



Growth and photosynthetic responses during *ex vitro* acclimatization of *Etilingera Elatior* (Jack) rm smith (torch ginger)

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ABSTRACT. Torch ginger (*Etilingera elatior* (Jack) RM Smith) can be propagated *in vitro*, but there is currently little information about the stages of acclimatization and adaptation for torch ginger in a greenhouse. The objective was to study the growth responses, survival and photosynthetic response during the acclimatization of plants maintained on three different substrates (Plantmax forestry®, sand and 1:1 Plantmax forestry® and sand) under three shading conditions (50% red and blue shading nets and control without shading nets). The highest leaf and shoot numbers per plant and survival rate were observed in treatments with the Plantmax forestry® type substrate in the absence of shading. *In vitro* culture plants behaved similarly or better than rhizome propagated control plants with regard to the net photosynthesis rate, carboxylation efficiency, stomatal conductance and transpiration rate. In general, for both conditions, *in vitro* and control plants had similar efficiency of biochemical functions and of photosystem II. These results show that plants derived from *in vitro* culture exhibit satisfactory physiological yield and greenhouse acclimation capacity.

Keywords: torch ginger, growth curve, gas exchange, photoautotrophy.

Crescimento e respostas fotossintéticas durante aclimação *ex vitro* de Bastão-do-imperador (*Etilingera elatior* Jack RM Smith)

RESUMO. O bastão-do-imperador (*Etilingera elatior* Jack Smith RM) pode ser propagado *in vitro*, mas não há informações sobre as fases de aclimação e adaptação no campo. O objetivo foi estudar as respostas de crescimento e sobrevivência durante a aclimação *ex vitro* da espécie, quando mantida em diferentes substratos: Plantmax florestal®; areia; Plantmax florestal® e areia 1:1; e malhas de sombreamento a 50% na cor vermelha; azul e controle sem a presença de malha, assim como estudar respostas fotossintéticas durante a aclimação em casa de vegetação. A maior taxa de sobrevivência das plantas, número de folhas e número de brotos por planta, foram observados no tratamento com substrato tipo Plantmax florestal® puro, na ausência de sombreamento. Quanto à taxa de fotossíntese líquida, eficiência de carboxilação, condutância estomática e taxa de transpiração, plantas de cultivo *in vitro* apresentaram comportamento semelhante ou superior quando comparadas com plantas controle obtidas a partir de rizomas. Em geral, a eficiência do funcionamento bioquímico e do fotossistema II foram semelhantes em plantas de cultivo *in vitro* e controle. Conclui-se que tais resultados mostram que as plantas derivadas do cultivo *in vitro* apresentam rendimento fisiológico satisfatório, conferindo capacidade de aclimação no campo.

Palavras-chave: Bastão-do-imperador, curva de crescimento, trocas gasosas, fotoautotrofia.

Introduction

Torch ginger (*Etilingera elatior* (Jack) RM Smith) belongs to the family Zingiberaceae; it has large red or pink inflorescences and is attractive and valued in the ornamental and landscaping plant sectors. Torch ginger is produced in tropical or subtropical regions, in full sun or partially shaded locations, and is currently propagated by seeds and rhizomes (UNEMOTO et al., 2012). Although the use of rhizomes is a traditional practice, this practice has caused the spread of pests and

diseases induced by plant pathogens, thus hampering the commercial production of torch ginger (ALMEIDA; PAIVA, 2005).

Due to the competitive nature of the market and demands of producers and consumers, recent research has focused on the development of healthy plants with phytosanitary control using *in vitro* propagation techniques (COLOMBO et al., 2010; LIMA-BRITO et al., 2011; PÊGO et al., 2013). Plants grown *in vitro* undergo various anatomical and

physiological phenotypic changes during propagation and require an acclimatization period (ARIGITA et al., 2002; KUBOTA; KOZAI, 1992; RODRIGUES et al., 2012; SANTANA et al., 2011a). However, the special environmental conditions inside the culture vessels and heterotrophic nutrition may generate anomalies at both anatomical and functional levels, such as hyperhydricity, poor water loss control, low photosynthesis, difficulty rooting and low functionality, in plants propagated using *in vitro* techniques (BADR; DESJARDINS, 2007; SANTANA et al., 2011b).

These anomalies are evident during the transfer of *in vitro* plants to a greenhouse or field, resulting in slow establishment and a low survival percentage compared to plants cultured by traditional propagation. Transferring plants from *in vitro* to *ex vitro* conditions requires adaptation to the new conditions (APÓSTOLO et al., 2005). One of the major changes that *in vitro* shoots must withstand is a substantial increase in irradiance, which challenges the photoprotective mechanisms of these plants. Excess irradiance can decrease photosynthesis and lead to the photo-oxidative destruction of the photosynthetic apparatus (OSÓRIO et al., 2010).

Plants have evolved a series of mechanisms that enable them to manage the absorption of excess radiation. In conventional propagation, *in vitro* plants are grown heterotrophically or photomixotrophically in a medium containing sucrose as the main carbon source. Such conditions may negatively affect biochemical processes related to both the quantity and the enzymatic activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. An insufficient supply of this enzyme may be related to an increased plant susceptibility to feedback inhibition associated with an excessive accumulation of hexoses and starch. Thus, the negative effect on the net photosynthetic rate may be compensated for by the promotion of *in vitro* plant growth by sugars supplied in the culture medium (LE et al., 2001).

Changes in environmental conditions and the substrate can cause specific changes in the distribution of photoassimilates in plants grown *in vitro* and affect metabolic processes such as photosynthetic efficiency, water transport and transpiration, directly affecting the production of seeds, fruits and flowers (LAISK et al., 2005; LE et al., 2001; TAEB; ALDERSON, 1990).

Some authors have reported marked effects on the photomorphogenesis of plants during the acclimatization process arising from changes in the

quantity or quality of the photosynthetic active radiation received. These changes can occur by using artificial lighting or roofing and colored nets in the greenhouse, which modifies radiation (MARTINS et al., 2009; OREN-SHAMIR et al., 2001; SHAHAK et al., 2004).

The morphological and physiological responses of plants depend on the presence, absence, quality and variation of radiation attenuation (LAISK et al., 2005). Some studies have noted the importance of radiation and substrate type in moisture retention during the acclimatization of plants cultivated *in vitro* (SILVA et al., 2008; STEFANELLO et al., 2009). Differences may also be observed in leaf and root cell differentiation (BRAGA et al., 2011; SILVA JÚNIOR et al., 2012). However, few studies have assessed the impacts of the quality of light spectra on the acclimatization phase and adaptation of *in vitro* plants in the field by collecting physiological data on metabolic efficiency.

This study aims to evaluate the vegetative growth of *E. elatior* var. Red Torch, derived from *in vitro* cultures, during the acclimatization process with different shading conditions and substrate types. Additionally, photosynthetic efficiency was compared between greenhouse-adapted *in vitro* plants and plants derived from rhizomes.

Material and methods

Seed collection, disinfection and *in vitro* culture

Seeds of torch ginger obtained from commercial planting were disinfected by serial immersion in 70% (v v⁻¹) ethyl alcohol for 1 min. under continuous agitation and 50% (v v⁻¹) bleach solution containing 5% sodium hypochlorite and three drops of Tween 20 per 100 mL for 5 min. The seeds were rinsed three times with sterile distilled water. A second disinfection step was implemented in the laminar flow cabinet by immersing the seeds in 70% (v v⁻¹) ethyl alcohol for 1 min. and then rinsing them three times with sterilized deionized water, under continuous agitation. Finally, the seeds were placed in test tubes (25 x 150 mm) containing 20 ml of culture medium (pH 5.8) and covered with polypropylene caps, in preparation for germination.

MS culture medium (MURASHIGE; SKOOG, 1962) was supplemented with 8.88 μ M N-6-benzylaminopurine (BAP) to induce the production of shoots. Four subcultures of shoots were performed every 30 days to obtain sufficient quantities of explants for the experiments. The explants were kept in a growth chamber at a temperature of $26 \pm 1^\circ\text{C}$ under a 16 hour photoperiod with fluorescent light irradiance of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$.

First Experiment - Acclimatization of plants in a growth chamber

Plants with a pair of leaves, a 3 cm shoot and a root system were acclimatized in plastic tubes (34 x 125 mm) and remained covered with transparent polyethylene plastic for the first 15 days. The experiment adopted a completely randomized factorial design consisting of three substrates (Plantmax forestry[®], sand and 1:1 Plantmax forestry[®] and sand) and 3 shade conditions (50% red shading net, 50% blue shading net and a control without shading) with 20 replicates per treatment. The commercial substrate, Plantmax forestry[®], consisted of an average composition of 60% pinus bark, 30% vermiculite and 10% humus.

Characteristics of vegetative growth, such as plant height, number of leaves, number of shoots and survival rate, were evaluated for 70 days. The results were analyzed by regression analysis, and growth curves were fitted using a sigmoidal fit.

Second Experiment - Acclimatization of plants in the greenhouse

After the 70 day period described in the first experiment, 60 plants were transferred to a greenhouse and were planted in pots containing 3 L of substrate composed of oxisol soil, cattle manure and washed sand in a 2:1:1 ratio. The analysis of gas exchange and chlorophyll fluorescence began 20 days after the transfer of the plants to the greenhouse; plants were maintained *ex vitro* for a total of 90 days. Plants obtained through conventional rhizome tillage for 120 days were used as a control. The experiment was conducted in a greenhouse with 50% black shade nets in a completely randomized design, composed of five plots with ten plants per plot, totaling 50 plants per treatment.

Every 15 days after the 20th day in the greenhouse, we assessed 1) the net photosynthetic rate (A); 2) carboxylation efficiency, by the ratio between net photosynthesis and carbon concentration in mesophyll (A/Ci); 3) the ratio of mesophyll (Ci) to atmospheric (Ca) CO₂ concentration (Ci/Ca); 4) stomatal conductance (gs); 5) transpiration (E); and 6) efficiency of photosystem II (PSII). An infrared gas exchange analyzer (IRGA) (LCA-4 ADC Hoddesdon, UK) was used, and evaluations were performed on days that were mostly clear, between 10:00 and 11:00 ours, from August to December 2011. Analysis was performed on fully expanded leaves of the third node. Fluorescence assessments were made at night using a portable fluorometer (Heinz Walz Mini-

PAM GmbH, Effeltrich, Germany), and the same leaves were evaluated in the morning for gas exchange.

Statistical analysis

The database was subjected to the Shapiro-Wilk test of normality ($p < 0.05$). Data were subjected to an analysis of variance, and the means were compared by Tukey post-hoc tests at 1% probability. Growth curve results were subjected to regression analysis and were fitted using the sigmoidal fit, which tends to be a finite upper asymptote, with biological significance. The adjusted equations were analyzed for significance by F tests of the variance ($p < 0.01$), and the fitted equations were analyzed for significance by t tests ($p < 0.01$).

Results and discussion

First Experiment

The growth of torch ginger during the acclimatization process was gradual and steady; however, the growth curves stabilized at certain periods of cultivation. The highest plant height (253 mm) was observed at 70 days when the Plantmax forestry[®] or Plantmax forestry[®] + sand substrate was used, in the absence of nets or when red shading nets were applied. The lowest plant height (98 mm) was observed for plantlets acclimatized in sand and placed under blue shading nets. We found that the growth in height stabilized after 50 days of the cultivation period (Figure 1).

Thus, the ranking of plant height by substrate type is as follows: Plantmax forestry[®] > 1:1 Plantmax forestry[®] + sand > sand. The ranking of plant height by shading conditions is as follows: control > red shading nets > blue shading nets.

The highest value for leaf number (6) was observed between 56 and 70 days of cultivation with the Plantmax forestry[®] substrate in the absence of shading nets. Plants grown with the same substrate maintained under red shading nets showed steady growth rates, with 5 leaves per plant after 70 days of cultivation. Blue shading nets also caused a decrease of leaf number, but this decrease was slower than under red shading net, reaching a mean value of 4 leaves per plant after 70 days of cultivation (Figure 2).

The lowest average number of leaves was observed in plants grown in sand with blue shading

nets. Under these conditions, there was an increase in leaf number after 21 days of cultivation, followed by a reduction in growth after 56 days this later resumed after 70 days of cultivation. Plants grown in the same substrate, in the absence of shading nets, maintained the formation of new leaves after 28 days, reaching a maximum value of 4 leaves per plant after 70 days of cultivation. Thus, the ranking for number of leaves follows the same ranking as described above for shoot height.

The difference in results may relate to the type of substrate used (Plantmax forestry®) and its composition, which allows for the retention of more water than traditional substrates, favoring the photochemistry phase. The lowest values were obtained with shading nets (red and blue), possibly due to the low incidence of photosynthetically active radiation. Similar results were reported for blue shading nets, which caused

a reduction in shoot height and overall biomass in *Anthurium andraeanum* (NOMURA et al., 2009). However, colored shading nets did not significantly influence the increase in height, leaf number, shoot number and height and leaflet height and width in raffia palm (*Rhapis excelsa*) (MEIRELLES et al., 2007).

The highest average number of shoots per plant (2 shoots plant⁻¹) was observed in plants grown in the Plantmax forestry® substrate in the absence of shading nets (Figure 3). The use of red or blue shading nets did not increase the shoot number in any of the treatments, regardless of the composition or type of substrate, demonstrating the negative influence of the shading nets. The nets may reduce the amount of incident radiation and/or change the light spectra, affecting the induction of multi-shoot plants of torch ginger (Figure 3).

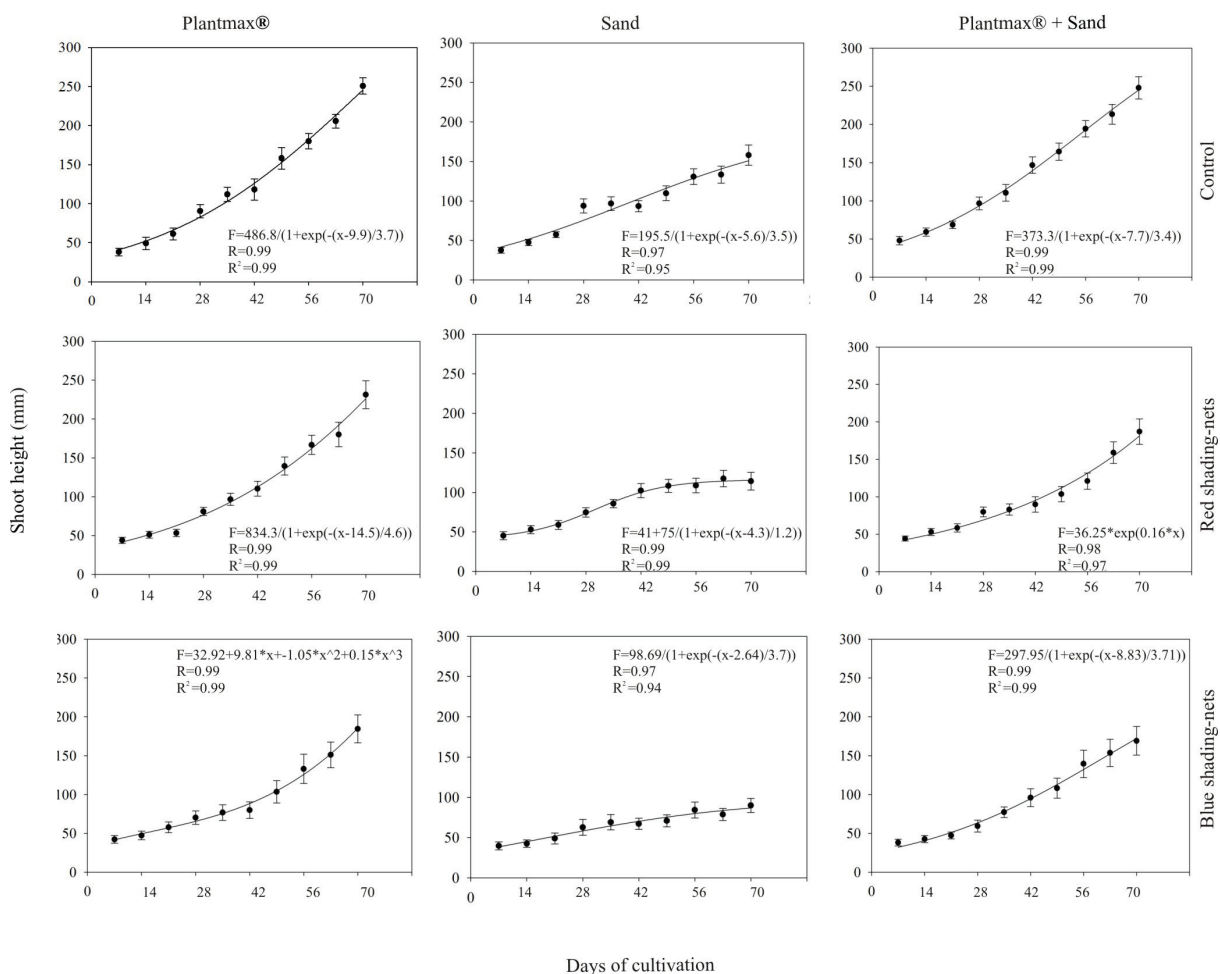


Figure 1. Temporal patterns of height growth of torch ginger plants acclimated with different substrate types and shading conditions. Standard deviations are shown by vertical error bars.

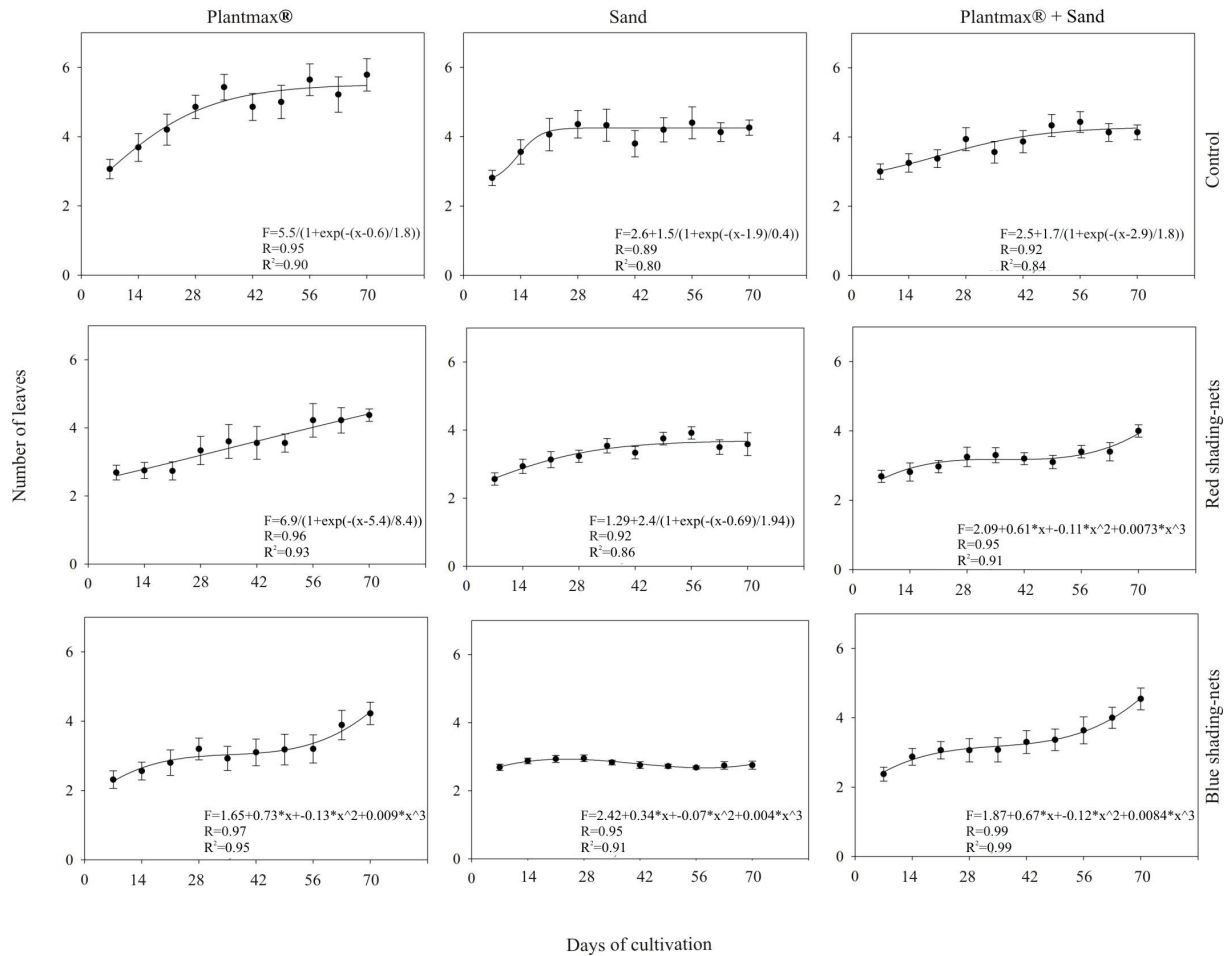


Figure 2. Temporal patterns of leaf number in plants of torch ginger acclimatized with different substrate types and shading conditions. Standard deviations are shown by vertical error bars.

The lowest average number of leaves was observed in plants grown in sand with blue shading nets. Under these conditions, there was an increase in leaf number after 21 days of cultivation, followed by a reduction in growth after 56 days this later resumed after 70 days of cultivation. Plants grown in the same substrate, in the absence of shading nets, maintained the formation of new leaves after 28 days, reaching a maximum value of 4 leaves per plant after 70 days of cultivation. Thus, the ranking for number of leaves follows the same ranking as described above for shoot height.

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A recent study on the impact of different substrates and 30% shading nets on the growth rates of *in vitro* cultures of torch ginger plants found that sand, powder coconut + sand and de-fibered coconut + sand provided the best results for survival rate, shoot height, fresh weight, shoot number and root dry weight (ASSIS et al., 2009).

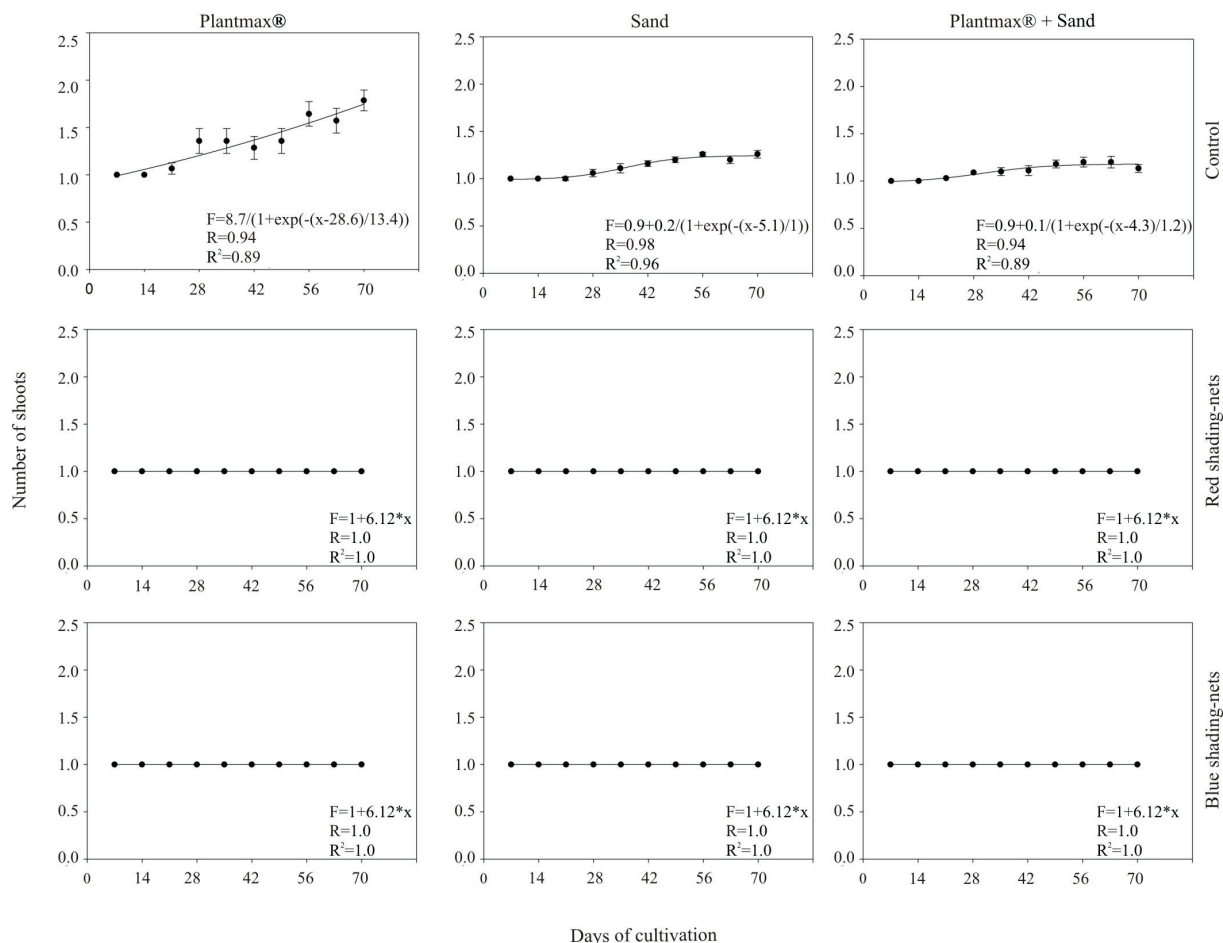


Figure 3. Temporal patterns of shoot numbers per plant of torch ginger acclimatized with different substrate types and shading conditions. Standard deviations are shown by vertical error bars.

The use of no shading net or a red shading net provided greater plant survival of torch ginger during the acclimatization period, with an average survival of 93 and 75%, respectively (data not shown). Thus, during the acclimatization process, the spectral quality of light and the type of substrate can significantly affect the growth, development and morphogenesis of vegetative organs of this species. Moreover, as observed in other studies, these factors greatly affect leaf expansion, showing a high degree of anatomical and physiological plasticity in plants during acclimatization (DIGNART et al., 2009; BRAGA et al., 2011).

The use of sand as a substrate was not favorable for vegetative growth in terms of height, number of leaves and induction of new shoots. The porosity of sand substrates, increased percolation and runoff may cause water stress and slow growth in plants (ALVINO; RAYOL, 2007).

Second Experiment

There was no temporal trend in gas exchange, with no increase or decrease in photosynthetic responses

over time of acclimatization, indicating that the *in vitro* plants are well established in the external environment. Furthermore, the *in vitro* plants and the control plants were responsive to environmental variations, since photosynthesis fluctuations were solely due to changes in photosynthetic active radiation.

The net photosynthesis (A) (Figure 4A) of the *in vitro* culture plants during acclimatization to greenhouse conditions was similar to that of the control plants derived from conventional tillage by rhizomes.

In assessments at 150, 165 and 180 days of acclimatization, the average rate of photosynthesis of the *in vitro* culture plants was higher than that in the control plants. The maximum net photosynthesis value reached by the *in vitro* plants was $8.3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at 150 days, while the control plants reached a maximum rate of $6.2 \mu\text{mol m}^{-2} \text{ s}^{-1}$ at 165 days (Figure 4A).

The carboxylation efficiency (A/Ci) of the *in vitro* culture plants was higher than that of the control plants in virtually all assessments, except in the 4th

evaluation. A maximum value of 0.056 at 180 days was recorded for the *in vitro* culture plants, compared to 0.038 at 165 days for the control plants. These results suggest that there may have been a limitation of carboxylation control, which may have been caused by differences in incident radiation (Figure 4B). These data are consistent with the non-significant results for the C_i/C_a ratio (Figure 4C)

and the observation of high rates of photosynthetically active radiation (PAR), which resulted in higher carbon assimilation rates and consequently higher rates of net photosynthesis. Thus, acclimatized plants were able to respond to environmental changes and increase net photosynthesis in response to increased photosynthetic photon flux.

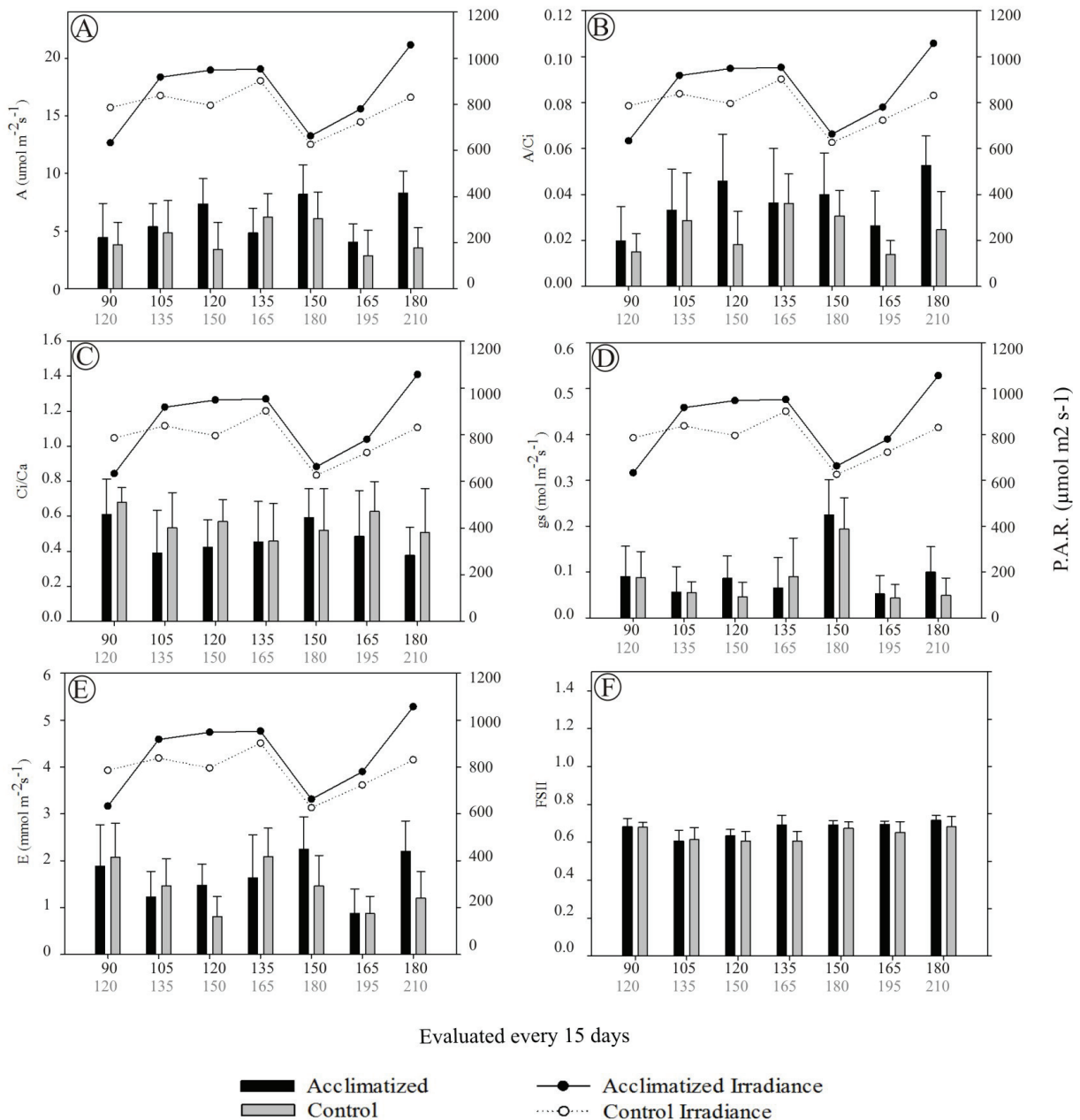


Figure 4. Gas exchange and fluorescence in acclimatized and conventionally propagated torch ginger plants. A) Photosynthesis [A]; B) carboxylation efficiency [A/C_i]; C) ratio of intercellular to atmospheric CO₂ concentration [C_i/C_a]; D) stomatal conductance [g_s]; E) transpiration [E]; F) efficiency of photosystem II [PSII]. Black bar = acclimatized; gray bar = control; continuous line = irradiance during acclimatization; dotted line = irradiance during growth of the control plants. The time scale of days (x axis) in black refers to the age of plants from *in vitro* cultivation, while gray refers to the age of control plants. Standard deviations are shown by vertical error bars.

Plants grown under shade or with low irradiance have a limited ability to increase their photosynthesis rates. The opposite occurs in plants grown with high irradiance. The factors that limit photosynthesis vary with the light regimes in the growth environment. Plants grown under low irradiance invest more energy in mechanisms to absorb light, whereas plants grown in full sun invest in Calvin cycle proteins and electron transport (LAISK et al., 2005).

Variations in the light regime during growth typically cause changes in the rates of photosynthesis due to differences in the maximum carboxylation velocity of Rubisco (V_{cmax}) and the maximum rate of regeneration of ribulose biphosphate, RuBP (J_{max}), which is dependent on transporting electrons, as well as differences in the rates of CO_2 diffusion into chloroplasts (AZEVEDO; MARENCO, 2012).

Acclimatized plants showed higher values of stomatal conductance (g_s) than the control plants at 120, 150, 165 and 180 days. The *in vitro* culture plants showed a mean maximum value of $0.23 \text{ mol m}^{-2} \text{ s}^{-1}$ at 150 days, while the control plants had an average of $0.19 \text{ mol m}^{-2} \text{ s}^{-1}$ at 180 days. The high values of g_s observed during the 5th evaluation for both treatments was probably due to the lower irradiance in the leaves, averaging $615 \mu\text{mol m}^{-2} \text{ s}^{-1}$ for this period but showing compared values greater than $800 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in other periods (Figure 4D). In contrast with results recently reported for *Minquartia guianensis*, traditionally classified as a late climax and shade tolerant species, high values for stomatal conductance were observed in plants acclimatized under high irradiance (MAGALHÃES et al., 2009).

The highest rates of transpiration for both treatments (Figure 4 E) were recorded under low irradiance, at about $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Stomatal closure probably occurred above this range, reducing the stomatal conductance and transpiration rate in both treatments.

Acclimatized plants showed higher transpiration rates compared to the control plants, with average values of 1.94, 2.97 and $2.75 \text{ mmol m}^{-2} \text{ s}^{-1}$ at 120, 150 and 180 days, respectively, for acclimatized plants and average values of 1.31, 2.05 and $1.72 \text{ mmol m}^{-2} \text{ s}^{-1}$ at 150, 180 and 210 days, respectively, for control plants (Figure 4E). There were no differences among the other treatments. Thus, the acclimatized plants had higher metabolic activity and water flow during these periods, which favored a higher rate of growth and development.

A recent study of gas exchange in *Castanea sativa* grown *in vitro* and *ex vitro* showed that the maximum

photosynthetic rate, carboxylation efficiency, stomatal conductance and transpiration rate were lower in plants grown *in vitro* than those acclimated in nurseries after 12 months of cultivation (SÁEZ et al., 2012). Thus, the period of acclimatization appears to be essential for adaptation to new environments for *in vitro* propagated plants.

Both the acclimated and control plants showed an average photochemical efficiency of photosystem II (PSII) between 0.69 and 0.72 during the evaluation period. There were no differences between the treatments, with the exception of the 4th cultivation evaluation, when the acclimatized plants had a higher average value of 0.76 at 165 days compared to an average of 0.63 for the control plants at 165 days (Figure 4F). Similar results were reported by MAGALHÃES et al. (2009) after exposing *Minquartia guianensis* plants to direct sunlight during the period of acclimatization. They also observed a reduction in the efficiency of photosystem II in the initial days of exposure, followed by gradual recovery in which the maximum values reached 0.93. A study on *Gevuina avellana* also showed maximum efficiency and rate of electron transport in PSII in acclimatized plants. The increase in photon flux (light treatment) resulted in a significant improvement in the fluorescence parameters of photochemical quenching, non-photochemical quenching, electron transport rate and photochemical efficiency of PSII, compared to the ventilation and control treatments. Nursery and light treatment plants showed similar values in microshoot comparisons, indicating that the light treatment plants managed to dissipate excess light (ALVAREZ et al., 2012).

Under conditions of low gas exchange, *in vitro* propagated plants usually show morphological and physiological anomalies that lead to high mortality rates during *ex vitro* acclimatization. Open systems allow natural ventilation to increase, favoring greater gas exchange, increased plant growth and photosynthetic pigment content. Thus, in *ex vitro* conditions, photoautotrophy provides greater physiological efficiency and biomass production (IAREMA et al., 2012; SALDANHA et al., 2012). This finding shows that the environmental conditions during plant growth can influence cell differentiation, resulting in anatomical and physiological adaptations. Direct implications in production may occur by modifying growth, increasing the number of leaves and roots, changing the leaf and root dry matter content, varying the contents of "a" and "b" chlorophyll and altering the thickness of mesophyll and leaf blades (SILVA JÚNIOR et al., 2013).

Conclusion

Based on the results, the acclimatization of Torch ginger using a substrate composed of Plantmax forestry® and sand was sufficient to retain water for plant growth.

The use of shading nets is not necessary for an increase in the shoot number, leaf number and shoot height. The production cost of the seedlings is lowered by reducing the material consumption.

Gas exchange and photosynthetic characteristics in plants from *in vitro* cultures were similar to those derived from conventional propagation using rhizomes (control). The results indicate that there was satisfactory acclimatization of plants derived from *in vitro* cultures.

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