ORIGINAL ARTICLE

Aerenchyma, gas diffusion, and catalase activity in *Typha domingensis*: a complementary model for radial oxygen loss

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Abstract

Radial oxygen loss is a physical phenomenon that occurs naturally in aquatic plants. *Typha domingensis* was chosen as a model plant because it possesses basic morphological characteristics, such as a stem (rhizome) that produces leaves and adventitious roots, which are present in many aquatic plants. This study aimed to evaluate the following: the relevance of the anatomy of *T. domingensis* on gas diffusion among organs; the influence of plant parts on radial oxygen loss; the role of catalase in radial oxygen loss; and the proposition of a novel explanation for the downward diffusion of oxygen through the organs of this aquatic macrophyte and into the environment. *Typha domingensis* plants were cultivated in a greenhouse under different conditions: plants with intact leaves, plants with leaves cut in half, and plants without leaves. Furthermore, we evaluated the percentage of aerenchyma in different vegetative organs, the minimum pressure required for radial oxygen loss, the daily variations of dissolved oxygen, and the roots' catalase activity. The results demonstrated that certain cellular features contributed to decreased oxygen diffusion among the organs, specifically, those found in the leaf-rhizome and root-rhizome interfaces as well as the suberin and lignin layers in these regions. Additionally, our experiments with a catalase activator and inhibitor validated that a significant amount of the oxygen released in radial oxygen loss could not, in fact, be exclusively supplied by the atmosphere. Thus, a complementary model is proposed in which catalase activity is an important component of radial oxygen loss.

Keywords Intercellular spaces · Antioxidant system · Aquatic macrophytes · Hypoxia · Anatomic barriers · Hydrogen peroxide

Introduction

Aquatic plants are important parts of wetlands, and they are distributed worldwide. These plants preserve the balance of aquatic ecosystems by constituting part of the local diversity (Bolduc et al. 2016). In addition, aquatic macrophytes have shown phytoremediation potential (Dai et al. 2017; Oliveira et al. 2017).

An important environmental function of aquatic macrophytes that warrants further study is their capacity to oxygenate their surroundings (Pang et al. 2016). To overcome the limitations imposed by low oxygen (O_2) concentrations in wetland soils, macrophytes have developed effective adaptations, such as an efficient antioxidant system (Alam and Ghosh 2018) and a tissue specialized to store and distribute gases within the plant structure, the aerenchyma (Seago Jr et al. 2005; Colmer et al. 2006). It is well-known that O_2 diffusion in an aqueous medium is 10,000 times slower than in a gaseous medium. Thus, the plants' internal gas diffusion reservoir plays a key role in wetland ecosystems.

Flooding is a potential source of reactive oxygen species (ROS) in aquatic organisms. ROS participate in deleterious processes, such as damage to cell structures, including lipids, membranes, proteins, and DNA (Spengler et al. 2017); therefore, plants' resistance to environmental pressures is linked to their antioxidant system. Catalase (CAT) is one of the main enzymes of this system, and it participates actively in the detoxification process consuming hydrogen peroxide (H_2O_2) (a ROS) and producing O_2 and H_2O (Alam and Ghosh 2018).

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This enzyme has been shown to be particularly efficient in aquatic macrophytes, such as *T. domingensis* (Corrêa et al. 2015).

Gas diffusion from intercellular spaces contributes to the supply of adequate amounts of O_2 for processes such as photosynthesis and respiration (Retta et al. 2016; Miao et al. 2020). In submerged tissues such as those in macrophyte roots, the three-dimensional structural arrangement of cells forming the tissue is critical for gas diffusion (Armstrong and Beckett 2011; Ho et al. 2011). Some specialized tissues such as the aerenchyma exhibit high porosity; thus, they favor internal gas diffusion in plants (Kordyum et al. 2017). On the other hand, the cellular arrangement of meristematic tissues is characterized by the absence of intercellular spaces, which favors the reduction or even blockage of gas diffusion between this tissue and other parts of the plant (Brulé et al. 2016).

Radial oxygen loss (ROL) is defined by Armstrong (1980) as the O_2 transfer from the root aerenchyma of aquatic plants to their rhizosphere. According to Dai et al. (2017), ROL is an important trait of aquatic plants and promotes tolerance to environments with low O_2 content. Previous studies showed the importance of rhizosphere aeration for the uptake and transport of heavy metals by hyperaccumulator plants (White and Ganf 2000) for the maintenance of microorganisms that interact with plant roots (Ma et al. 2018) and for normal plant growth (Mano et al. 2006). Consequently, researchers have investigated ROL for a wide range of applications, such as environmental conservation, crop plant improvement, wastewater treatment, and heavy metal tolerance (Cheng et al. 2010; Rehman et al. 2017; Zhang et al. 2017). Some studies have tried to explain the origin and production of the O_2 released in ROL (Zhang et al. 2014) and the mechanisms involved in gas diffusion and ROL (Colmer 2003).

Armstrong and Armstrong (1988), Armstrong (1980), and Brix (1993) investigated the physical principles involved in gas exchange dynamics and the mathematical models that could explain them. Furthermore, Colmer (2003) and Tanaka et al. (2007) re-examined and improved models for gas transport over long distances inside plants. These findings have contributed to the improvement of our understanding of gas diffusion dynamics and gas release by the plant; therefore, these models previously described can be improved by recent scientific findings. For instance, these models evaluate only the aerenchyma tissue, instead of the entire anatomical structure of the plant. Studies have demonstrated the presence of some tissues such as meristems (Kaul 1974) and the exodermis (Armstrong et al. 1992; Corrêa et al. 2015) in cattail. According to Brix et al. (1992), some species do not exhibit internal pressurization for convective flow, although there is evidence that connections between plant organs may permit airflow since meristems are not present as they can occlude lacunar spaces. Tornberg et al. (1994) estimated that the resistance in the leaf-rhizome connection is very high in *Typha latifolia* and *T. angustifolia* and both plant species have intercalary meristems at their leaf bases, which are related to increased resistance. However, previous studies did not provide any images of intercellular spaces in the connections. Additionally, the anatomical constraints of other macrophytes have not been sufficiently explored in previous studies; this is particularly true for research examining the anatomical connections among different plant organs, which is essentially nonexistent.

In this study, we hypothesized that the presence of tissues with few intercellular spaces will increase significantly the gas diffusion resistance among the different plant organs as well as into the soil. Additionally, we hypothesized that at least a significant part of the O_2 released in ROL originates in CAT activity in the roots of macrophytes, such as *T. domingensis*.

Therefore, the aim of this study was to investigate the anatomical barriers for O_2 diffusion and clarify the origin of O_2 involved in ROL by using *T. domingensis*, an aquatic macrophyte distributed worldwide. The objectives of this study were (1) to investigate the role of anatomical traits in gas flow in *T. domingensis*, (2) to test the role of CAT in the O_2 released from ROL in *T. domingensis*, and (3) to propose a complementary model to study the origin of O_2 released from the roots in ROL.

The results of the present study showed that anatomical barriers constrained the O_2 pathway from the shoot to the roots in aquatic macrophytes and that root CAT was sufficiently active and had adequate substrate to provide enough O_2 to fill aerenchyma chambers. Thus, we suggested a complementary model for ROL in *T. domingensis*.

Material and methods

Plant material

Cattail plants (*T. domingensis* Pers. − Typhaceae) were collected from natural populations in wetlands located at Alfenas, Minas Gerais (21° 25′ 44″ S, 45° 56′ 49″ W) in the southeast region of Brazil. The collected plants contained rhizomes and approximately five leaves each (they were 1.5 m long). These plants were immersed into a 50% hypochlorite [commercial sodium hypochlorite (NaClO) solution and distilled water (v v^{-1}); the final NaClO concentration was 3% (w v^{-1})] solution for 10 min and then washed with tap water before being cultivated in the greenhouse. The plants were grown in 60-L plastic pots containing 10 L of a nutrient solution (Hoagland and Arnon 1940) at 40% ionic strength to obtain acclimatized clone plants. The Hoagland and Arnon nutritive solution contained the following salts: $NH_4H_2PO_4$, $Ca(NO_3)_2$, $Mg(NO_3)_2$, KNO_3, K_2SO_4, $FeSO_4 \cdot 7H_2O$, H_2BO_3, $MnSO_4 \cdot$

H_2O, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $H_2MoO_4.H_2O$. All clone plants used in the experiments described below were of matching size and age (15 cm tall and 60 days old), and the rhizomes were 4.5 cm long and had a 0.9-cm diameter.

Experiments

Different experiments were conducted to investigate (1) the role the anatomy of *T. domingensis* played in gas diffusion in different plant organs, (2) the influence of plant parts in ROL, and (3) the role of CAT in ROL.

Anatomy and morphology of *T. domingensis*

We analyzed the anatomy of vegetative organs (leaves, rhizomes, and roots) while searching for tissues related to both enhanced (aerenchyma) and reduced gas diffusion (meristems and suberized tissues). The leaf base of *Typha* species has an intercalary meristem (Kaul 1974), and the roots of *T. domingensis* have an external cortex lacking intercellular spaces (Corrêa et al. 2017). The anatomical traits evaluated in roots were the area of the root section, the area occupied by aerenchyma chambers, and the percentage of aerenchyma chambers in the root (calculated as the ratio of the aerenchyma chambers area to the root area). Additionally, the anatomical traits evaluated in the rhizomes were the area of the rhizome section, the area occupied by aerenchyma chambers, and the percentage of aerenchyma chambers in the rhizome (calculated as the ratio of the aerenchyma chambers area to the rhizome area). Lastly, the anatomical traits evaluated in the leaves were the area of the leaf section, the area occupied by aerenchyma chambers, and the percentage of aerenchyma chambers in the leaf (calculated as the ratio of the aerenchyma chambers to the leaf area).

For anatomical analysis, plant parts were fixed in an FAA 70% solution (formaldehyde, acetic acid, and 70% ethanol at a 0.5:0.5:9 ratio) for 48 h and then stored in 70% ethanol until further analysis (Johansen 1940). Furthermore, the samples were dried with increasing ethanol concentrations (70%, 80%, 90%, and 100%) at 2-h intervals and embedded in historesin according to the manufacturer's instructions (Leica Microsystems, Wetzlar, Germany). The application of an ethanol series, with increased concentrations of this compound, is an efficient method for dehydration (Johansen 1940) and is necessary to achieve the necessary water content to permit resin infiltration in the material. Transversal sections were obtained using a semi-automated rotary microtome (Yidi YD-335; Jinhua Yidi Medical Appliance CO., LTD, Zhejiang, China). Then, the sections were stained with toluidine blue 1% (w v^{-1}) and mounted on slides with Entellan (Merck, Darmstadt, Germany). The slides were photographed using a microscope attached to an image capturing system (CX31; Olympus, Tokyo, Japan), and quantitative anatomical analysis was performed using UTHSCSA ImageTool software.

Fluorescence microscopy was performed to identify suberized or lignified tissues that may form barriers to gas diffusion. Cross and longitudinal sections of transition regions between the leaf and rhizome and the rhizome and root were placed in a solution containing distilled water and 0.1% berberine hemisulphate (w v^{-1}) for 1 h and then washed in distilled water. Thereafter, the sections were kept in a 0.5% aniline blue (w v^{-1}) solution for 30 min and then washed twice with distilled water. The sections were mounted in a 0.1% $FeCl_3$ (w v^{-1}) solution in 50% glycerol (w v^{-1}) (Brundrett et al. 1988). Then, the slides were analyzed with a fluorescence microscope (BX60; Olympus) equipped with a cooled monochrome camera (Olympus). Images were captured with ultraviolet excitation/emission wavelengths of 358–461 nm (Brundrett et al. 1988).

In addition to the anatomical analysis, *T. domingensis* plants were analyzed to measure the total air volume that filled the aerenchyma spaces in different organs. The volumes of roots, rhizomes, and leaves of 10 plants were measured via the water displacement method using a measuring cylinder (data not shown). Based on the volume of each organ and its aerenchyma percentage, we calculated the air space volume in each plant part (the organ volume multiplied by the aerenchyma proportion). These air-filled spaces inside each organ are important for the calculation of the amount of gas necessary to provide enough pressure for gas movement across the barriers between plant organs (leaf-rhizome and rhizome-root interfaces) and from the root to the soil (mainly the exodermis and epidermis).

The anatomical analysis was used to identify the gas diffusion resistance along the entire plant structure. By incorporating some adaptations and considerations, we assumed a model similar to that used for CO_2 diffusion in leaves, according to Terashima et al. (2011). In our model, the following assumptions regarding resistance to gas diffusion were necessary: (a) the intercellular spaces and aerenchyma cause low resistance; (b) tissues with primary cell walls and without lignin or suberin deposition (the epidermis, meristems, and the parenchyma) generate the greatest resistance on their plasma membranes, thin walls, and cytosol; (c) meristems and the epidermis generate high resistance owing to the absence of intercellular spaces; and (d) lignified or suberized tissues cause the highest resistance owing to their thick walls and the deposition of these substances on their secondary cell walls.

Determination of the pressure limit necessary for ROL

To determine the pressure limit required for noticeable ROL, plants were placed individually in plastic pots containing 3.5 L of deionized water and a sealed rubber hose that was attached to *T. domingensis* leaves and to an air compressor.

These leaves were cut on the apical third, so that the pressurized air entered the leaf and passed through the leaf-rhizome and rhizome-root interfaces and from the root to the solution. A multiparameter probe (5565 MPS, version 1.12; YSI, Yellow Springs, Ohio, USA) that detects dissolved O_2 in the water was used to determine ROL. The air pressure was increased in a gentle manner until the limit required for ROL detection was obtained, and it was then registered. The experiment was replicated 10 times, and the mean ± standard deviation (SD) was calculated for the experimental plants.

The influence of plant parts on ROL

One of the hypotheses that may explain ROL in macrophytes is that O_2 enters the plant via stomata, broken stems, or leaves and then finds its way through leaves, towards the rhizome, then to the roots, and finally into the soil (Colmer 2003). Therefore, leaves and stems (shoots) may be crucial parts in the mechanism by which O_2 enters the plant, if this is the only way that this process takes place. We examined *T. domingensis* plants in three distinct conditions to test this hypothesis: intact plants, leafless plants (all leaves were carefully removed leaving only rhizomes and roots, which were completely submerged), and plants with leaves cut in half (in the middle). Leafless plants were kept underwater for the entire duration of the experiment (they had no air contact). The dissolved O_2 was measured using the multiparameter probe containing an O_2 electrode (previously mentioned). All data sampling was performed in the morning between 7 a.m. and 9 a.m., while we implemented a 12-h photoperiod, and the daily mean air temperature and water temperature were 22.5 ± 1 °C and 14.5 ± 1 °C, respectively. The mean oxygen level was 4.21 mg L^{-1}, while during the experiment, all measured values remained below the saturation level (calculated as 6.85 mg L^{-1}). In order to stabilize the electrode, we kept it resting in the solution for 3 min before every measurement. To ensure sanitization and avoid the presence of microalgae, the pots were previously washed with deionized water and sodium hypochlorite; additionally, we avoided adding a nutrient solution during the 10-day duration of the experiment, which prevented algal growth. The experimental design was completely randomized with three treatments and 10 replicates. Dissolved O_2 measurements were obtained for 10 consecutive days. The following equation was used for ROL calculation: $ROL = O_2D2 - O_2D1$, where O_2D1 is the O_2 concentration in a given day and O_2D2 is the O_2 concentration in the next day. ROL was expressed in mg L^{-1} day^{-1}. During the 10 days of the evaluation, two main ROL events were detected, and calculations were performed when O_2 concentration increases were observed. During the 10 days of the evaluation, four or five increases were detectable in consecutive days, and ROL calculations were performed for each increase. Data were further averaged for each replicate.

Polynomial curves were predicted for O_2 concentrations with periodic increases and depletions (Benson and Krause 1976; Benson et al. 1979; Wetzel 2001). Detectable increases in the O_2 concentration were considered ROL events.

CAT activity in *T. domingensis* roots and its role in ROL

The CAT activity in *Typha* species is remarkably high (Corrêa et al. 2015), and O_2 is a product of this enzyme's reaction, whose substrate (H_2O_2) increases under flooding conditions (Voesenek and Bailey-Serres 2015). Thus, we performed experiments containing substances that modify CAT activity to determine the role of CAT as a complementary source of O_2 for ROL. To this end, we used both intact and leafless plants (using a method similar to that previously described) and subjected them to three treatments: (1) sodium nitroprusside (SNP; Êxodo Científica, São Paulo, Brazil), described by Fu et al. (2016) as a CAT activator; (2) 3-amino-1,2,4-triazole (AT; Sigma Aldrich, St. Louis, MO, USA), described by Aver'yanov et al. (2015) as a CAT inhibitor; and (3) a control treatment (distilled water), without modifying CAT activity. All plants were placed in plastic pots containing 3.5 L of distilled water and either 0.1 mM SNP, 0.1 mM AT, or only distilled water (for the control plants). The experiment was conducted using a 2×3 factorial design with 10 replicates, and the entire experiment was repeated three times.

The O_2 dissolved in the solution was measured using the multiparameter probe previously mentioned. Measurements were taken 12 h after the addition of CAT activity modifiers. Then, their difference was calculated and expressed as ΔO_2.

The roots were collected 2 h after the application of CAT activity modifiers, placed in liquid nitrogen, and stored at −80 °C until they were analyzed. CAT was extracted as follows, according to the method proposed by Biemelt et al. (1998): 0.2 g of fresh root mass was ground in liquid nitrogen and homogenized in 1.5 mL of an extraction buffer that contained 1.47 mL of potassium phosphate buffer (0.1 M; pH 7.0), 15 μL of EDTA (0.1 M; pH 7.0), 6 μL of dithiothreitol (DTT) (0.5 M), 12 μL of phenylmethylsulfonyl (PMSF) (0.1 M), ascorbic acid (0.001 M), and 22 mg of polyvinylpolypyrrolidone. The extract was centrifuged at 12,000 *g* for 30 min at 4 °C, and the supernatant was collected and stored at −20 °C until further analysis. Moreover, CAT activity was evaluated according to Havir and McHale (1987) as follows: 10 μL aliquots of the enzyme extract was added to 170 μL of an incubation medium that contained 90 μL of potassium phosphate (200 mM; pH 7.0), 71 μL of water, and 9 μL of H_2O_2 (250 mM) and then incubated at 28 °C. The enzyme activity was determined by the decrease in absorbance at 240 nm measured every 15 s for 3 min, by monitoring the consumption of H_2O_2. The molar extinction coefficient used was 36 mM^{-1} cm^{-1}. The specific activity of CAT was calculated based on the total amount of proteins in the samples

determined according to Bradford (1976). We calculated the enzymatic activity in triplicate, and the mean was calculated for each replicate.

An additional experiment was conducted to test the direct effects of CAT modifiers (AT and SNP) on the enzymatic activity under in vitro conditions (to ensure these modifiers were acting on CAT extracted from *T. domingensis* roots). For this experiment, roots sampled from intact plants were used and subjected to CAT extraction as described above (Biemelt et al. 1998). Further, CAT activity was evaluated according to Havir and McHale (1987) using the same method described above. However, in addition to all the described components of the incubation medium, in this experiment, we applied three more treatments: (1) control (the incubation medium and the CAT extract), (2) the incubation medium and the CAT extract plus 0.1 mM of AT (CAT inhibitor), and (3) the incubation medium and the CAT extract plus 0.1 mM of SNP (CAT activator). The experiment was conducted on a completely randomized design with 10 replicates for each plant.

H_2O_2 was determined according to Velikova et al. (2000) as follows: 200 mg of fresh roots were ground in liquid nitrogen and 20% polyvinylpolypyrrolidone (w m^{-1}) and further homogenized in 1.5 mL of trichloroacetic acid 0.1% (w v^{-1}). The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. The H_2O_2 content was determined measuring the absorbance at 390 nm in a reaction medium that contained 500 μL of extract, 500 μL of potassium phosphate buffer (10 mM; pH 7.0), and 1 mL of potassium iodide (1 M). We calculated the H_2O_2 content in the roots in duplicate, and the data were averaged to one replicate.

Statistical analysis

The data were submitted to one-way or two-way ANOVA using the SISVAR 5.0 software (Ferreira 2011). Prior to parametric analysis, the data were tested for a normal distribution using the Shapiro-Wilk test, and the means were compared using the post hoc Scott-Knott test; $p < 0.05$ was considered statistically significant.

Results

Anatomy of *T. domingensis*

The leaves of *T. domingensis* comprised a single epidermal layer without intercellular spaces. Internally, 3–5 layers of palisade parenchyma were followed by 3–6 layers of large ground parenchyma cells, both having particularly small intercellular spaces (Fig. 1). According to our assumption, these three tissues represented limited gas diffusion capacity, and this pattern was found on both the adaxial and abaxial leaf sides. Large aerenchyma chambers were found on the central part of the leaf (Fig. 1a). Furthermore, the leaf-rhizome interface included an intercalary meristem that preserved the mitotic capability of the leaf base and promoted continuous growth (Fig. 1 c and d). However, this meristem and the region where cells were still differentiating lacked intercellular spaces, thereby limiting gas diffusion from the leaf aerenchyma to the rhizome (Fig. 1 c and d).

Similarly, the rhizome of *T. domingensis* comprised a single epidermal layer without intercellular spaces (Fig. 2 a and b). Internally, three or four exodermal layers, which had thick walls and lacked intercellular spaces, were detected. The aerenchyma was detected on the innermost part of the rhizome cortex; this area had small vascular bundles and parenchyma. The innermost part of the rhizome comprised an atactostelic cylinder with ground parenchyma and large, scattered vascular bundles; this part had few intercellular spaces (Fig. 2a).

Typha domingensis roots comprised a single epidermal layer without intercellular spaces. Internally, three layers of parenchyma cells with thick walls formed the exodermis (Fig. 2 c and d). The middle cortex consisted of large aerenchyma chambers, while the innermost part of the cortex contained parenchyma cells with few intercellular spaces. Moreover, the vascular cylinder (with the xylem and phloem) was found at the center of the roots. Intercellular spaces were nearly absent in this part of the root (Fig. 2b). The root-rhizome interface consisted of external root tissues as well as external rhizome parts, namely, the exodermis and epidermis, but lacked direct connections between the two organs along the root axis (Fig. 2e). This interface had scarce intercellular spaces (Fig. 2e), and fluorescence microscopy revealed a significant deposition of lignin and suberin on the exodermis of both the rhizome and the root (Fig. 2f).

The *T. domingensis* leaves had the highest aerenchyma percentage among the vegetative organs, followed by the root and the rhizome. In contrast, the low aerenchyma percentage found in the rhizome indicated that few parts were filled with air, although this organ had a large area section measuring 6.1 mm^2. In fact, the largest and smallest air spaces were found on the leaves and rhizomes, respectively, of *T. domingensis* (Table 1). The leaf pressure threshold required to promote ROL was 0.08 ± 0.01 (SD) MPa.

We found miniscule intercellular spaces and deposition of compounds restrictive to air diffusion in the leaf-rhizome and rhizome-root interfaces. Furthermore, the deposition of lignin and suberin was remarkably abundant in the cell walls of the root exodermis and in the external layers of the rhizome. The connection between the root and rhizome included large areas with lignified and suberized tissues and lacked intercellular spaces (Fig. 2 e and f). Additionally, the leaf base had scarce intercellular spaces because of the intercalary meristem found in this region (Fig. 1 c and d).

Fig. 1 Anatomical structure of *Typha domingensis* leaves in cross sections (**a** and **b**) and leaf-rhizome connection in longitudinal sections (**c** and **d**). *ade* adaxial epidermis, *abe* abaxial epidermis, *st* stomata, *pp* palisade parenchyma, *ae* aerenchyma, *gp* ground parenchyma, *vb* vascular bundle, *fb* fibers, *rhi* rhizome, *le* leaf, *asterisk* leaf intercalary meristem. Bars = 300 μm (**a**), 50 μm (**b**), and 200 μm (**c** and **d**)
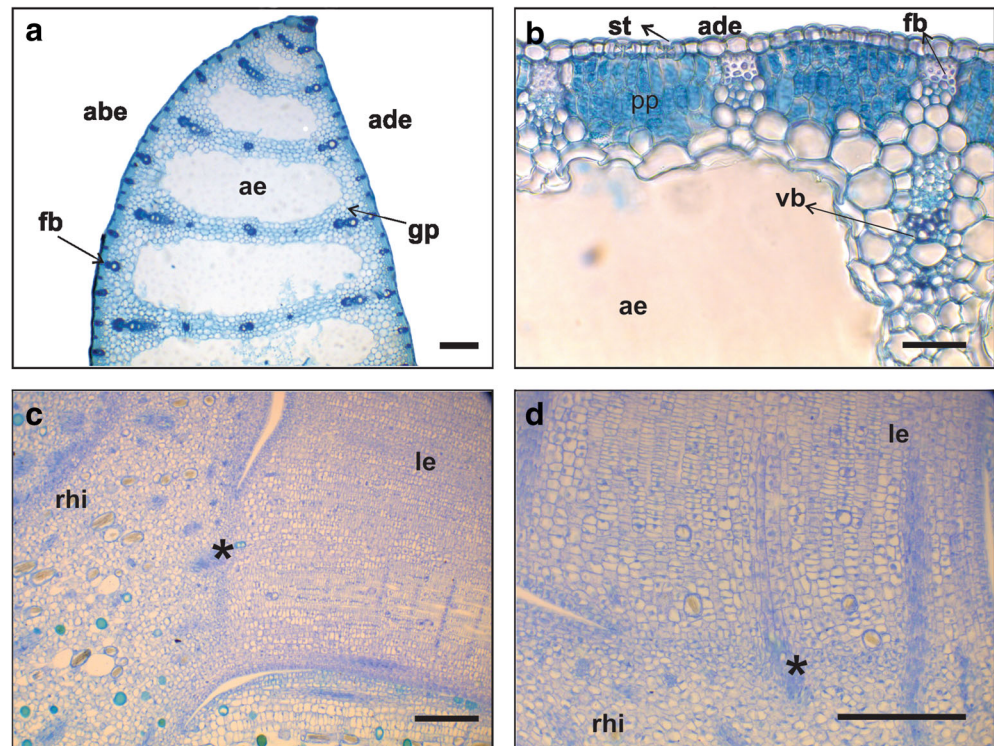
The influence of plant parts on ROL

The *T. domingensis* plants subjected to different experimental conditions regarding their leaves (the plants were either intact, leafless, or with their leaves cut) exhibited ROL; however, the differences among them were not statistically significant ($p = 0.08$) (Fig. 3a). In addition, the O_2 concentration did not increase constantly throughout the experiment; two main ROL events were detected for all plant conditions in days with increased dissolved O_2 levels followed by days with decreased O_2 levels (Fig. 3 b, c, and d).

CAT activity on *T. domingensis* roots and its role in ROL

No interaction was found between the effect of the plant parts and enzyme modifiers ($p = 0.98$), and no effect of intact or leafless plants was found ($p = 0.05$); however, a significant effect of the CAT modifiers was detected ($p < 0.01$). Both intact and leafless plants showed similar rates of ROL (Fig. 4a). The highest and lowest ROL means were detected in plants treated with SNP and AT, respectively, whereas the means of control plants were in the intermediate range (Fig. 4b).

The CAT activity of intact plants was modified after the application of SNP and AT. In order to demonstrate the efficiency of the modifiers, the in vitro experiment (which proved the effects of both SNP and AT) showed that SNP increased CAT activity by 3.3 times compared to the control treatment,

and AT reduced the CAT activity to values close to zero (Fig. 5a). In addition, intact plants always showed higher CAT activity than leafless plants (Fig. 5b). The H_2O_2 concentration on *T. domingensis* roots was not significantly different between leafless and intact plants. However, plants subjected to the AT treatment had the highest H_2O_2 levels in their roots (Fig. 5c), which was later consumed as the substrate for CAT under this treatment.

Discussion

Does ROL depend on macrophyte shoots?

The models that have been proposed to explain ROL argue that O_2 enters the plant via broken shoots, then diffuses throughout the rhizome and roots, and is finally released into the soil (Armstrong and Armstrong 1988; Armstrong 1980; Konnerup et al. 2011). According to Colmer (2003), O_2 enters the plants via stomata or broken shoots and then diffuses from the shoot to the roots, where ROL occurs. However, our results indicated that the origin of O_2 may not rely exclusively on the shoot air uptake, because even leafless and submerged plants exhibited ROL. In addition, the pressure threshold to promote noticeable ROL on *T. domingensis* (0.08 MPa) was considerably higher than the estimated values of internal pressure in other macrophytes; for instance, Konnerup et al. (2011) found an average internal pressure of 0.0004 MPa in *Cyperus* L. species. It seems unlikely that the high pressure on

Fig. 2 Anatomical structure in cross sections of the rhizome (**a** and **b**), root (**c** and **d**), and the rhizome-root interface in longitudinal sections (**e** and **f**) of *Typha domingensis*. The bright areas on the F image show lignin/suberin deposition on fluorescence microscopy and staining with cyanide blue. *Ep* epidermis, *ex* exodermis, *ae* aerenchyma, *gp* ground parenchyma, *vb* vascular bundle, *vc* vascular cylinder, *fb* fibers, *rhi* rhizome, *ro* root, *arrow* rhizome-root interface. Bars = 300 μm (**a**, **b**, **c**, and **e**), 100 μm (**d**), and 200 μm (**f**)
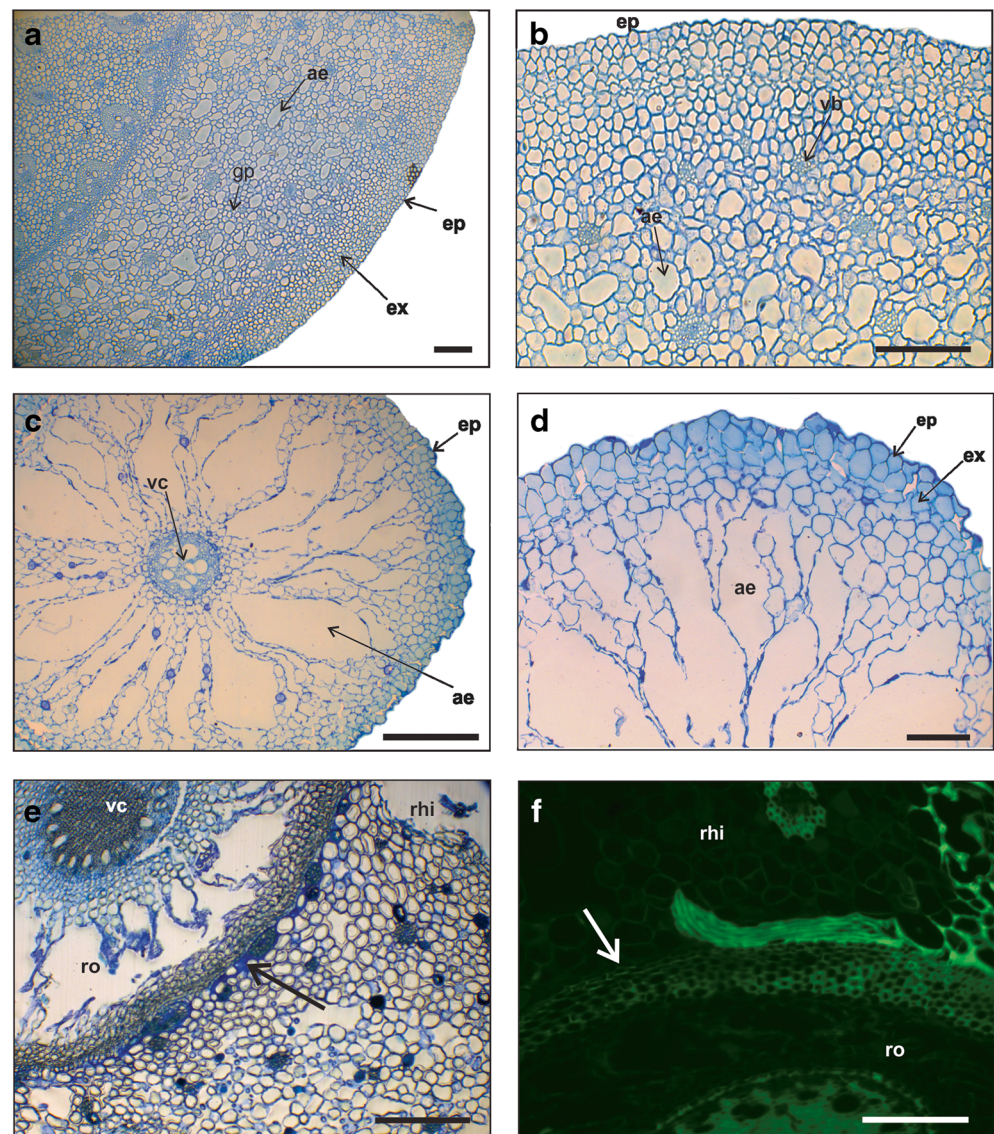
the leaf mesophyll necessary to promote ROL could be reached only by the air uptake of leaf stomata.

In this study, we measured O_2 concentrations for 10 days for each experiment, which certainly exceeded the time required to deplete the aerenchyma-stored gas; two ROL events were detectable, and the second event that occurred from the sixth to the eighth day was higher. In fact, the average amount of O_2 released was 1.15 ± 0.3 mg L^{-1} day^{-1}, which was calculated as the mean of all treatments at the second ROL event (Fig. 3). This mean was too high to be sourced only from aerenchyma-stored gas. In this experiment, the van't Hoff equation could be used to estimate the amount of O_2 in *T. domingensis* roots: $n = PV/RT$, where P is the pressure for ROL in standard atmospheres (atm), V is the aerenchyma gas

Table 1 Aerenchyma and internal air volume characterization of the vegetative organs of *Typha domingensis* Pers. Data are shown as the mean ± standard deviation

Organ	Organ section (mm^2)	Aerenchyma area (mm^2)	Aerenchyma (%)	Air volume (mL)
Root	1.35 ± 0.50	0.51 ± 0.38	37.77 ± 13.75b	0.56 ± 0.16b
Rhizome	6.1 ± 0.89	0.49 ± 0.14	8.03 ± 2.56c	0.33 ± 0.17b
Leaf	8.18 ± 6.03	4.29 ± 3.48	52.44 ± 12.86a	2.41 ± 0.98a

Means followed by the same letters in columns do not differ according to the Scott-Knott test at $p < 0.05$

Fig. 3 Root oxygen loss (ROL) expressed as the mean difference of the dissolved oxygen between two consecutive days on the solution containing *Typha domingensis* plants that were either intact, leafless, or with their leaves cut (**a**). Dissolved oxygen content and ROL events throughout the 10-day evaluation of plants that were intact (**b**), leafless (**c**), and with their leaves cut (**d**). Bars indicate a *p* value ≤ 0.05. Columns with the same letters do not differ according to the Scott-Knott test at *p* < 0.05
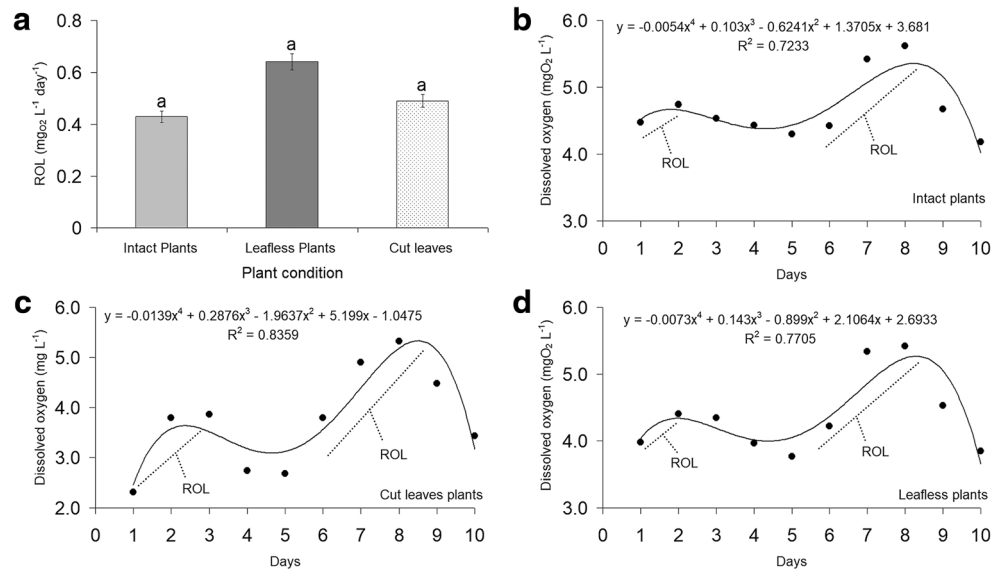
volume in liters (L), R is the gas constant, and T is the temperature in kelvin degrees (K). In our experimental conditions, we could consider the values for the pressure for ROL (measured), root aerenchyma volume (Table 1), average temperature, and gas constant to be 0.7 atm (0.07 MPa), 0.00056 L (where 0.0001288, or 23%, is the estimated O_2), 293.15 K (20 °C), and 0.082, respectively. Therefore, $n = (0.7 \times 0.00012)/(0.082 \times 293.1)$, which is approximately equal to 0.0000375 M of O_2; this suggests that approximately 1.2 mg of O_2 was present in the roots. Notably, this O_2 amount is released on a daily basis (1.15 ± 0.3 mg L^{-1}), which means the entirety of O_2 present in the roots could be exhausted in a single day. Furthermore, if we consider pressure values as those predicted by convective models for internal pressurization that reach up to 800 Pa in two *Typha* species (Tornberg et al. 1994), which equal 0.0079 atm, the van't Hoff equation will be: $n = (0.0079 \times 0.00012)/(0.082 \times 293.1)$. The results of this calculation will be 0.00000004 M of O_2, which is equal to 0.00128 mg of O_2 present in the roots. This calculation,

considering convective flow pressures, means that the entirety of O_2 present in the roots will be exhausted in just 1 min and 26 s. Consequently, the total amount of O_2 stored in the roots of *T. domingensis* will be insufficient to provide constant O_2 release for more than 1 day, thereby implying that, particularly in leafless plants, O_2 in the root aerenchyma must be replenished by a mechanism different from those previously proposed (shoot uptake and transport).

The ROL was not affected by the removal of the shoot and was measured as the difference of dissolved O_2 in the solution between consecutive days (Fig. 4). In addition, no differences were detected between intact and leafless plants (Figs. 3 and 4). The analysis of these results showed that ROL occurred independently of the shoot integrity.

Conclusively, our results showed that ROL was independent of macrophyte shoots and occurred even in the absence of this part of the plant. Moreover, these results suggest that a mechanism different from shoot air diffusion is necessary to replenish the O_2 released in ROL.
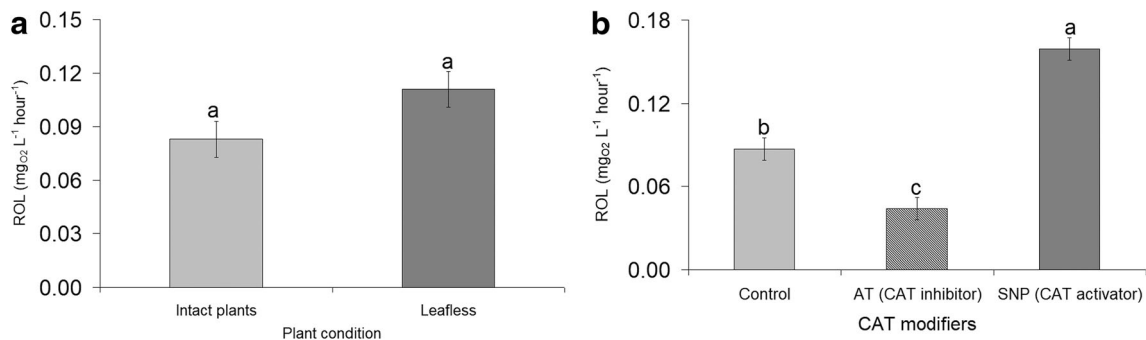

Fig. 4 Root oxygen loss (ROL) expressed as the mean difference of the dissolved oxygen 12 h after the application of the treatments. The ROL was calculated in the solution containing intact and leafless *Typha domingensis* plants subjected to 3-amino-1,2,4-triazole (AT) [catalase (CAT) inhibitor] and sodium nitroprusside (SNP) (CAT activator). As no significant effect was detected ($p = 0.92$), data are shown for different plant types (**a**) and CAT modifiers (**b**). Bars indicate a *p* value of ≤ 0.05. Columns with the same letters do not differ according to the Scott-Knott test at *p* < 0.05
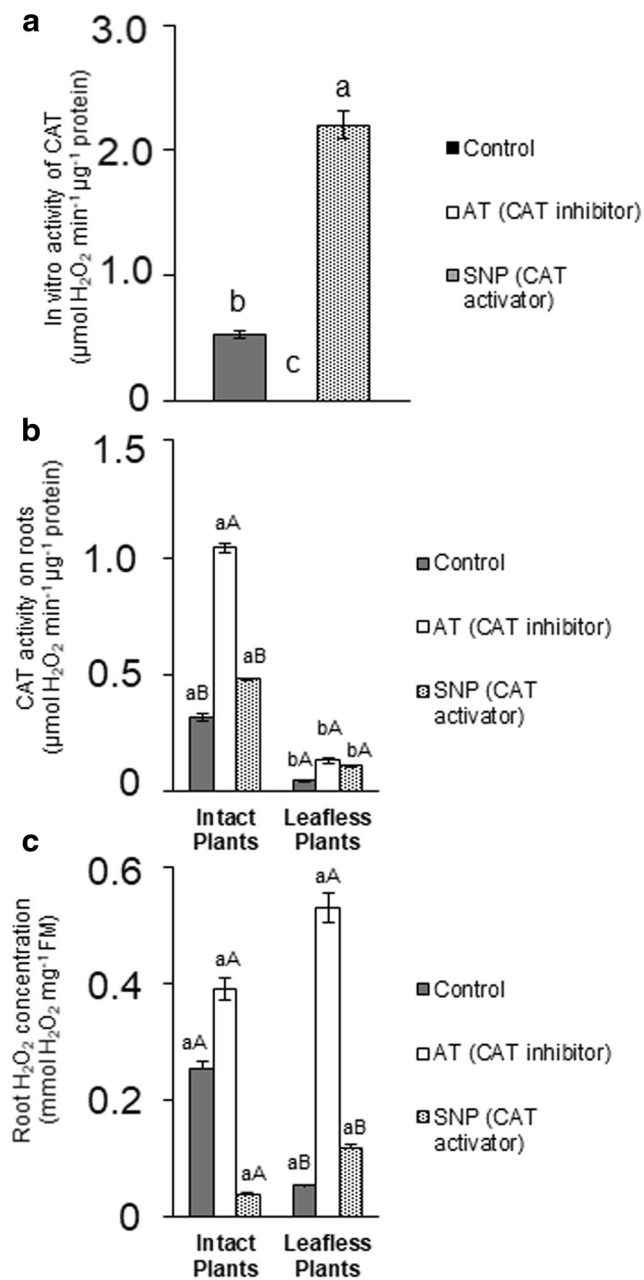
Fig. 5 Catalase (CAT) activity and H_2O_2 concentration in the roots of *Typha domingensis* after the application of 3-amino-1,2,4-triazole (AT) (CAT inhibitor) and sodium nitroprusside (SNP) (CAT activator) or neither (control). (**a**) In vitro activity of CAT, (**b**) CAT activity for the experiment performed ex vitro with samples taken 2 h after the application of CAT modifiers, and (**c**) H_2O_2 activity for the experiment performed ex vitro with samples taken 2 h after the application of CAT modifiers. Bars indicate a *p* value of ≤ 0.05. Columns with the same letters do not differ according to the Scott-Knott test at $p < 0.05$

Anatomical barriers for O_2 diffusion throughout the shoot-root-soil continuum

Most studies concerning O_2 diffusion through the plant and its role in ROL disregard many anatomical traits that could significantly limit the plant's internal airflow. Different plant tissues show variable permeability to O_2 depending on the abundance of intercellular spaces. The resistance to gas diffusion in intercellular spaces is low, but it increases greatly in the presence of cell membranes and cell liquid compartments (Terashima et al. 2011). Thus, tissues that typically have a low percentage of intercellular spaces show a high resistance to O_2 diffusion.

Anatomical barriers present in specific tissues of *T. domingensis* have been previously reported; however, their role in O_2 diffusion has not been discussed or considered when suggesting the models to explain ROL. For instance, Kaul (1974) identified the intercalary meristem on the leaf base. Furthermore, the root exodermis of several aquatic plants limits O_2 diffusion from the roots in order to allow its use in root respiration (Armstrong and Armstrong 1988; Armstrong et al. 1992; Brix and Schierup 1990; Corrêa et al. 2017). According to Pi et al. (2009), gas diffusion occurs naturally under low resistance conditions within one of the plant's organs containing abundant intercellular spaces. The high percentage of aerenchyma present and the volume of air contained in it provided the necessary conditions for gas distribution in the roots and leaves of *T. domingensis*. However, the aerenchyma percentage found in the rhizome was significantly lower than that in roots and leaves; thus, this organ has a limited gas diffusion capacity.

The multiseriate exodermis in the roots and the suberin deposition in the root-rhizome interface further impede O_2 diffusion. The hydrophobic property of suberin (Song et al. 2011) is another factor that limits (or even blocks) gas diffusion through the root-rhizome interface as well as the release of O_2 from the external root cortex and the epidermis.

In addition to the aforementioned anatomical barriers hindering O_2 diffusion throughout the plant organs, leaf tissues also present difficulties for the air uptake to reach the aerenchyma and for the gas diffusion between consecutive chambers. For instance, stomatal resistance is very well-known (Terashima et al. 2011) and is largely reduced when stomatal pores open. This is the way air enters leaves by providing carbon dioxide (CO_2) for photosynthesis. However, in *T. domingensis* leaves, the stomata are located just above the palisade parenchyma that lacks (or has unnoticeable) intercellular spaces (Fig. 1), which constitutes a second resistance barrier for air diffusing into the aerenchyma. Successively, ground parenchyma creates a third resistance barrier. The sum of these resistances comprises a strong barrier for air to fill the aerenchyma chambers sufficiently to reach the high pressure required for ROL (0.08 MPa).

Figure 6 indicates the locations on the structure of *T. domingensis* where significant resistance occurred. Considering the main tissue and organ interfaces, the total resistance for O_2 diffusion through the plant body could be defined as follows: $R_{TO2} = R_l + R_t + R_{lri} + R_{riro} + R_{ee}$, where R_{TO2} is the total resistance to O_2 diffusion, R_l is the leaf

$$R_{tO_2} = R_l + R_t + R_{lri} + R_{riro} + R_{ee}$$

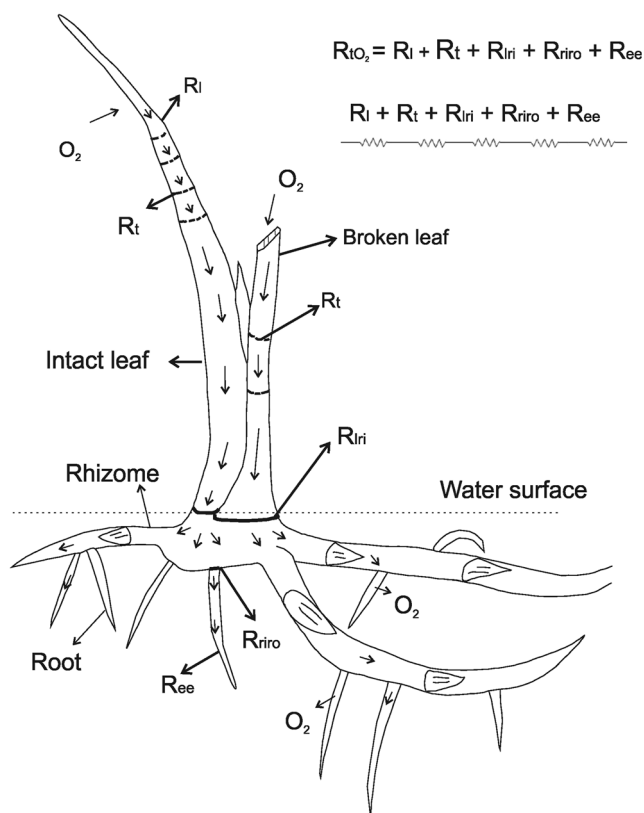$$R_l + R_t + R_{lri} + R_{riro} + R_{ee}$$

Fig. 6 Scheme of the path to O_2 diffusion from the atmosphere to the soil throughout the plant body, when considering anatomical resistance. R_{TO2} total resistance to O_2 diffusion, R_l leaf resistance, R_t trabecular resistance, R_{lri} resistance of the leaf-rhizome interface, R_{riro} resistance of the rhizome-root interface, R_{ee} resistance of the exodermis and epidermis of the roots

resistance, R_t is the trabecular resistance, R_{lri} is the resistance of the leaf-rhizome interface, R_{riro} is the resistance of the rhizome-root interface, and R_{ee} is the resistance of the root exodermis and epidermis. Consequently, these accumulated resistances may limit severely the process in the current O_2 diffusion model (Colmer 2003). Thus, the anatomy of aquatic macrophytes (such as T. domingensis) is adapted to store O_2 in the aerenchyma and to diffuse it within an organ; nonetheless, the diffusion between organs is limited by barriers in their interfaces.

CAT activity is an O_2 source for ROL

The CAT enzyme consumes H_2O_2 to produce O_2 and H_2O; its activity is associated with the scavenging of H_2O_2 (a ROS) to protect plant cells against oxidative stress (Møller 2001). Flooding causes O_2 deprivation that promotes ROS formation; thus, the aerenchyma tissue has evolved to store O_2 to avoid this effect (Voesenek and Bailey-Serres 2015). Therefore, aquatic macrophytes experience constant O_2 deficiency, thereby promoting the increase of H_2O_2 in roots, which are detoxified by CAT activity. This provides a

continuous source of H_2O_2 for CAT. Consequently, this enzyme is able to operate steadily in the roots of aquatic macrophytes, such as T. domingensis.

In aquatic macrophytes, CAT activity is increased by several environmental factors such as heavy metals (Pereira et al. 2014), population density (Corrêa et al. 2015), UV radiation (Xu et al. 2014), and chemical oxygen demand (Xu et al. 2011). Thus, this enzyme is exceptionally responsive to environmental conditions. The CAT modifiers used in this study were efficient in altering this enzyme's activity (in vitro assay shown in Fig. 5a), and these changes were sufficient to shift the levels of ROL in the plants (Fig. 4). The role of this enzyme in ROL was evidenced in a convincing manner by the 81.9% increase and 49.5% reduction of ROL obtained from treatments with a CAT activator and inhibitor, respectively. In fact, changes in CAT activity were proportionally related to ROL.

These results support the effects of CAT modifiers and the enzyme's use of H_2O_2 as a substrate. CAT activity was reduced by its inhibitor (AT) as is shown in Fig. 5, and during the effect of the latter, H_2O_2 was accumulated in T. domingensis tissues. Then, the excess H_2O_2 in the roots increased CAT activity. Therefore, the plants treated with the inhibitor showed the highest CAT activity, 2 h after application, because the enzyme's substrate accumulated, which confirm the Michaelis-Menten kinetics model that states that enzyme activity rates depend on the substrate concentration (Reuveni et al. 2014).

Another issue was whether CAT would be able to provide sufficient O_2 to match the levels of ROL that we measured (1.15 mg L^{-1}). The average CAT activity for control plants was 0.8 µmol H_2O_2 min^{-1} $µg^{-1}$ protein (data not shown). The method to assess CAT activity is based on H_2O_2 consumption; however, the analysis of the equilibrated equation showed that for every mol of this substrate, 0.5 mol of O_2 would be produced (Møller 2001). Thus, 0.4 µmol O_2 min^{-1} $µg^{-1}$ protein is produced from CAT activity in T. domingensis roots. Furthermore, 12 g of fresh mass was the average mass of the root system of the T. domingensis experimental plants measured at a similar growth stage and size (data not shown). The protein proportion found on T. domingensis roots was 0.08 mg protein g^{-1} fresh mass. Therefore, the total protein in the root system of these plants was 0.96 mg (960 µg^{-1}). Furthermore, the O_2 production capacity of the T. domingensis root system was 384 µmol O_2 min^{-1}, which is equivalent to 12.3 mg O_2 min^{-1} of enzyme activity. Thus, this enzyme has an exceptional capacity to produce O_2, and a single minute of its operation could provide sufficient O_2 to maintain the ROL measured in T. domingensis roots. Additionally, this enzyme activity rate was similar to those previously reported for Typha species and for other aquatic macrophyte species (Corrêa et al. 2015; Yang and Ye 2015). This shows that, in aquatic macrophyte roots, CAT could be a reliable source of O_2 to

supplement the aerenchyma and the root's aerobic metabolism. In addition, CAT could support ROL rates found in aquatic macrophyte roots.

Complementary model to explain the aerenchyma O_2 origin and ROL

The O_2 supplied via transport from the shoots may be insufficient for ROL because of anatomical barriers. Complementary CAT acting as an O_2 source in the roots may be a plausible way to replenish the gas lost in ROL. Moreover, O_2 produced directly in the roots avoided all anatomical barriers except for the root exodermis (R_{ee} resistance), which facilitated substantially O_2 in reaching the soil. Consequently, we propose a complementary model.

Conclusion

In summary, anatomical barriers consisting of tissues lacking intercellular spaces constrain the O_2 pathway from the shoot to the roots in aquatic macrophytes. The root CAT was sufficiently active and had adequate substrate to provide enough O_2 to fill aerenchyma chambers and to support ROL. Moreover, the O_2 released by aquatic macrophytes in wetlands was partially originated in CAT activity. *Typha domingensis* roots produced sufficient O_2 for ROL, independently of the aerial plant part examined.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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