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Initial in vitro plant establishment of seeds and nodal segments from bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch differs in respiratory rates and shoot formation

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Título resumido: Respiration of seeds and nodal segments maintained in vitro

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ABSTRACT – (Initial in vitro plant establishment of seeds and nodal segments from bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch differs in respiratory rates and shoot formation). We aimed to investigate the morphological and respiratory differences during in vitro shoot formation from seeds and nodal segments (NS) of *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch, due to differences in plants obtained by micropropagation. During 35 days of culture, seeds resulted in full plants 14 days earlier than NS, with longer leaves and more roots. Nevertheless, NS plantlets exhibited shoot multiplication. Peaks in O_2 consumption and CO_2 release were detected at 7 and 14 days for NS and seeds, respectively, suggesting that initial growth has a high energetic requirement. However, the respiration peak was higher in NS than in seeds, possibly due to high energy consumption required for multiple bud breaks. After peaking, respiration decreased, reaching similar values between propagules by 35 days, indicative of an ongoing increase in photosynthesis in both seed and NS plants, possibly due to shoot growth. In conclusion, the development process of NS plants may affect the energy and respiratory demand differently than in seedlings.

Keywords: carbon dioxide, lateral buds, micropropagation, oxygen, respiration

RESUMO – (Estabelecimento in vitro inicial de plantas de sementes e segmentos nodais da bromélia *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch difere em taxas respiratórias e formação de brotos). Buscou-se investigar as diferenças morfológicas e respiratórias durante a formação in vitro de brotos a partir de sementes e segmentos nodais (NS) de *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch, devido às diferenças entre as plantas obtidas pela micropropagação. Durante 35 dias de cultura, as sementes resultaram em plantas completas 14 dias antes do NS, com folhas mais longas e mais raízes. No entanto, as plantas NS exibiram multiplicação de brotos. Picos no

consumo de O2 e liberação de CO2 foram detectados aos 7 e 14 dias para NS e sementes, respectivamente, sugerindo que o crescimento inicial tem alta demanda energética. No entanto, o pico de respiração foi maior em NS do que em sementes, possivelmente devido ao alto consumo de energia necessário para as múltiplas quebras de gemas. Após o pico, a respiração diminuiu, atingindo valores semelhantes entre os propágulos aos 35 dias, indicativo de um aumento contínuo da fotossíntese em plantas de sementes e NS, possivelmente devido ao crescimento de brotos. Em conclusão, o processo de desenvolvimento das plantas de NS pode afetar a demanda energética e respiratória de forma diferente daquelas de sementes.

Palavras-chave: dióxido de carbono, gemas laterais, micropropagação, oxigênio, respiração

Introduction

An efficient conservation method for endangered plant species is the development of in vitro germplasm banks, since they allow the storage of a great number of plants in small spaces, without excessive maintenance as required for plants in the field or greenhouses (Imarhiagbe *et al.* 2016, Oseni *et al.* 2018). This technique can be applied to seeds or tissue explants, such as nodal segments (NS, Pilatti *et al.* 2011). The ornamental bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch (Bromeliaceae) can be propagated in vitro by seeds and NS isolated from the stems of elongated plants that are subcultured to obtain new plants (Santos *et al.* 2010).

Micropropagation has been used in the cultivation of diverse bromeliads species to meet the ornamental plants market demand, which aids in preventing illegal extraction (Mercier & Nievola 2003, Negrelle *et al.* 2012). Due to the possibility of maintaining cultures in controlled conditions (light, temperature, nutrients, etc.), this

technique has been used for studies of basic physiology in diverse species (Narayani & Srivastava 2017, Bakhshipour *et al.* 2019, Chetty *et al.* 2020), including bromeliads (Carvalho *et al.* 2013, Freitas *et al.* 2015, Andrade & Tamaki 2016, Santos *et al.* 2017, Silva *et al.* 2017, Andrade-Santos *et al.* 2020, Silva *et al.* 2020).

The cultivation of plants under sealed flasks could lead to restriction of gas exchange between the internal and external environment, causing an accumulation of gases such as CO_2 and O_2 inside the flasks (George *et al.* 2008). Thus, the gaseous composition of the isolated in vitro atmosphere reflects the respiratory and photosynthetic activities of micropropagated plants (Chen 2006). When plants are cultivated under photomixotrophic conditions, defined by the use of high sugar concentration in the culture medium, low irradiance, high relative humidity and reduced gas exchange due to enclosed vessels, the rate of photosynthesis is diminished while respiration is less affected due to the carbon supply (Lucchesini *et al.* 2001, George *et al.* 2008, Ševčíková *et al.* 2019).

The consumption of gases and respiratory activity of seeds and NS would inevitably differ since shoot development from each propagule undergo very distinct processes: germination involves the imbibition of the seed, followed by increased respiration and metabolic activity that result in root emergence from the seed testa (Taiz *et al.* 2017).

NS lead to shoot formation from an axillary bud meristem that is stimulated after apical dominance is broken due to the node isolation from the mother plant (George *et al.* 2008). However, no studies about respiration during in vitro plantlet development from lateral buds and how it compares to seed germination were found.

Considering the developmental differences between seeds and NS, we hypothesize that these propagules of *A. strobilacea* have distinct rates of respiration

during plant formation. Hence, to evaluate our hypothesis, we investigated the morphological development during initial in vitro shoot formation from seeds and NS of *A. strobilacea* and the progression of respiratory rates during 35 days by assessing CO_2 and O_2 levels inside culture vessels. By evaluating the progression of respiratory rates during in vitro plant formation, it would also be possible to detect differences in photosynthetic activity between propagules. The obtained results may indicate the metabolic state of plants from seeds and NS and provide information on the period that plants initiate photosynthetic activity and thus, have higher capability to endure acclimatization (Ševčíková *et al.* 2019). Therefore, this study may aid in the improvement of the maintenance of germplasm collections and plant production of *A. strobilacea*, while also presenting the original method of a simultaneous evaluation of the time course of the initial development of these kinds of explants during micropropagation.

Materials and methods

Seeds of *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch were harvested at the Reserva Biológica de Mogi Guaçu in São Paulo State, Brazil, and stored under 8°C for approximately 12 months prior to the experiments. NS were obtained as described by Santos *et al.* (2010). The seeds were surface sterilized in 100% (v/v) commercial sodium hypochlorite (2% of active chlorine) containing 0.1% (v/v) Tween 20® for 20 min and then transferred to 25% (v/v) of hydrochloric acid for 10 min to remove a large part of the mucilage. Seeds were immersed in 70% (v/v) ethanol for 5 min, in the fungicide Benomyl 0.1% (w/v) for an additional 15 min, and in 100% (v/v) commercial sodium hypochlorite containing 0.1% (v/v) Tween 20® for 1 h (Santos *et al.* 2010). The seeds were transferred aseptically to 250 mL flasks containing 40 mL of

Murashige & Skoog (1962) medium (MS) with 1/5 of the original macronutrient concentration (MS/5), 100% of MS micronutrients, sucrose 2% (w/v), 100 mg L^{-1} of myo-inositol, and 0.1 mg L^{-1} of thiamine. The pH was adjusted to 5.8, and agar (5 g L^{1}) was added before autoclaving for 15 min at 121°C. The seeds were kept in a growth room at 25±2°C, 12-h photoperiod and 14 μmol m^{-2} s^{-1} to induce elongation of the stem axis of the plants (Santos *et al.* 2010). After three months, NS were isolated from the elongated plants (figure 1a) and transferred aseptically to flasks containing 20 mL of the same culture media described previously, closed with caps containing a 2-mm hole covered by a rubber septum for the harvesting of air samples (Lamarca & Barbedo 2012). The same was performed for a batch of surface-sterilized seeds. Flasks were kept in a growth room under 12-h photoperiod, 25±2 °C and 30 μmol m^{-2} s^{-1} irradiance. Plant growth evaluation and air samples were analysed during the light period from five flasks containing five propagules each at 0, 7, 14, 21 and 35 days.

The percentage of oxygen (O_2) and carbon dioxide (CO_2) in the flasks was determined by a gas analyser (model ILL6600, Illinois Instruments, Inc., Johnsburg, IL, USA) as described (Lamarca & Barbedo 2012). These analyses allow an indirect assessment of respiratory activity by the calculation of O_2 consumption and CO_2 release rates. The percentage values of O_2 and CO_2 were converted to partial pressure of gas through the equation: $p1\ P^{-1} = v1\ V^{-1}$, where: p1: partial pressure of gas (atm), P: local atmospheric pressure (= 0.9 atm), v1: gas volume (%) and V: total volume (= 100%) (Atkins & De Paula 2001). These values were then converted to μmoles of O_2 and CO_2 through the equation: $p1\ V = n\ RT$, where: V: total volume of air of flasks (L), n: number of moles of gas, R: gas universal constant (0.082 atm L mol^{-1} K^{-1}), T: temperature (in Kelvin) (Atkins & De Paula 2001).

The following growth parameters were evaluated in 20 plants separated at random from the pool of 25 propagules, per period: number of leaves and roots; longest leaf and root length. Only roots over 0.5 cm and expanded leaves were measured. The selection was performed due to the presence of NS that did not sprout or seeds that did not germinate during the experiment.

For the evaluation of number of shoots per propagule, a separate batch of NS and seeds were cultivated in 250 mL flasks containing 40 mL of MS/5 culture media and maintained in a growth room under 12-h photoperiod, 25±2 °C and 30 μmol m^{-2} s^{-1} irradiance. Shoot production was assessed every 15 days of in vitro culture up to 75 days in 15 plants per period (five plants per flask in a total of three flasks).

Results and discussion

Leaf formation was first detected in seeds at 14 days of culture, concomitantly to roots (figures 1d and 2). The same occurred for leaves of NS, although roots were detected at 28 days (figure 2). Accordingly, it was reported that *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch seedlings develop roots earlier than leaves (Pereira 1988) while in node cultures, shoots are generally formed prior to roots, requiring hormonal addition to the media to induce rooting (George *et al.* 2008) – although this is not the case for *A. strobilacea* (Santos *et al.* 2010, 2017).

NS showed shoot multiplication after two weeks of culture (table 1, figures 1b and c), which was not present in seeds (table 1, figures 1d and 2e). However, seedlings had higher leaf elongation, more and longer roots than NS plants by 35 days of culture (figure 2). The more intense root and leaf growth in seedlings might be the result of reserve mobilization from the seed, which provides nutrients for the plant until it is nutritionally independent (Taiz *et al.* 2017). Meanwhile, shoot multiplication in NS may

be due to multiple bud breaks induced by apical dominance release after node excision from the mother plant (George *et al.* 2008).

The O_2 consumption and CO_2 release rates in seeds of *A. strobilacea* peaked at 14 days of culture (figure 3), corresponding to full seedling formation (figure 2). Contrarily, respiratory rates in NS peaked at 7 days (figure 3), being five times higher than seeds and before the detection of leaf emergence at 14 days. Therefore, we may assume that NS showed a more intense and earlier requirement for energy generation through respiration than seeds for initiating plant formation. The isolation of nodes and apical dominance release stimulates cytokinin accumulation in the excised tissue, leading to cell proliferation in the lateral buds and new shoots formation (Souza *et al.* 2010, Buchanan *et al.* 2015, Li *et al.* 2018). Thus, respiratory activity would increase accordingly to provide sufficient energy for cell division during shoot formation and multiplication process (Siqueira *et al.* 2018). Accordingly, it is reported that sugar accumulates in NS cultivated in vitro as to promote bud outgrowth and provide substrates for respiration to sustain the intense cell division (see review by Schneider *et al.* 2019). A similar pattern was previously described for the bromeliad *Ananas comosus* (Souza *et al.* 2010). Furthermore, the higher respiration rates in NS than in seeds may be due to the multiple bud breaks in the former (table 1, figures 1b and c).

The peaks in respiration during plant formation may increase the flux of electrons in the mitochondria and consequently, formation of reactive oxygen species (ROS) (Møller 2001). ROS are involved in cell signaling pathways and specific gene expression related to seed germination, seedling formation and bud dormancy release (Bailly *et al.* 2008, Kranner *et al.* 2010, Considine & Foyer 2014). Hence, it is possible that the respiration pattern of seeds and NS observed herein is associated with

fluctuations in mitochondrial ROS that directly influence seedling and plant formation from NS.

The O_2 consumption and CO_2 release rates progressively decreased in NS and seeds after peaking at 7 and 14 days, respectively, reaching similar values by 35 days (figure 3). This decrease in respiration rate might have derived from an ongoing increase in photosynthetic activity due to the development of leaves and multiple shoots in seeds and NS, respectively, and due to depletion of the carbon source of the media. Overall, in vitro plants cultivated under photomixotrophic conditions as in the present study show photosynthetic activity, although at lower rates than respiration (Ševčíková *et al.* 2019). It has been reported that protocorm-like bodies of the *Cymbidium* Melody Fair 'Marilyn Monroe' orchid showed an increase in photosynthetic rates concomitant to decreases in respiration between 20 and 60 days of in vitro culture in MS medium containing 2% sucrose (Ogasawara *et al.* 1995). Studies also indicate that an increase in photosynthesis can lead to higher cytosolic ATP/ADP rates, inhibiting respiration (Krömer 1995; Buchanan *et al.* 2015). The decrease in O_2 consumption observed herein may also reflect the cellular energy demand, which was possibly lower after seedling establishment and full morphogenesis in NS plants - both highly energetic processes (Yaseen *et al.* 2012, Taiz *et al.* 2017). Finally, the occurrence of some photosynthetic capacity observed in seed and NS plants by 35 days would possibly facilitate ex vitro acclimatization at that period (Ševčíková *et al.* 2019). Indeed, our research group observed a 100% survival rate after transplanting one-month-old *A. strobilacea* seedlings (De Carvalho *et al.* 2014) and NS plants (unpublished data) to *Pinus* bark substrate, under the same environmental conditions described.

To our knowledge, the present study is the first to compare the time course of in vitro plant development from distinct propagules simultaneously. This approach

allowed us to conclude that the developmental process of NS plants formation may affect the cell energy demand and, thus, respiration activity differently than in seedlings. Nevertheless, both seeds and NS plants of *A. strobilacea* can form viable plants with leaves and roots in a short period of time, which broadens the applicability of in vitro culture for both conservation and commercial production of this species. Indeed, the use of seeds ensures genetic variability, desirable for germplasm conservation (Pence 2010), while the shoot multiplication in NS enables a large-scale clone production, which is of considerable value for commercial production aiming at the ornamental plants market (Kyte *et al.* 2013).

Finally, considering the involvement of mitochondrial ROS production and carbohydrate mobilization in seed germination and bud outgrowth (Considine & Foyer 2014, Taiz *et al.* 2017, Signorelli *et al.* 2018), it may be valuable to investigate the effects of redox status and carbohydrate composition during shoot generation from seeds and NS of *A. strobilacea* to provide more insight on the developmental discrepancies between them.

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Author contributions

Victória Carvalho: Substantial contribution in data collection, analysis, interpretation, and manuscript preparation.

Camila Pereira Carvalho: Contribution in data analysis, interpretation, and manuscript preparation; Contribution to critical revision, adding intellectual content.

Daniela Soares dos Santos: Substantial contribution in the concept and design of the study; Substantial contribution in data collection; Contribution in analysis, interpretation, and manuscript preparation; Contribution to critical revision, adding intellectual content.

Catarina Carvalho Nievola: Substantial contribution in the concept and design of the study; Contribution to critical revision, adding intellectual content.

Conflicts of interest

There is no conflict of interest.

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Table 1. Number of shoots of *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch plants obtained from seeds and nodal segments (NS) cultivated in vitro. Values are means±s.d. (n=15).

Time (days)	Seed	NS
0	0 ± 0	0 ± 0
15	0 ± 0	2 ± 1
30	0 ± 0	4 ± 1
45	0 ± 0	4 ± 1
60	0 ± 0	5 ± 1
75	0 ± 0	5 ± 1

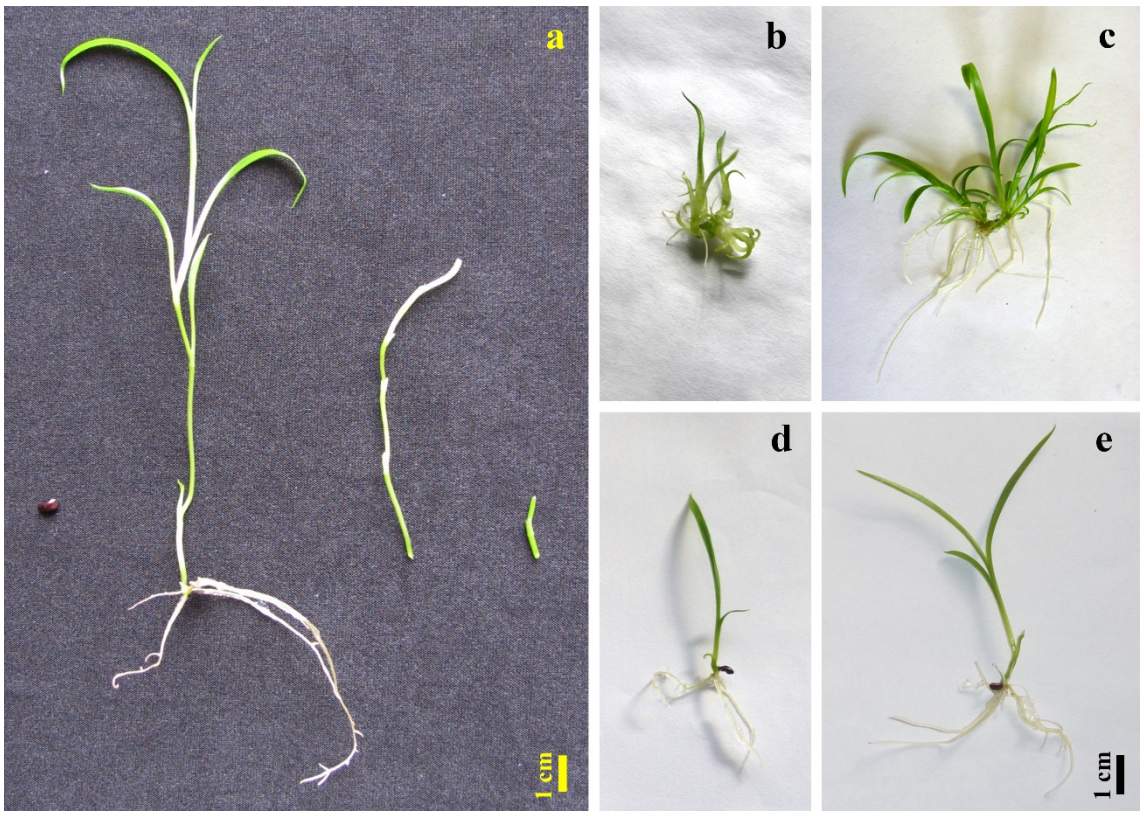

Figure 1. a. General aspect of the micropropagation procedure of *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch, indicating the seed, elongated plants, defoliated stem, and isolated nodal segments (from left to right). Visual aspect of nodal segment plants with multiple shoots at b. 15 days and c. 35 days. Seed plants at d. 15 days and e. 35 days. Bar in figure e applies to figures b-d.

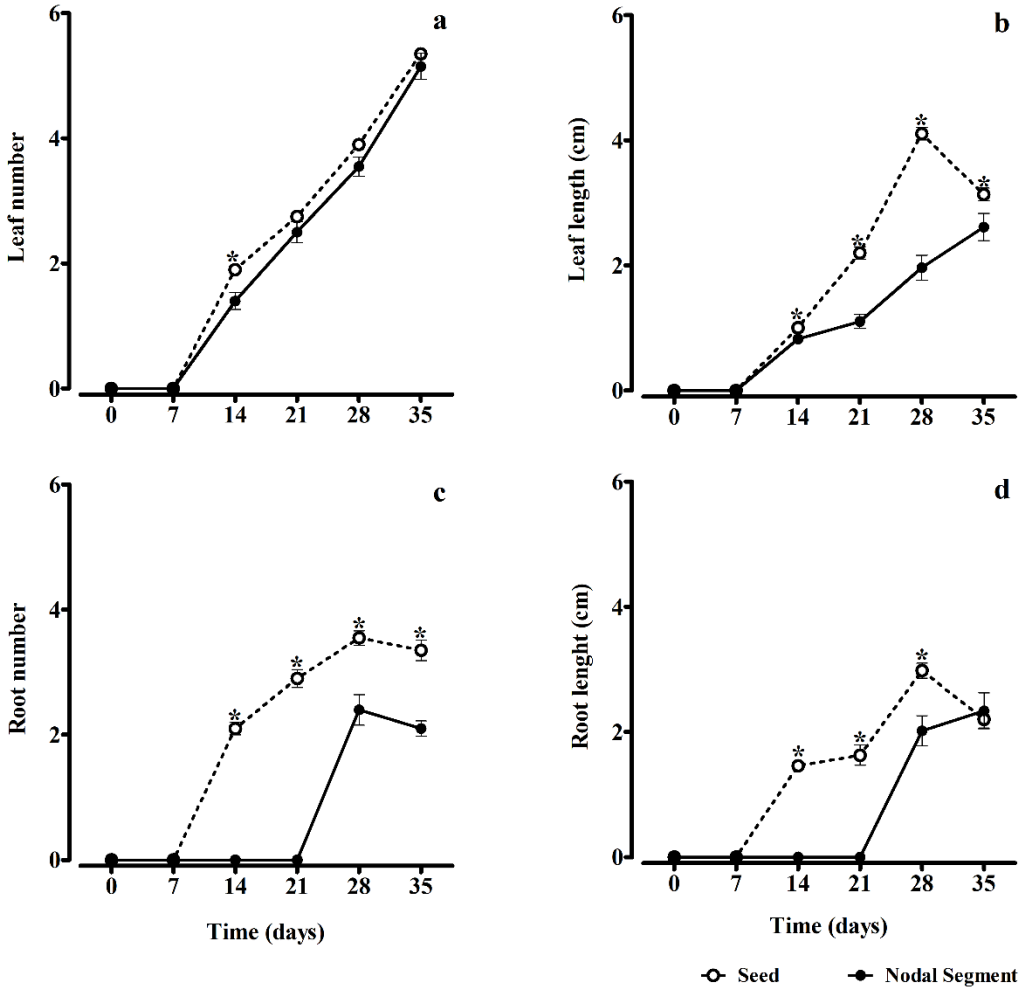

Figure 2. Leaf and root number and length in *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch plants obtained from seeds and nodal segments cultured in vitro. Values are means±s.e. and asterisks show significant differences according to the *t* test for samples taken at the same time ($P<0.05$, n=20).

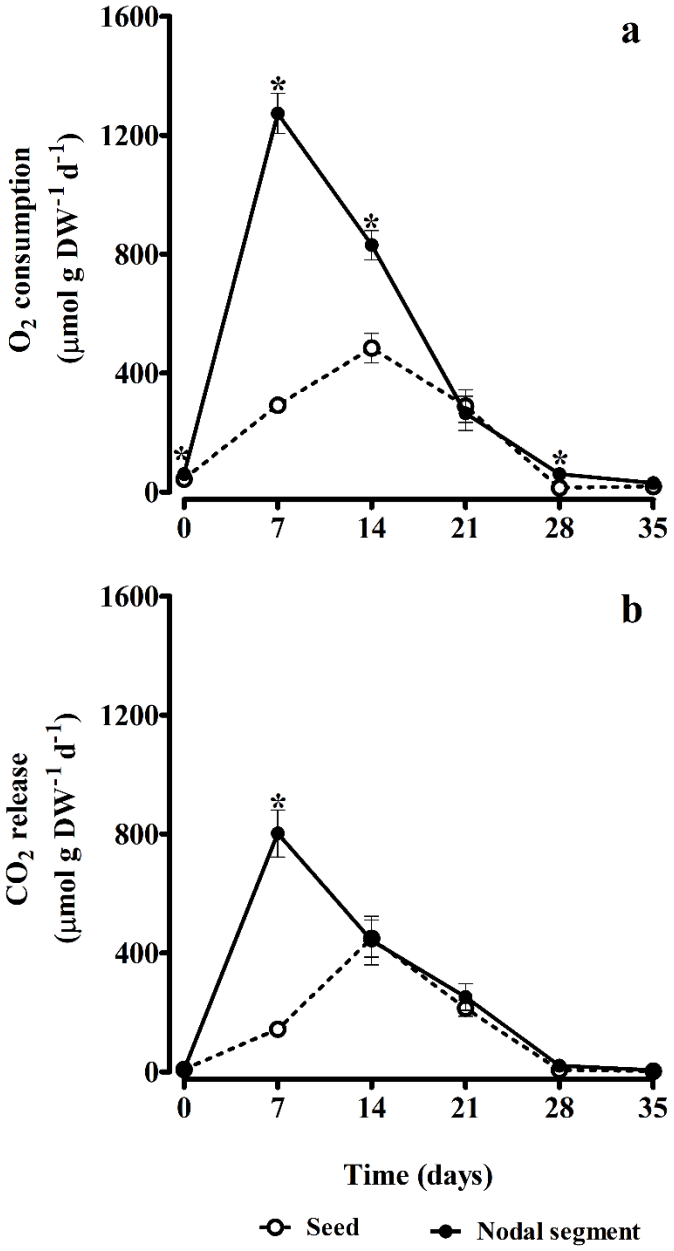

Figure 3. a. Oxygen (O_2) consumption and b. carbon dioxide (CO_2) release in *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch plants obtained from nodal segments and seeds cultured in vitro. Values are means±s.e. and asterisks show significant differences according to the *t* test for samples taken at the same time ($P<0.05$, n=5).

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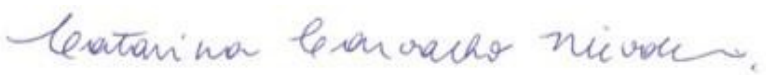

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