOLAD, 2007). In addition, Nanjo et al. (2003), studying *Arabidopsis*, related the use of proline (1g·L^{-1}) with the promotion of normal plant growth. In alfalfa callus (*Medicago sativa*) cultures, 1g·L^{-1} of exogenous proline was very effective at reducing the effects of salt stress and promoting culture growth (EHSANPOUR; FATAHIAN, 2003). Similar results were observed for the use of proline (1g·L^{-1}) added to the culture medium, promoting greater proline contents in plant tissue (14.69 µg·mg^{-1} leaf) (Table 2) and better growth of plants in NV (Table 1). Further, as described in the water content section below, the use of this osmoprotector reduced water loss in the early hours of the acclimatization process (Figure 1).

The use of proline (2g·L^{-1}) had inhibitory effects on plants' in vitro growth, mainly in PDM in both micropropagation systems (Table 1) and lower proline content in foliar tissue (Table 2). Heuer (2003) also mentioned that proline concentrations higher than 3 g·L^{-1} reduced growth in rice and tomato plantlets. It seems that high proline concentrations in the culture medium may be harmful to plants, including having inhibitory effects on growth or cellular metabolism (EHSANPOUR; FATAHIAN, 2003; NANJO et al., 2003).

Chlorophyll content

Chlorophyll *a* content ranged from 3.04 to 3.80 µg·mg^{-1}leaves, and there were no differences among treatments (Table 3). The NV system promoted higher chlorophyll *b* content (4.01 µg·mg^{-1}leaves) than in CM (2.87 µg·mg^{-1}leaves), when plantlets were grown without proline (Table 3). The use of proline (1 g·L^{-1}) increased chlorophyll *b* content in CM (4.52), and this result did not differ from NV system treatments (Table 3).

Table 3. The chlorophyll content of leaves of orchid plantlets grown in different *in vitro* micropropagation systems and proline levels.

MS(*)	Proline (g·L^{-1})					
	0	1	2	0	1	2
	--Chlorophyll *a* (µg·mg^{-1} leaves)---			- Chlorophyll *b* (µg·mg^{-1} leaves)--		
CM(1)	3.04Aa	3.32Aa	3.11Aa	2.87Bb	4.52Aa	3.26Ab
NV(2)	3.59Aa	3.14Aa	3.80Aa	4.01Aa	3.85Aa	3.82Aa

Means followed by the same capital letter in a column or lower case letter in a row do not differ by the Scott–Knott test at 5% of probability. (*)Micropropagation system (MS); (1)Conventional Micropropagation (CM), (2)Natural Ventilation Micropropagation (NV).

Zobayed et al. (1999) suggested that ethylene normally accumulates in bottles containing in vitro cultures, and this reduces the chlorophyll content in plants. Lower chlorophyll content of in vitro grown plants has been also reported in conventional micropropagation (MOHAMED; ALSADON, 2010; IVANOVA; VAN STADEN, 2010). Probably, gas exchange in NV reduces the ethylene concentration in the culture bottle, increasing the chlorophyll b concentration, which was observed in plants grown in this system when compared with CM without proline (Table 3). This could have contributed to the increased photosynthetic rate and greater biomass accumulation observed in NV without proline addition (Table 1).

The use of proline (1g·L^{-1}) in CM promoted higher chlorophyll b content (Table 3). Ben Ahmed et al. (2010) also observed that exogenous proline induced an increase in photosynthetic pigments, and suggested that this might have increased net photosynthesis. Probably, this is related to the fact that proline is involved in the protection of cellular structures and chloroplasts (MOUSTAKAS et al., 2011).

Water content

Plants grown in NV with proline (0, 1 or 2 g L^{-1}) tended to have a higher percentage water content, even 120 min after they were transferred to ex vitro conditions than plants in CM without proline (Figure 1A, B and C). This was due to improved stomatal functionality and tissue foliar organization promoted by culture in NV, which prevented water loss in plants after they were transferred to the natural environment (IVANOVA; VAN STADEN, 2010).

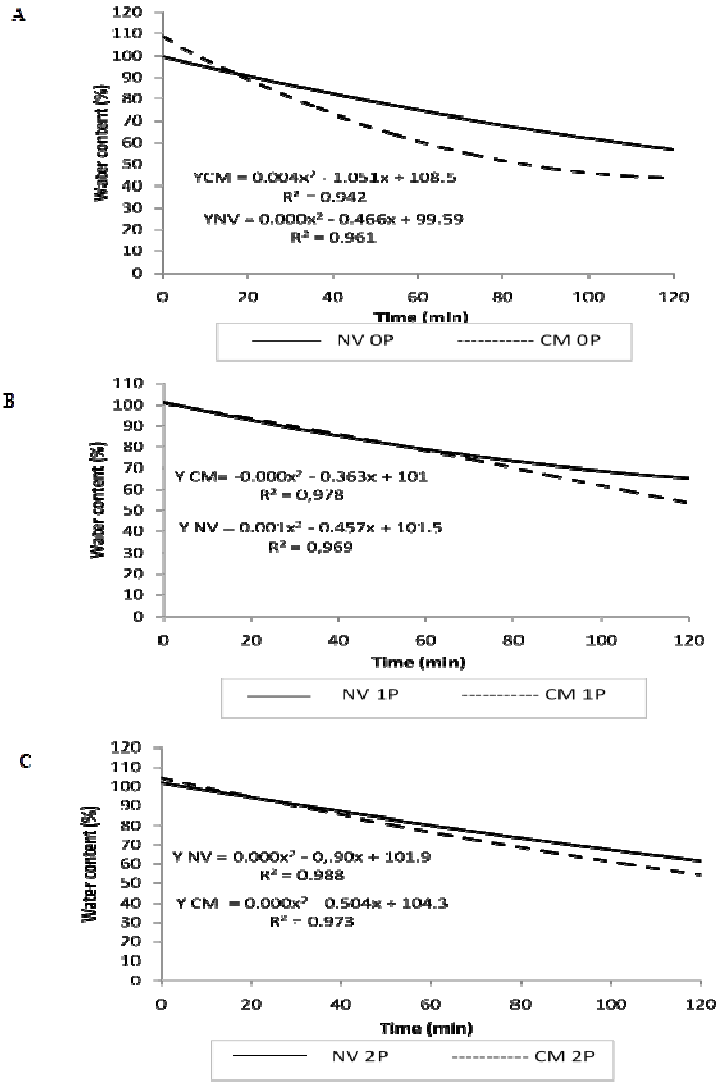

Figure 1. Water content. **A**) Without proline (0 g·L^{-1}) or 0P; **B**) proline (1 g·L^{-1}) or 1P; **C**) proline (2 g·L^{-1}) or 2P. Conventional micropropagation (CM); natural ventilation system (NV).

Excessive water loss by plantlets during the acclimatization process has been reported as the main cause of their poor survival rate in this phase of micropropagation (MOREIRA et al., 2013). Sáez et al. (2012) reported better stomatal functionality and improved water loss control in *Castanea sativa* grown in NV. Moreira et al. (2013) observed lower water loss of orchids cultivated in NV than in conventional micropropagation.

Plants grown in CM without proline had worse water loss control (45%) when transferred to *ex vitro* conditions than those grown in CM with the use of proline (56–60%). Similar results were observed for plants cultured in NV with proline (65–70%) when compared with a micropropagation system without proline (55%). In addition, 1 $g \cdot L^{-1}$ of proline was the best concentration in CM and NV after 120 min of transfer (Figure 1 A, B, and C). In both cases, the use of proline induced an increase of 10–15% in the water content when compared to plants grown without proline. However, plants grown in NV with proline (1 $g \cdot L^{-1}$) had 25% higher water content than those grown in CM without proline.

Proline is associated with an osmoregulatory response and the protection of cellular structures (MOUSTAKAS et al., 2011; BEN AHMED et al., 2010; MUNNS; TESTER, 2008). Thus, it plays an important role in the biochemical defense system—it is accumulated in several plant species in response to abiotic stress, might act as a reactive oxygen species (ROS) scavenger (MOUSTAKAS et al., 2011), and increase the activities of catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidases (APX) enzymes (BEN AHMED et al., 2010). These enzymes are involved in H_2O_2 and O_2 detoxification, reducing damages in cellular structures, such as the membrane system (BEN AHMED et al., 2010). These effects are responsible for cellular integrity and the organization of leaf tissues, which are essential to reduce plants' water loss during the acclimatization phase of micropropagation (MAJADA, 2002, MOHAMED; ALSADON, 2010). Thus, these functions are probably related to improvement of the observed hydric status of the plants by endogenous proline (Figures 1A, 1B, and 1C).

Moustakas et al. (2011), working with *Arabidopsis thaliana*, observed better hydrated leaves in plants treated with proline ($1g \cdot L^{-1}$) than control plants grown on drought soil. Ben Ahmed et al. (2010), studying salt-stress in olive trees, observed a 20% increase in leaf water content in proline treated plants compared to a control, and increased activities of CAT, SOD, and APX enzymes and preservation of appropriate water content in olive leaves due to exogenous proline application. Probably, all of the mentioned factors could explain the better water control of orchid plants treated with exogenous proline.

CONCLUSION

The NV with gas exchange is a better micropropagation system than CM. Also, the use of exogenous proline ($1\ g \cdot L^{-1}$) had positive effects on the in vitro growth and leaf water control of orchid plants.

RESUMO: A micropropagação é uma alternativa para a produção de plantas de orquídeas em larga escala. Entretanto, este processo apresenta perdas durante a fase de aclimatização. O emprego de prolina exógena na cultura de tecidos vegetais é uma alternativa para reduzir o estresse das plantas na fase de aclimatização. O objetivo da presente pesquisa foi verificar o crescimento de orquídeas em diferentes sistemas de micropropagação com prolina adicionada no meio de cultura. Plantas de *Cattleya walkeriana* foram obtidas a partir da germinação de sementes em meio de cultura. Sementes foram germinadas em meio MS, adicionado de 20 g. L^{-1} de sacarose, solidificado com 6 g. L^{-1} de ágar e pH ajustado para 5,8. Após 5 meses, plântulas com 1 cm de comprimento foram inoculadas nos frascos de cultivo de acordo com os tratamentos, os quais foram compostos por dois sistemas de micropropagação (convencional e ventilação natural) em combinação com prolina (0, 1 e 2 g L^{-1}). O experimento foi conduzido em esquema inteiramente casualizado, constando de um fatorial 2x3, totalizando 6 tratamentos com 5 repetições. O sistema de ventilação natural com o uso de prolina (1 g L^{-1}) promoveu o maior acúmulo de massa seca e melhor controle da perda de água das plantas.

PALAVRAS-CHAVE: Orquídeas. Micropropagação. Estresse abiótico.

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