VINÍCIUS POLITI DUARTE

THE RADIAL OXYGEN LOSS IN *Typha domingensis* AS RELATED TO AERENCHYMA, GAS DIFFUSION AND CATALASE ACTIVITY

Lavras-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Botânica Aplicada, área de concentração em Botânica Aplicada, para a obtenção de título de Doutor.

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Orientador

Prof. Dr. Evaristo Mauro de Castro

Coorientador

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RESUMO

A perda radial de oxigênio (PRO) é um fenômeno físico que ocorre naturalmente nas plantas, majoritariamente àquelas que habitam zonas úmidas ou ambientes alagados. Apesar desse fenômeno estar presente em quase todas as macrófitas, a PRO pode variar entre essas espécies. Foi utilizada nesse trabalho *Typha domingensis* PERS. como planta modelo pois a mesma apresenta características morfológicas básicas encontradas na maioria das plantas aquáticas tais como, folhas emergindo de um caule (rizoma) e dele partem raízes adventícias. Esse trabalho teve como objetivo investigar: o papel da anatomia de *T. domingensis* na difusão dos gases entre diferentes órgãos; a influência de partes de plantas na PRO e o papel da catalase na PRO, além de fornecer um modelo como forma alternativa para a explicação da difusão descendente de oxigênio entre a planta e o meio no qual está inserida. As plantas de *T. domingensis* foram cultivadas em solução de Hoagland em casa de vegetação em diferentes condições: Plantas com folhas intactas, plantas com folhas cortadas ao meio e plantas sem folhas. Foram avaliadas a porcentagem de aerênquima nos diferentes órgãos vegetativos, a pressão mínima necessária para ocorrer a PRO, as variações diárias do oxigênio dissolvido e a atividade da enzima catalase (CAT) nas raízes. Os resultados demonstraram que as características celulares nas conexões folha/rizoma e na interface rizoma/raiz juntamente com uma camada de suberina/lignina nessas regiões contribuem para a diminuição da difusão do oxigênio entre os órgãos. Os resultados com o ativador e inibidor da CAT também contribuíram para comprovar que uma parte significativa do oxigênio liberado nas raízes pela PRO não pode ser de fato, unicamente fornecido pela atmosfera, conforme sugerem as teorias.

Palavras-chave: Perda Radial de Oxigênio, Macrófitas, Anatomia, Catalase e Difusão de Gases.

ABSTRACT

Radial oxygen loss (ROL) is a physical phenomenon that occurs naturally in plants, mostly those live in wetlands or flooded environments. Although this phenomenon is present in almost all macrophytes, ROL can vary among these species. *Typha domingensis* PERS. was used as a model plant because it presents basic morphological characteristics found in most aquatic plants such as leaves emerging from a stem (rhizome) and from it adventitious roots. This work aimed to investigate: the anatomy of *T. domingensis* on gas diffusion between different organs; the influence of plant parts on ROL and the catalase role in ROL, besides providing a model as an alternative way to explain the downward diffusion of oxygen between the plant and the environment in which it is inserted. The plants of *T. domingensis* were cultivated in Hoagland solution in greenhouse under different conditions: Plants with intact leaves, plants with leaves cut in half and plants without leaves. The percentage of aerenchyma in the different vegetative organs, the minimum pressure required for ROL, the daily variations of dissolved oxygen and the catalase (CAT) activity enzyme in the roots were evaluated. The results demonstrated that the cellular traits in the leaf-rhizome connection and the root-rhizome interface besides suberin/lignin layer in these regions contribute to the decrease of the oxygen diffusion of between the organs. The results with the CAT activator and inhibitor also contributed to prove that a significant amount of the oxygen released into the roots by ROL can not, in fact, to be only supplied by the atmosphere, as suggested by the theories.

Keywords: Radial Oxygen Loss, Macrophytes, Anatomy, Catalase and Gas Diffusion

SUMÁRIO

1 INTRODUÇÃO

A perda radial de oxigênio PRO é um fenômeno físico descrito por Armstrong (1980) que consiste na liberação de oxigênio gasoso das raízes de plantas que habitam ambientes úmidos ou alagados para a rizosfera. Estudos recentes revelaram o papel da PRO na tolerância a alguns metais pesados (WU et al., 2017) além da sua comprovada importância para as plantas em manter processos vitais como a respiração das raízes e a oxigenação das áreas adjacentes à rizosfera (SASIKALA et al., 2009).

Nesse sentido, alguns modelos matemáticos e biofísicos de difusão dos gases tentam explicar o processo de PRO pelas macrófitas desde a obtenção, o percurso por todo o corpo da planta, até a lixiviação do oxigênio pelas raízes na rizosfera (ARMSTRONG, ARMSTRONG; BECKETT, 1990; BRIX, 1993; COLMER, 2003). Apesar desses modelos serem consagrados em toda a literatura científica, eles não são suficientemente claros e apresentam inconsistências ao tentar explicar a origem e geração do oxigênio e principalmente, a relação entre a quantidade de ar que entra nas plantas e a que é librada pelas raízes no processo da PRO.

Contudo, sabe-se que algumas macrófitas aquáticas apresentam modelos e mecanismos de PRO diferentes de outras (DAI et al., 2017) e nesse sentido, nosso trabalho demostra que para *Typha domingensis* Pers., esse mecanismo se mostrou particularmente distinto dos trabalhos publicados até o momento.

Typha domingensis, popularmente conhecida como taboa é uma macrófita aquática distribuída por todo o mundo em ambientes de terras úmidas como lagos, canais de drenagem e várzeas (MARTINS et al., 2007). Estudos recentes comprovam sua alta capacidade fitorremediadora (OLIVEIRA et al., 2017) além de ser usualmente relacionada com a melhoria da drenagem e infiltração de água além de promover habitat para a vida selvagem ao seu redor (HOULAHAN et al., 2006).

Tradicionalmente, *T. domingensis* é considerada uma espécie invasiva de ambientes aquáticos, porém, o trabalho realizado por Lewis (1995) descrevendo a capacidade de suas raízes em fornecer oxigênio em sedimentos anóxicos destacou algumas de suas importâncias ecológicas em seu habitat. Nesse sentido, o baixo suprimento de oxigênio disponível em ambientes alagados é, sem dúvida, um dos principais fatores limitantes abióticos para o crescimento e sobrevivência das plantas (PIMENTEL et al., 2014).

Ambientes com pouca aeração podem alterar o equilíbrio entre os antioxidantes e as espécies reativas de oxigênio, resultando em estresse oxidativo, para tolerar esses ambientes,

as macrófitas desenvolveram diversos mecanismos de defesa do sistema antioxidante que respondem ao estresse por hipóxia (CHEN et al., 2018; ROMERO-OLIVA; CONTARDO-JARA; PFLUGMACHER, 2015). Desse forma, a enzima catalase se mostra uma promissora fonte fornecedora de oxigênio para as raízes pois, o mecanismo de ação da enzima se dá pela desmutação do peróxido de hidrogênio (espécie reativa de oxigênio que provoca danos ao metabolismo vegetal) em água e oxigênio molecular (FEKI et al., 2015; XU et al., 2015).

Outro mecanismo é o desenvolvimento do aerênquima. Um tecido com grandes espaços intercelulares conectados que aumenta a tolerância ao alagamento, pois favorece o acúmulo, circulação e a difusão dos gases entre parte aérea e raiz além de fornecer uma via de baixa resistência à difusão de gases para a planta (ARMSTRONG et al., 2000).

Dessa forma, o presente trabalho traz uma série de caracterizações anatômicas, morfológicas e enzimáticas de *Typha domingensis* com a finalidade de comprovar a origem do oxigênio liberado por suas raízes. Dentre elas, a caracterização anatômica dos órgãos vegetativos, relacionando suas estruturas internas com a difusão dos gases, a caracterização da atividade da enzima catalase e também foram realizados experimentos que indicam a pressão necessária para a que ocorra a PRO nas raízes de *T. domingensis*. Além disso, os experimentos com alteradores específicos da atividade da catalase tiveram por finalidade comprovar a origem do oxigênio que é difundido para o meio externo. Para finalizar, foi proposto um mecanismo que explica o a origem, produção e liberação de gases das raízes de *T. domingensis* desde o alagamento até a difusão dos mesmos no ambiente alagado.

As descobertas do presente trabalho revelam que as propostas mais aceitas na literatura científica para a liberação de gases por macrófitas aquáticas, especificamente para a taboa, não são totalmente compreendidas e que nossos resultados colaboram com o crescimento científico no sentido de trazer clareza quanto a origem da produção do oxigênio lixiviado pelas raízes para o ambiente.

2 HIPÓTESE

O presente estudo fundamenta-se nas seguintes hipóteses: As características anatômicas nas regiões de conexão folha-rizoma e na interface rizoma-raiz de *T. domingensis* cria uma barreira física, limitando o fluxo de difusão do oxigênio entre os órgãos da planta. Devido à essas resistências anatômicas, a enzima catalase pode ser uma provável fonte fornecedora de O_2 para a PRO.

3 REFERENCIAL TEÓRICO

3.1 Perda Radial de Oxigênio (PRO)

Os estudos do transporte de gases em macrófitas aquáticas têm seus primeiros registros em meados do século XIX com as observações de Raffeneau-Delile fornecendo as primeiras evidências de pressurização de gases (GROSSE; ARMSTRONG; ARMSTRONG, 1996) e desde então, apesar do crescente progresso acerca das descobertas das teorias que regem esses fluxos (COULT; VALLANCE, 1958; SORRELL; DROMGOOLE, 1987; ARMSTRONG; ARMSTRONG, 1991), o caminho percorrido por esses gases e, principalmente a origem dos mesmos ainda não é clara.

Já no início do século XX, as principais investigações sobre a difusão de gases se deu em estudos de Ohno em 1910 (GROSSE; ARMSTRONG; ARMSTRONG, 1996) que percebeu uma grande quantidade de ar saindo das folhas de *Nelumbo nucifera* Gartn. Ele confirmou que pela quantidade de ar observada, a circulação do mesmo que ocorria naquela espécie advinha da atmosfera e que esses gases não poderiam ser resultado do produto da fotossíntese, porém, essas conclusões foram comprovadas apenas para *N. nucifera*, uma espécie enraizada com folhas flutuantes.

Do início do século XX até o final da década de 70, nenhum trabalho de grande impacto relacionando o movimento de gás pressurizado com plantas aquáticas foi publicado até que Drew, Jackson e Giffard (1979) descreveram a formação do tecido aerenquimático como uma das respostas ao ambiente inundado e a partir de então, muitos trabalhos investigando os fluxos gasosos e a perda radial de oxigênio (PRO) pela raiz começaram e ser publicados (ARMSTRONG; ARMSTRONG, 1990; KOTULA et al., 2017; WHITE; GANF, 2001).

A PRO é um fenômeno físico que ocorre naturalmente nas plantas, majoritariamente naquelas que habitam zonas úmidas ou ambientes alagados (GROSSE; ARMSTRONG; ARMSTRONG, 1996). De acordo com Tanaka et al., (2007), uma fração do oxigênio produzido pela fotossíntese e o ar atmosférico entram pelas folhas via estômatos e chegam até os rizomas pelo processo de difusão. Além disso, o oxigênio atmosférico também pode ser transportado para os órgãos subterrâneos por meio de rizomas quebrados e mortos, sendo que parte desse oxigênio é consumido pela planta e parte é liberado pelas raízes por difusão para a rizosfera. O fluxo de oxigênio que escapa das raízes e difunde-se para o substrato através da

rizosfera é portanto, denominado perda radial de oxigênio (MEI et al., 2014; MEI; YE; WONG, 2009).

Apesar desse fenômeno estar presente em quase todas as macrófitas, a PRO pode variar entre as espécies de ambientes alagados e essa variação está relacionada com sua morfologia, seu porte e principalmente, pela necessidade de demanda de oxigênio dos organismos encontrados na rizosfera (DAI et al., 2017). Por exemplo, Lemoine et al., (2012) demostraram que enquanto *Myriophyllum spicatum* L. e *Vallisneria spiralis* L. apresentaram plasticidade funcional em ambientes anóxicos aumentando a porosidade da raiz e, portanto, aumentando a PRO, *Potamogeton coloratus* Horne, *Elodea canadensis* Michx e *Sparganium emersum* Michx. não foram capazes de tolerar as mesmas condições por não investirem em estratégias de aclimatação como no aumento da PRO.

De acordo com Colmer (2003), existem quatro características que podem aumentar a PRO nas raízes de plantas aquáticas:

A primeira diz respeito às características anatômicas dos tecidos. As grandes câmaras de aerênquima, a estrutura poliédrica das células agrupadas de parênquima e uma região cortical relativamente grande proporciona maior porosidade à raiz (ARMSTRONG, 1980; BECKETT; ARMSTRONG, 1987; JUSTIN; ARMSTRONG, 1987), enquanto que uma barreira a PRO no córtex diminui as perdas de O2 da raiz para a rizosfera.

A segunda característica está relacionada com a morfologia da raiz. Raízes mais espessas (ARMSTRONG, 1979; AGUILAR; TURNER; SIVASITHAMPARAM, 1999; ARMSTRONG; HEALY; WEBB, 1982), pequeno número de raízes laterais (ARMSTRONG et al., 1983; SORRELL; MENDELSSOHN; MCKEES, 2000) e raízes emergindo da base da planta (ARMSTRONG; ARMSTRONG; BECKETT, 1990) também contribuem para o aumento e a manutenção da quantidade da PRO.

A terceira característica se relaciona com aspectos fisiológicos das plantas. Embora não haja evidências cientificamente comprovadas de que as plantas tolerantes ao alagamento tenham demandas mais baixas de O_2, raízes com grande quantidade de aerênquima têm taxas de respiração mais baixas (ARMSTRONG, 1980) e isso colabora com a manutenção das concentrações de gases na planta.

Por fim, a última característica listada é a ambiental. Temperaturas mais baixas diminuem o consumo de O_2 pela planta (ARMSTRONG, 1980). Além disso, a difusão do O_2 para o meio externo diminui com a redução da temperatura.

Além de manter processos vitais para a planta como a respiração das raízes e a oxigenação das áreas adjacentes à rizosfera (SASIKALA et al., 2009), estudos recentes

demonstram como a PRO pode afetar a absorção de nutrientes pela planta e alterar a tolerância aos metais pesados em ambientes anóxicos (MEI et al., 2014; WU et al., 2017). As espécies *Cyperus flabelliformis* Rottb. e *Canna indica* L. apresentaram as maiores taxas de porosidade e PRO exibiram maior eficiência na absorção e na adsorção de nutrientes. Com relação à tolerância aos metais pesados, os pesquisadores concluíram que o aumento da PRO proporcionou menor acúmulo de arsênio pelas raízes de plantas de arroz em zonas alagadas (MEI et al., 2014).

A liberação de oxigênio pelas raízes também está relacionada com o tratamento de águas residuais pois é o oxigênio liberado pelas mesmas que mantém ativo processos de oxidação bacteriana na rizosfera, fundamental para que esse processo ocorra (TANAKA et al., 2007). Além disso, a compreensão dos processos de aeração envolvidos nas macrófitas fornece aplicações práticas para o manejo das zonas úmidas e áreas de tratamento de águas residuais e para o aumento da tolerância de espécies cultivadas em locais inundados (COLMER, 2003a).

Embora existam muitos trabalhos demonstrando a existência de barreiras à perda radial de oxigênio da raiz para o solo, estas são estudadas em camadas celulares mais externas como na epiderme ou exoderme. Estudos que envolvam a circulação de gases, a comunicação da parte aérea/raiz e sua posterior liberação para o ambiente no entanto, ainda são escassos.

3.2 Modelos de difusão de gases pela planta

Assim como as teorias que explicam a PRO, o século XX teve papel fundamental na discussão e na elucidação de fenômenos como a difusão de gases pelas plantas. Esses trabalhos tiveram relevância com Armstrong (1980, 1982) descrevendo que os espaços intercelulares de ar intercelular atuam de forma a aerar e transportar gases para órgãos subterrâneos e exercem papel promovendo a oxidação da rizosfera da maioria das macrófitas vasculares. Posteriormente, Brix (1993) trouxe um enfoque físico para esse assunto e afirmou que o transporte interno de gases pode ocorrer basicamente por meio da difusão molecular ou pelos fluxos de convecção.

A difusão molecular é um processo de transporte de matéria de molécula a molécula, partindo da maior pressão para a menor (BRIX, 1993). Essa difusão depende de uma série de fatores como as condições do meio, o peso da molécula e a temperatura. Para o cálculo da difusão molecular, inclusive no interior do corpo das plantas, utiliza-se a equação de Fick que leva em conta o coeficiente de difusão do ar, a concentração do ar ao longo da planta e a distância percorrida pelo ar (ARMSTRONG, 1980) e pode ser descrita genericamente como: $\vec{j} = -D\nabla c$, onde $\vec{j}$ representa o fluxo (em mol m^{-2} s), D é o coeficiente de difusão da espécie (em m^2s^{-1}) de concentração c (mol m^{-3}) e v é a distância percorrida.

Contudo, em muitas espécies de plantas aquáticas, os fluxos de convecção que foram descritos desde a década de 1980 também desempenham papel importante na aeração dos tecidos e órgãos alagados que podem ser contínuos ou não. Esse fluxo foi descrito como sendo dirigido pela diferença da temperatura (também chamado de transpiração térmica) e de pressão de vapor da água entre a parte interna das folhas e o ar atmosférico circundante. Brix (1993) aborda esses mecanismos de transporte de massa para Nymphaeaceae, no qual o ar entra pelas folhas jovens (na maioria das vezes pelos estômatos), são conduzidos por convecção até os órgãos alagados e então segue a direção contraria chegando nas folhas mais velhas até a atmosfera.

Posteriormente, Armstrong, Armstrong e Beckett (1996) relatam também a possibilidade dos fluxos de gás ocorrerem internamente em *Phragmites australis* (Cav.) Trin. ex Steud. em função do sopro do vento. As hastes mais velhas e mortas expostas ao vento promovem uma variação de pressão entre o ar atmosférico e o sistema radicular da planta. O ar então, entra por essas hastes, percorrem todo o corpo da planta até as raízes e retornam à atmosfera pelas hastes e folhas mais jovens. Esse mecanismo é chamado de Efeito Venturi.

Por fim, embora essas descobertas tenham contribuído para o melhor entendimento da dinâmica da perda e circulação de gases pela planta, essas teorias ainda deixam brechas no entendimento de algumas questões. Por exemplo, não há trabalhos na literatura que expliquem como ocorre a passagem de gás entre os rizomas e as raízes, uma vez que essa região apresenta inúmeras barreiras que tornam essa passagem dificultada.

3.3 Aerênquima

A abordagem dada à anatomia vegetal contemporânea trata das variações e respostas de órgãos e tecidos das plantas em função da pressão exercida pelo meio em que elas estão inseridas (CASTRO; PEREIRA; PAIVA, 2009), dentre esses, o meio inundado e de terras alagadas.

Os solos alagados podem afetar o crescimento e o desenvolvimento de muitas espécies de plantas devido à baixa disponibilidade de oxigênio (KORDYUM et al., 2017). Sob condições anaeróbias ou de baixa concentração deste gás, a maioria das macrófitas aquáticas (dentre elas a taboa), podem desencadear dois principais mecanismos de resistência à essas pressões: os bioquímicas (que serão abordados no tópico 3.4) e os anatômicas (EVANS; GLADISH, 2017).

Um desses mecanismos anatômicos é o desenvolvimento do aerênquima. Um tecido com grandes espaços intercelulares conectados que aumenta a tolerância ao alagamento, pois favorece o acúmulo, circulação e a difusão dos gases entre parte aérea e raiz (ARMSTRONG et al., 2000). Esse tecido que pode ser encontrado tanto em folhas como rizomas e raízes também confere capacidade aos órgãos das plantas de flutuar nos ambientes alagados (BONA; MOÇO; MASTROBERTI, 2018).

Além de ser o principal tecido responsável por evitar o estresse por alagamento devido às condições de baixo teor de oxigênio do meio, o aerênquima também foi descrito como um tecido capaz de conferir tolerância em diferentes situações de estress como a alta temperatura, seca e a deficiência de nutrientes (KORDYUM et al., 2017).

Outra característica desse tecido está relacionada com sua formação. Pode ser constitutiva (como ocorre em muitas espécies de ambiente aquático), sendo o aerênquima formado como parte de seu desenvolvimento, ou induzida, quando as condições ambientais/estresse induzem a formação do tecido (EVANS, 2003).

Muito embora existam diversos trabalhos que subcategorizem o aerênquima de acordo com seu desenvolvimento (LEITE et al., 2017; SEAGO et al., 2005), de maneira geral, esse tecido é usualmente classificado de duas maneiras conforme seu processo de desenvolvimento: Lisígeno, causado pela morte seletiva e degradação das células presentes no córtex, e Esquizogênico, processo em que ocorre a separação das células pela lamela média durante seu processo de desenvolvimento (HAQUE; ABE; KAWAGUCHI, 2010).

Outro aspecto observado no aerênquima, é que suas características anatômicas promovem uma via interna de baixa resistência para o armazenamento e circulação de gases

dentro da planta. Essa circulação fornece oxigênio necessário para a manutenção de células, tecidos e até da própria raiz (PI et al., 2009).

Assim, esse tecido se revela como uma das principais características anatômicas presente nas plantas submetidas a ambientes alagados ou com baixa concentração e oxigênio (SILVEIRA et al., 2016). Apesar de seu papel no mecanismo de tolerância ao alagamento em plantas ser claro, sua função no processo de difusão de gases da planta para o ambiente ainda necessita de estudos mais detalhados.

3.4 Catalase

As catalases são enzimas que abrangem várias isoformas e três tipos diferentes: as catalases monofuncionais (ou catalases típicas), as catalases bifuncionais (peroxidases) e as catalases de manganês. Todas elas evoluíram de duas famílias de proteínas diferentes: as do tipo I, contendo um agrupamento heme e, do tipo II, que são as catalases não heme (GRIGORAS, 2017). Dentre as diversas isoformas que podem ser encontradas nas plantas, a mais comum é a Catalase 1 que se localiza nos peroxissomos e é responsável por 80% da atividade desta enzima, sendo assim, considerada a mais importante (ACEVEDO; SCANDALIOS, 1990).

O déficit de oxigênio é limitante para o crescimento e desenvolvimento de plantas que estão expostas à ambientes alagados ou inundados. O dano oxidativo é portanto, uma consequência à essa condição e se revela através do acúmulo de espécies reativas de oxigênio (EROs). Essas EROs em altas concentrações podem causar prejuízos severos nas plantas como lesão e morte celular (FEKI et al., 2015). Como forma de amenizar esses danos, as plantas utilizam o sistema antioxidante, formado por diversas enzimas dentre elas a catalase e outros compostos não enzimáticos.

A enzima catalase (EC 1.11.1.6, CAT), apresenta estrutura tetramérica com um anel porfirínico ligado a átomos de ferro, responsável pela aceleração da desmutação do peróxido de hidrogênio (H_2O_2) em água e oxigênio molecular (FRUGOLI et al., 1996). Essa enzima é amplamente sintetizada por diversos grupos de bactérias, fungos, animais e plantas e desempenha um papel importante na proteção contra os efeitos tóxicos do H_2O_2 que, em excesso, são subprodutos tóxicos para os organismos vivos (XU et al., 2015).

Além disso, estudos anteriores demonstraram outras funções exercidas pela CAT como no processo de embriogênese e apoptose celular em *Pinus sylvestris* L. (ACEVEDO; SCANDALIOS, 1990), na atuação de uma isoforma da CAT (CAT2) na regulação negativa durante a senescência foliar (ALAM; GHOSH, 2018) e em processos de indução da quebra de dormência de sementes de *Avena fatua* L. (CEMBROWSKA-LECH; KOPROWSKI; KEPCZYŃSKI, 2015).

Embora o mecanismo de ação da catalase não seja totalmente conhecido, sabe-se que o H_2O_2 reage com o elemento ferro do agrupamento heme da catalase e forma o peróxido de ferro. Sob baixa concentração do H_2O_2, o peróxido de ferro é reduzido a etanol e ácido ascórbico e, sob alta concentração, o peróxido de ferro reage com outra molécula de H_2O_2 formando como produtos a água e o oxigênio molecular (GRIGORAS, 2017).

Existem substâncias capazes de alterar a atividade da catalase de modo a inibir ou aumentá-la. Por exemplo os ácidos salicílico, isonicotínico e benzotiadiazol são reconhecidos com mecanismo de ação anti-infecciosos que levam ao acúmulo de peróxido de hidrogênio causada pela inibição da catalase (AVER'YANOV et al., 2015). Já o ácido 5-aminolevulênico é um conhecido doador de óxido nítrico, aumentando dessa maneira a atividade da CAT (FU et al., 2016).

Dentre esses alteradores, o 3-Amino-1,2,4triazole (AT) e o óxido nítrico (nitroprussiato de sódio-SNP, como molécula doadora de NO) são compostos específicos e bem conhecidos que atuam de forma a diminuir e aumentar, respectivamente, a velocidade de reação da CAT (HALLIWELL; GUTTERIDGE, 2007).

O AT é uma molécula tóxica que realiza ligação covalente no lado distal do grupamento heme inibindo unicamente a atividade catalítica da CAT (METODIEWA; DUNFORD, 1991). É concebível que outros inibidores de enzimas antioxidantes atuem de maneira análoga ao AT (AVER'YANOV et al., 2015).

Por outro lado, o óxido nítrico (SNP como doador de NO) atua como uma molécula sinalizadora da cascata de reações bioquímicas que leva à expressão de genes responsáveis pelo aumento da atividade da CAT (FU et al., 2016). Essa proteção está relacionada com a capacidade do NO em regular os níveis das EROs nas plantas. Assim, o NO pode exercer ação protetora contra o estresse oxidativo provocado pelas EROs (LAMATTINA et al., 2003).

3.5 *Typha domingensis* Pers.

Typha domingensis Pers. é uma espécie vegetal cosmopolita, nativa do Brasil e conhecida como taboa. Pertence à família Typhaceae e ao gênero *Typha (*L.) sendo que esse gênero é composto por ervas perenes e aquáticas. Apresentam folhas alternas, reunidas em um escapo simples e sem nós que suporta as inflorescências. Sua morfologia foliar, aliada ao padrão de crescimento vertical minimizam o aoutosombreamento. Embora *T. domingensis* seja uma planta C3, as taxas fotossintéticas são tão altas quanto as encontradas em plantas tipo C4 (DICKERMAN; WETZEL, 1985). A taboa é encontrada com muita frequência em terras úmidas e alagadas como margens de lagos, reservatórios, canais de drenagem e várzeas (MARTINS et al., 2007).

Os rizomas por sua vez, formam um suporte denso e quase monoespecífico que promove um vigoroso crescimento vegetativo. A unidade de crescimento vegetativo é o ramete, que por sua vez é composto de rizomas submersos, raízes e folhas associadas. A folha pode exceder 2 m de altura (DICKERMAN; WETZEL, 1985). *T. domingensis* pode propagar-se por sementes ou por brotos vegetativos (MARTINS et al., 2007).

A taboa faz parte de um grupo de plantas denominado de macrófitas aquáticas. Essas plantas são caracterizadas por apresentar seus órgãos fotossintetizantes localizados total ou parcialmente submersas em água (GRASIELE; HEGEL; TORTO, 2016). Segundo Lewis (1995), as espécies de *Typha*, como a taboa, desempenham um papel importante nos ciclos dos nutrientes, no controle da qualidade das águas, na produção de oxigênio (PANG et al., 2016), dentre outros.

Por muito tempo essa espécie foi considerada uma erva invasiva em seu habitat (NEWMAN; GRACE; KOEBEL, 1996; WOO; ZEDLER, 2002). Estudos recentes mostraram a importância das macrófitas aquáticas, bem como a taboa, desempenhando papel fundamental contra inundações, promovendo melhor infiltração de água no solo e provendo habitat para vida selvagem (HOULAHAN et al., 2006).

Além disso, estudos demonstram sua utilidade em vários campos ecológicos, como a restauração da diversidade em zonas úmidas (BOLDUC; BERTOLO; PINEL-ALLOUL, 2016), a purificação de água poluída pelas indústrias (GUITTONNY-PHILIPPE et al., 2015) e por compostos farmacêuticos (DORDIO et al., 2009) além de ter demonstrado potencial para a fitorremediação (HEGAZY; ABDEL-GHANI; EL-CHAGHABY, 2011; OLIVEIRA et al., 2017; SANTOS et al., 2015).

Por ser uma macrófita aquática, a presença de tecidos especializados como o aerênquima proporciona à mesma, alta capacidade adaptativa aos ambientes alagados e também a facilitação de circulação de gases no seu interior (BONA; MOÇO; MASTROBERTI, 2018).

Contudo, apesar de haver inúmeros estudos que tratam a respeito de fluxos de gases em macrófitas (ARMSTRONG et al., 2000; ARMSTRONG; ARMSTRONG; BECKETT, 1996; BECKETT; ARMSTRONG; ARMSTRONG, 2001; WHITE; GANF, 1998), tais modelos não são capazes de explicar claramente a origem da geração do oxigênio que é liberado pelas raízes e tão pouco fornecer um modelo que atenda aos pressupostos físicos para esse fenômeno em *Typha domingensis*.

4 CONCLUSÃO

São conhecidos diferentes modelos que tentam explicar a difusão do oxigênio lixiviado pelas raízes de macrófitas aquáticas no processo de perda radial de oxigênio. Contudo, os mesmos modelos não levam em consideração características morfofisiológicas encontradas nessas plantas que podem limitar essa difusão. Não se conhece ainda um modelo suficientemente claro que aborde tanto as características do ambiente como a estrutura básica do corpo das plantas que podem influenciar a dinâmica da difusão interna do oxigênio em macrófitas.

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SEGUNDA PARTE – ARTIGO

AERENCHYMA, GAS DIFFUSION AND CATALASE ACTIVITY IN *Typha domingensis*: A NEW MODEL FOR RADIAL OXYGEN LOSS

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Abstract

Radial oxygen loss (ROL) is a physical phenomenon that occurs naturally in plants, mostly those live in wetlands or flooded environments. Although this phenomenon is present in almost all macrophytes, ROL can vary among these species. *Typha domingensis* PERS. was used as a model plant because it presents basic morphological characteristics found in most aquatic plants such as leaves emerging from a stem (rhizome) and from it adventitious roots. This work aimed to investigate: the anatomy of *T. domingensis* on gas diffusion between different organs; the influence of plant parts on ROL and the catalase role in ROL, besides providing a model as an alternative way to explain the downward diffusion of oxygen between the plant and the environment in which it is inserted. The plants of *T. domingensis* were cultivated in Hoagland solution in greenhouse under different conditions: Plants with intact leaves, plants with leaves cut in half and plants without leaves. The percentage of aerenchyma in the different vegetative organs, the minimum pressure required for ROL, the daily variations of dissolved oxygen and the catalase (CAT) activity enzyme in the roots were evaluated. The results demonstrated that the cellular traits in the leaf-rhizome connection and the root-rhizome interface besides suberin/lignin layer in these regions contribute to the decrease of the oxygen diffusion of between the organs. The results with the CAT activator and inhibitor also contributed to prove that a significant amount of the oxygen released into the roots by ROL can not, in fact, to be only supplied by the atmosphere, as suggested by the theories.

Keywords: Radial Oxygen Loss, Macrophytes, Anatomy, Catalase and Gas Diffusion

1 INTRODUCTION

Aquatic plants are important part of wetlands and they are distributed worldwide. These plants maintain the balance of aquatic ecosystems by constituting part of the local diversity (BOLDUC; BERTOLO; PINEL-ALLOUL, 2016) and promoting the nutrient cycling (LEWIS, 1995). In addition, aquatic macrophytes have shown potential for phytoremediation (DAI et al., 2017; OLIVEIRA et al., 2017).

Among the environmental function of the aquatic macrophyte, one important but less investigated trait is the capacity of these plants to provide oxygen to its surroundings (PANG et al., 2016). To overcome the limitations imposed by the low dissolved oxygen in the soil, macrophytes developed effective adaptations such as an efficient antioxidant system (ALAM; GHOSH, 2018) and the aerenchyma which is a tissue specialized to store and distributing gases within the plant structure (COLMER, 2003a; VOESENEK; BAILEY-SERRES, 2015). Thus, the plant internal gas diffusion reserve plays a key role at wetlands. It is well known that O_2 diffusion in the aqueous medium is 10 000 times slower than in the gaseous medium.

Flooding is a potential source of reactive oxygen species (ROS) in aquatic organisms in which ROS participate in plant-deleterious processes as damage to cell structures, including lipids and membranes, proteins and DNA (SPENGLER; WANNINGER; PFLUGMACHER, 2017), so the resistance of plants to environmental pressures is linked to the capacity of their antioxidant system. One of the main enzymes of this system, the catalase enzyme, actively participates in this process consuming the hydrogen peroxide (from ROS) and producing O_2 and H_2O (ALAM; GHOSH, 2018). This enzyme has been shown to be particularly efficient in aquatic macrophytes, among them *T. domingensis* (CORRÊA et al., 2015).

In plant tissues, the intercellular spaces can be classified into two groups, filled by liquid or gas. The gas diffusion from intercellular spaces contribute to supply of adequate amounts of O_2 to processes such as photosynthesis and respiration (MIZUTANI; KANAOKA, 2017). In submerged organisms such as macrophyte roots, gas diffusion depends critically on the three-dimensional structural arrangement of cells and tissues (HO et al., 2016). Specialized tissues such as aerenchyma exhibit higher porosity, thus favoring the 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 and this favors the reduction or even the blocking of gas diffusion between this tissue and other part of the plant (BRULÉ et al., 2016; EVANS; GLADISH, 2017).

The radial oxygen loss (ROL) is defined by Armstrong (1980) as the oxygen transfer from root aerenchyma of aquatic plants to the rhizosphere. According to Dai et al. (2017),

ROL is an important trait of aquatic plants and promotes the tolerance to low environments with low oxygen content. Recent studies showed the importance of the rhizosphere aeration for the uptake and transport of heavy metals by hyperaccumulator plants (WANG et al., 2015; WHITE; GANF, 2000), for the maintenance of the microorganisms which interact with plant roots (MA et al., 2018) as well as for the normal plant growth (MANO et al., 2006). Thus, researchers investigate the ROL in a wide range of applications such as: environmental conservation, improvement crop plants, wastewater treatment and the tolerance to heavy metals (CHENG et al., 2010; REHMAN et al., 2017; ZHANG et al., 2017). There are studies which try to explain the origin and production of this O_2 (HEADLEY; TANNER, 2008; ZHANG; WU; HU, 2014) and the mechanism involved in the gas diffusion and ROL (CARDOSO; JIMÉNEZ; RAO, 2014; COLMER, 2003a).

Armstrong, Armstrong (1988); Armstrong (1980); Brix (1993) investigate the physical principles involved in the dynamics of gas exchange and mathematical models to explain this principles. Further, Colmer (2003) and Tanaka et al., (2007) proposed models for the gas transport over long distances inside the plant. However, although these findings have contributed to a better understanding of the dynamics of the gas diffusion and its release by the plant, these models still have issues. One of the problems is that these models do not evaluate the whole plant anatomical structure but just the aerenchyma tissue. There are studies which showed the presence of some tissues such as meristems (KAUL, 1974), exodermis (ARMSTRONG; ARMSTRONG; BECKETT, 1992; CORRÊA et al., 2015). However, these anatomical constrains were never explored in previous works, particularly, no investigation is found considering the connections between different plant organs.

Therefore, the purpose of this study was to clarify the O_2 origin in ROL using the *Typha domingensis*, a worldwide distributed aquatic macrophyte as a model to investigate the anatomical resistances to O_2 diffusion and a new model for the origin of the O_2 released by ROL. *T. domingensis* was chosen as a model because it has a basic morphological traits found in most aquatic plants such as leaves emerging from stem (rhizome) and adventitious roots. The objectives of this study were: (i) to investigate the role of anatomical traits on gas flow in *T. domingensis*; (ii) to test the role of the catalase in O_2 generating for ROL in *T. domingensis*; and (iii) propose an alternative model to the origin of the O_2 released by the roots in the ROL.

2 MATERIAL AND METHODS

2.1 Plant material

Cattail plants (*Typha domingensis* Pers. − Typhaceae) were collected from the natural populations in wetlands located at Alfenas − MG (21° 25' 44" S, 45° 56' 49" W) in the southeast region of Brazil. The collected plants were comprised of rhizomes and approximately five leaves (1.5 m in length). These plants were subjected to hypochlorite 50% [commercial sodium hypochlorite solution and distilled water (v/v) as the final NaClO concentration was 3% (w v^{-1})] for 10 min and then washed with tap water before further cultivation 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. Hoagland and Arnon nutritive solution contains 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 $H_2MoO_4.H_2O$. So, all clone plants used in the experiments described below were of similar size and age (15 cm tall and 60 days old).

Experiments

Different experiments were conduced to investigate: I) the role of the anatomy of *T. domingensis* in the gas diffusion between different organs, II) the influence of plant parts on the ROL, III) the catalase role on ROL.

2.2 - Anatomy and morphology of *T. domingensis*

We analyzed the anatomy vegetative organs (leaves, rhizomes and roots) searching for the tissues which are related both to enhance gas diffusion (aerenchyma) as well to block this process (meristems and suberized tissues). The leaf basis of the *Typha* species show an intercalary meristem (Kaul, 1974) and the roots of *T. domingensis* show a external cortex lacking intercellular spaces (CORRÊA et al., 2017). Therefore, we hypothesize that the presence of such tissues will significantly increase the resistances on the transition between leaves and stem as well as the root cortical aerenchyma (RCA) and soil. The anatomical traits evaluated in roots were: the area of the root section, the area occupied by the aerenchyma chambers, the percentage of aerenchyma chambers in the root (calculated by the ratio of the area occupied by aerenchyma chambers by the root area). The anatomical traits evaluated in rhizomes were: the area of the rhizome section, the area occupied by the aerenchyma chambers, the percentage of aerenchyma chambers the rhizome (calculated by the ratio of the area occupied by aerenchyma chambers by the rhizome area). The anatomical traits evaluated in leaves were: the area of the leaf section, the area occupied by the aerenchyma chambers,

the percentage aerenchyma chambers in the organ (calculated by the ratio of the area occupied by aerenchyma chambers by the leaf area).

For anatomical analysis, plant parts were fixed in F.A.A.$_{70\%}$ solution (formaldehyde, acetic acid and 70% ethanol at the 0.5:0.5:9 proportion) for 48 h and then stored in 70% ethanol until further analysis (JOHANSEN, 1940; JENSEN, 1962). Further, 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). Transversal sections were obtained using a semi-automated rotary microtome Yidi YD-335 (Jinhua Yidi Medical Appliance CO., LTD, Zhejiang, China). 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 capture 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 which can form barriers for gas diffusion. Cross and longitudinal sections of the leaf-rhizome and rhizome-root transition regions 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. Further, sections were kept in 0.5% aniline blue (w v^{-1}) solution for 30 min and then washed twice with distilled water. Sections were mounted in a solution of 0.1% $FeCl_3$ (w v^{-1}) in 50% glycerol (w v^{-1}) (BRUNDRETT et al., 1988). 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 volume of air which filled the aerenchyma spaces in the different organs. The volume of roots, rhizomes and leaves from ten plants were measured by the water displacement method using a measuring cylinder (data not shown). Based on the aerenchyma percentage on each organ and the volume of this organ, we calculate the air space volume of each plant part (aerenchyma proportion multiplied by the organ volume). This air filled spaces inside each organ is important to calculate the necessary amount of gas necessary to provide the limit pressure on the leaves for gas movement across the tissue barriers on the interface between plant organs (leaf-rhizome and rhizome-root) and the barrier from RCA to the external substrate (mainly exodermis and epidermis).

The anatomical analysis was used to identify the resistances to gas diffusion along all plant structure. We assumed a similar model to that used in the carbon dioxide (CO_2) diffusion in the leaf according to Terashima et al., (2011) with some adaptations and considerations. In our model the following premises were necessary: a) the intercellular spaces and aerenchyma show low resistance to gas diffusion; b) tissues with primary cell walls and no lignin or suberin deposition shows the major resistances on the plasma membrane, thin walls and cytosol, these tissues are epidermis, meristems and parenchyma; c) meristems and epidermis show high resistance due to the absence of intercellular spaces; d) lignified or suberized tissues show highest resistances due to thick walls and the deposition of these substances on the secondary cell walls, the tissue with more relevant role in our work is the root exodermis.

Determination of the pressure limit to provide ROL

To determine the pressure limit required for noticeable ROL, plants were individually placed in plastic pots containing 3.5 L of deionized water and a sealed rubber hose which was attached to an air compressor and to the *T. domingensis* leaves. These leaves were cut on the apical third so the pressurized air enter the leaf and pass through the connections of leaf-rhizome and rhizome-root and than from roots to the solution. The pressure was gently increased until the limit required for ROL detection was found. To determine ROL, a multiparameter probe (YSI, 5565 MPS, Yellow Springs, Ohio-USA version 1.12) that detects dissolved oxygen in the water was used. The pressure limit to provide ROL was then registered. The experiment was replicated ten times and the mean ± standard deviation was calculated for the experimental plants.

2.3 The influence of plant parts on the ROL

One of the hypothesis that try to explain ROL in macrophyte is that the oxygen (O_2) enters the plant by broken stems/leaves or stomata and then follows its way through leaves to rhizome and then to roots and, finally, to the soil (COLMER 2003). Therefore, if this is the only way by which O_2 enters the plant, leaves and stems (shoots) are crucial for the mechanism. To test this hypothesis, in this experiment we used *T. domingensis* plants at three different situations: intact plants, leafless plants (leaves were removed carefully lasting only rhizomes and roots) and that had their leaves cut at the median part. Leafless plants were kept underwater all the time (no contact with air). The dissolved oxygen was measured using a multiparameter probe containing an oxygen electrode (YSI, 5565 MPS, Yellow Springs, Ohio-USA, version 1.12). All data sampling was performed in the morning, between 7-9 a.m. and for each measurement the electrode was kept resting on the solution by three minutes to

stabilize and then the data log was performed. Experimental design was completely randomized with three treatments and ten replicates. Data was sampled by ten days and averaged to each replicate.

2.4 The CAT activity on *T. domingesis* root and its role on ROL

The novel model which we state in this work is related to the hypothesis that, at least a significant part of the O_2 released by ROL, has an origin on the CAT activity in the roots of macrophytes such as *T. domingensis*. This hypothesis comes from that one of the products of the reaction of this enzyme is the O_2 by using hydrogen peroxide (H_2O_2) as the substrate which is increased under flooding (VOESENEK et al., 2006; MØLLER et al., 2007;) and that the CAT activity on *Typha* species is remarkable high (CORRÊA et al., 2015).

To determine the role of CAT as source of oxygen by ROL, we performed experiments containing substances which modify the CAT activity. We used intact and leafless plants (similar to experiment II). We used a control treatment (no modifiers), SNP (Sodium Nitroprusside – Êxodo Científica, São Paulo, Brazil) described by Fu et al., (2016) as a CAT activator; and AT (3-amino-1,2,4-triazole - Sigma Aldrich, St. Louis, MO, USA) which was described by Aver`yanov et al., (2015) as a catalase inhibitor. All plants were placed in plastic pots containing 3.5 L of distilled water and 0.1 mM SNP, 0.1 mM AT or distilled water only for the control plants. The experiment was conduced on a factorial 2x3 design with ten replicates. The entire experiment was repeated three times.

The dissolved O_2 on the solution was measured with a probe containing an oxygen electrode YSI, 5565 MPS, Yellow Springs, Ohio-USA version 1.12. The dissolved O_2 was measured passed 12 h from the addition of the modifiers, The difference between dissolved O_2 concentration on the day before and after the addition of the modifiers was then calculated and expressed as ΔO_2.

The roots were collected passed 2 hours from the application of the modifiers and placed in liquid nitrogen and further stored at -80° C until analysis. CAT was extracted according to Biemelt et al., (1998) as follows: 0.2 g of fresh root mass was ground in liquid nitrogen and homogenized in 1.5 mL of extraction buffer containing 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 DTT 0.5 M, 12 µL of PMSF 0.1 M, ascorbic acid 0.001 M and 22 mg polyvinylpolypyrrolidone (PVPP). The extract was centrifuged at 12,000 g for 30 minutes at 4° C, and the supernatant was collected and stored at -20° C until further analysis. CAT activity was evaluated according to Havir & McHale (1987) as follows: aliquots (10 µL) of enzyme extract were added to 170 µL of incubation medium containing 90 µL of potassium phosphate 200 mM (pH 7.0), 71 µL of

water and 9 µL of hydrogen peroxide 250 mM, incubated at 28 °C. Enzyme activity was determined by the decrease in absorbance at 240 nm every 15 seconds for 3 minutes, monitored by the consumption of hydrogen peroxide. The molar extinction coefficient used was 36 mM^{-1} cm^{-1}. The specific activity of the CAT was calculated based on the total amount of proteins of the samples determined according to Bradford (1976). We calculated the enzymatic activity in triplicate and the mean was calculated to each replicate.

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% PVPP (w m^{-1}) and further homogenized in 1500 µL of trichloroacetic acid (TCA) 0,1% (w v^{-1}). The homogenate was centrifuged at 12,000 g for 15 minutes at 4° C. The H_2O_2 was determined by measuring the absorbance at 390 nm in a reaction medium containing 500 µL of extract, 500 µL of 10 mM (pH 7.0) potassium phosphate buffer and 1000 µL of 1 M potassium iodide. We calculated the H_2O_2 content in the roots in duplicate and data was averaged to one replicate.

Statistical Analysis

The data were submitted to one-way (experiment I and II) or two-way ANOVA (experiment III) using the SISVAR 5.0 software (FERREIRA, 2011). Prior to parametric analysis, the data were tested for a normal distribution by using the Shapiro-Wilk test mean compared by the post-hoc Scott-Knott test to $p < 0.05$.

3 RESULTS

3.1 Anatomy of *T. domingensis*

The leaves of *T. domingensis* are comprised of one-layered epidermis with no intercellular spaces. Internally, 3-5 layers of palisade parenchyma are found and this tissue has very small intercellular spaces and is followed by 3-6 layers of large ground parenchyma cells which show very small intercellular spaces (FIGURE 1). These three tissues show limited gas diffusion capacity and this pattern can be found both on adaxial and abaxial leaf sides. The large leaf aerenchyma chambers are found on the central part of the leaf (FIG. 1A). The leaf-rhizome interface is comprised of an intercalary meristem which maintain the mitotic capacity of the leaf base and promotes continuous growth (FIG. 1C and D). However, this meristem and the region where cells are still differentiating show no intercellular spaces limiting the gas diffusion from the leaf aerenchyma to the rhizome (FIG. 1 C and D).

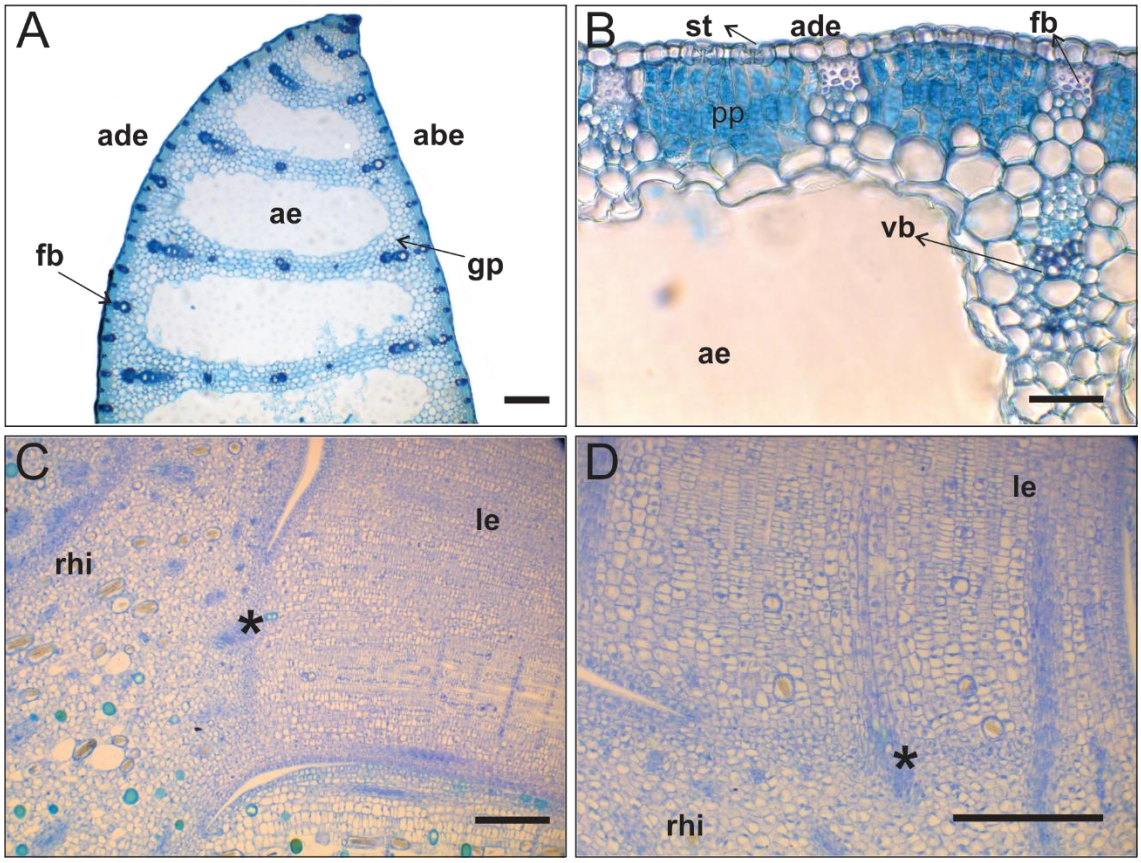

Figure 1. Anatomical structure of *Typha domingensis* leaves (A and B) and leaf-rhizome connection (C and D). ade= adaxial epidermis, abe= abaxial epidermis, pp= palisade parenchyma, ae= aerenchyma, is= intercellular space, gp= ground parenchyma, vb= vascular bundle, fb= fibers, rhi= rhizome, le= leaf, asterisk= leaf intercalary meristem. Bars= 300 µm (A), 50 µm (B) and 200 µm (C and D).

The rhizome of *Typha domingensis* is comprised of one layered epidermis with no intercellular spaces (FIGURE 2 A and B). Internally three or four layers of exodermis are found with no intercellular spaces and thick walls. Aerenchyma is found on their innermost part of the rhizome cortex, this area shows small vascular bundles and parenchyma trabecular. The innermost part of the rhizome is comprised of an atactostelic cylinder with ground parenchyma and scattered large vascular bundles, this part shows few intercellular spaces (FIG. 2A).

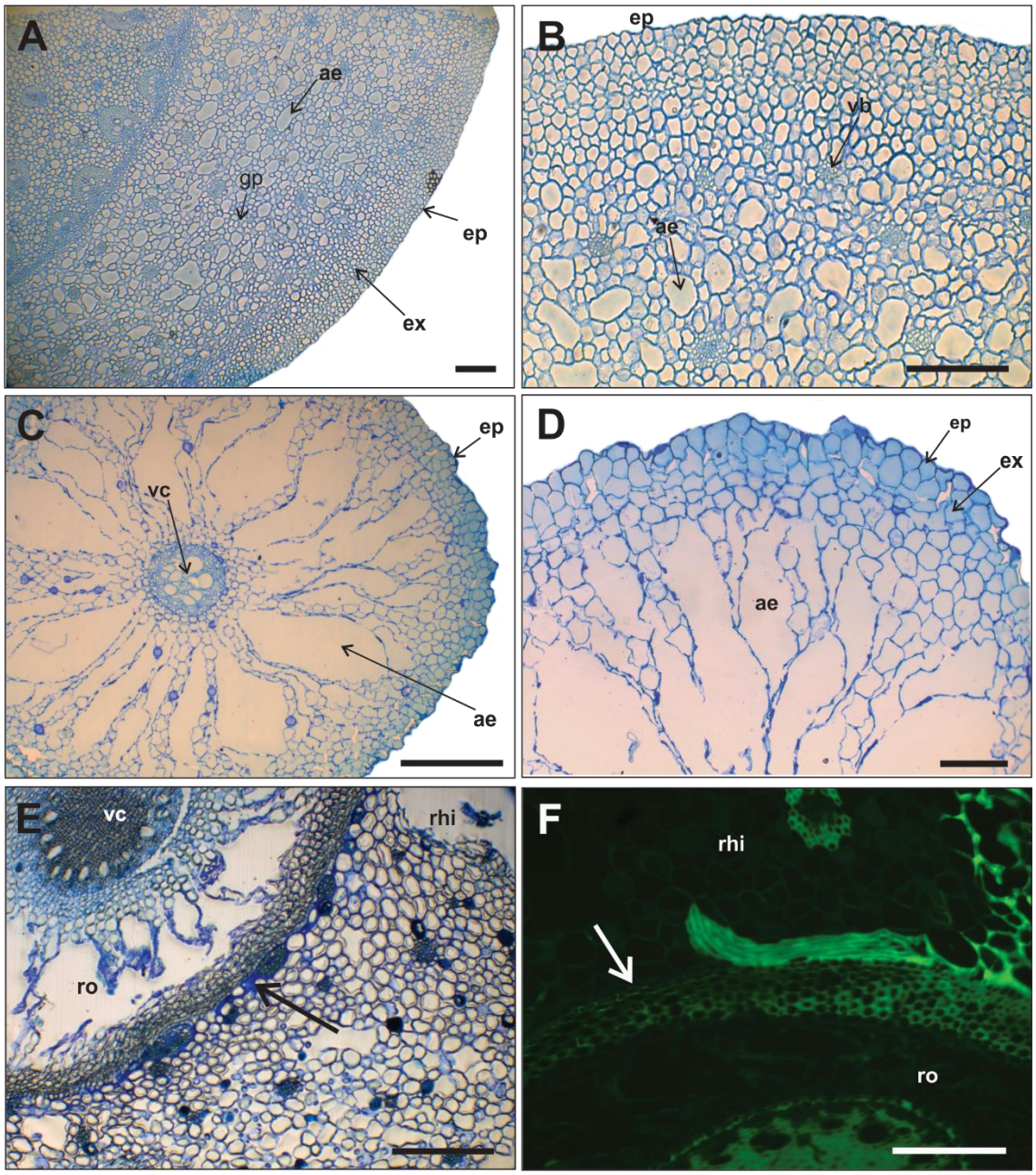

Figure 2. Anatomical structure of the rhizome (A and B), root (C and D) and the rhizome-root interface (E and F) of *Typha domingensis*. The bright areas on the F image show lignin/suberin deposition on fluorescence microscopy and the staining with cyanide blue. ep= epidermis, ex= exodermis, ae= aerenchyma, is= intercellular space, 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).

Typha domingensis roots are comprised of one-layered epidermis with no intercellular spaces and internally three layers of parenchyma cells with thick walls forming the exodermis (FIG. 2 C and D). The middle cortex is comprised of large aerenchyma chambers and the innermost part of the cortex is comprised of parenchyma cells with few intercellular spaces. At the center of the roots the vascular cylinder is found with xylem and phloem, almost no

intercellular spaces are found in that part of the root but it unlikely has a significant role on the ROL (FIG. 2B). The root-rhizome interface is comprised by the root external tissues (exodermis and epidermis) and also the rhizome external parts (exodermis and epidermis) with no direct connections between the two organs along the root axis (FIG. 2E). This interface between root and rhizome show very little intercellular spaces (FIG. 2E) and the fluorescence microscopy revealed strong deposition of lignin/suberin on the exodermis of both rhizome and roots (FIG 2F).

The leaf of *T. domingensis* shows the highest aerenchyma percentage among the vegetative organs followed by the root and then the rhizome. In contrast, although the rhizome has a large area section with 6.1 mm^2, the lower percentage of aerenchyma found in this organ indicates that there are less spaces filled with air. In fact, the largest air spaces were found on the leaves of *T. domingensis* and the smallest on the rhizomes (TABLE 1). The leaf pressure limit required to promote ROL was 0.077±0.01 MPa.

TABLE 1. Aerenchyma and internal air volume characterization of the vegetative organs of *T. domingensis* Pers. Data are shown as 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 $p < 0.05$.

The regions of two vegetative organs are connected, the interface of leaf/rhizome and rhizome/root shows very small intercellular spaces and the deposition of restrictive compounds to air diffusion. The deposition of lignin/suberin in the cell walls of the root exodermis as well as in the external layers of the rhizome is very intense. The connection between root-rhizome shows large areas with lignified/suberized tissues and no intercellular spaces (FIG. 2 E and F). The leaf base shows very few intercellular spaces because of the intercalary meristem found on this region (FIG. 1C and D).

3.2 The influence of plant parts on the ROL

Typha domingensis plants showed ROL at all conditions (TABLE 2). However, No significant differences (p=0.08) were found to the ROL for intact, leafless or cut leaves *T. domingensis* plants (TAB. 2).

TABLE 2. Root oxygen loss (ROL) expressed as the mean difference of the dissolved oxygen between two consecutive days on the solution containing intact, leafless and *T. domingensis* plants with cut leaves. Data is shown as mean ± standard deviation.

	ROL **(mg L^{-1})**
Intact Plants	0.43± 0.4a
Leafless Plants	0.64± 0.4a
Cut leaves	0.49± 0.4a

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

3.3 The CAT activity on *T. domingesis* root and its role on ROL

No interaction was found for the two factors (p= 0.98) and no effect of intact or leafless plants was found (p= 0.05) but significant effect was found to the CAT modifiers (p<0.01). Both intact and leafless plants showed similar ROL rates (TABLE 3). The solution containing the SNP showed the highest means for the ROL, followed by control plants and the lowest means were found for the plants exposed to the AT solution (TAB. 3).

TABLE 3. Root oxygen loss (ROL) expressed as the mean difference of the dissolved oxygen between two consecutive days after the application of treatments. The ROL was calculated in the solution containing intact and leafless *T. domingensis* plants subjected to AT (CAT inhibitor) and SNP (CAT activator). Data are shown as mean ± standard deviation.

Treatment	ROL (mgO_2 L^{-1})
Intact plants	1.00 ± 0.3a
Leafless	1.33 ± 0.4a
Treatment	ROL (mgO_2 L^{-1})
Control	1.05 ± 0.1b
AT (CAT inhibitor)	0.53 ± 0.2c
SNP (CAT activator)	1.91 ± 0.2a

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

The CAT activity was modified passed a period after the application of SNP and AT on the intact plants and plants exposed to AT showed higher CAT activity. In addition, intact plants always showed higher CAT activity as compared to leafless plants (TABLE 4). The hydrogen peroxide concentration on the roots of *T. domingensis* showed no significant differences between leafless and intact plants. However, plants submitted to AT showed a higher H_2O_2 levels in their roots as compared to the control and SNP treatments (TAB. 4).

TABLE 4. CAT activity and H_2O_2 concentration in roots of *T. domingensis* evaluated from intact or leafless plants passed 2 h after the application of AT (CAT inhibitor) and SNP (CAT activator). Data is shown as mean ± standard deviation.

CAT activity on roots (µmol H_2O_2 min^{-1} ug^{-1} protein)			
	Control	**AT**	**SNP**
Intact Plants	0.0315±0.002aB	0.1041±0.02aA	0.0481±0.03aB
Leafless	0.0046±0.003bA	0.013±0.001bA	0.0108±0.004bA
Root H_2O_2 concentration (mmol H_2O_2 mg^{-1} FM)			
	Control	**AT**	**SNP**
Intact Plants	0.255±0.13aA	0.390±0.21aA	0.040±0.11aA
Leafless	0.053±0.12aB	0.530±0.50aA	0.118±0.16aB

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

4 DISCUSSION

Does ROL depend on macrophyte shoots?

The models proposed to explain ROL argue that all the O_2 enters the plant by broken shoots and diffuses throughout the rhizome and roots finally releasing to the soil (ARMSTRONG; ARMSTRONG, 1988; ARMSTRONG, 1980; BRIX, 1997; KONNERUP; SORRELL; BRIX, 2011). According to Colmer, (2003), the O_2 enters the plants by stomata or broken shoots diffusing from shoot to roots, where it will be released by ROL. However, our results show that even leafless plants showed ROL which indicates that the origin of the O_2 may not exclusively rely on the shoot air uptake. In addition, the measured pressure to promote noticeable ROL on *T. domingensis* (0.077 MPa) is much higher than estimated values of internal pressure to other macrophytes as described by Konnerup; Sorrell; Brix, (2011) which found average 0.0004 MPa to the internal pressure of *Cyperus* L. species. This high pressure on the leaf mesophyll as necessary to promote ROL seems unlikely to be reached only by the air uptake on leaf stomata.

In this work we measured the ROL during 10 days on each experiment which is too long to support O_2 release only with aerenchyma stored gas. The amount of O_2 released between two consecutive days (1.15 ±0.3 mg L^{-1}, Tab. 2) is too high to be supported only by the aerenchyma stored oxygen. The Van't Hoff equation can be used to estimate the amount

of O_2 in the *T. domingensis* roots in this experiment. The equation (n = PV/RT) were P is the pressure in atm for the ROL, V is the volume of the gas (aerenchyma, in liters), R is the gas constant and T the temperature in K. For the experimental conditions we can estimate that the pressure for ROL (measured) was 0.7 atm (0.07 MPa); the root aerenchyma volume is 0.00056 L (TAB. 1) were 0.0001288 (23%) is the estimate O_2; the average temperature was 293.15 K (20 ºC) and the gas constant 0.082. Therefore, n= (0.7x0.00012)/(0.082x293.15), which goes to ~ 0.0000375 M of O_2; the equivalent to 1.2 mg of O_2 present of the roots. This amount of O_2 is released on a daily basis (1.15 ±0.3 mg L^{-1}), which means that the total O_2 present on the roots can be exhausted just in one day. Therefore, the total O_2 stored on the roots of *T. domingensis* can not provide the constant release of this gas during 10 days, meaning that this gas must be replenished on the root aerenchyma by some mechanism other than shoot uptake and transport in the leafless plants.

The dissolved O_2 on the solution was slightly lower on the solution containing leafless plants (TAB. 2) however the ROL, as measured by the difference on the dissolved O_2 on the solution between consecutive days was not affected by the removal of the shoot (TAB. 3). In addition, no difference can be found to intact or cut shoots (TAB. 2 and 3). The analysis of these results shows that ROL occur independent of the shoot integrity.

Therefore, overall the results of the present work show that the ROL is independent of macrophyte shoots and is maintained even in the absence of this part of the plant. It suggests necessity of a different mechanism to replenish the O_2 released by ROL other than the air diffusion from shoots.

Anatomical limitations to O_2 diffusion throughout shoot-root-soil continuum

Most of the works concerning on the O_2 diffusion through the plant and this role on the ROL disregard many anatomical traits which can significantly limit this internal air flow. Different plant tissues show variable permeability to O_2 depending on the abundance of intercellular space. The resistance to gas diffusion is low in the intercellular spaces but increase greatly in the presence of cell membranes and liquid compartments on the cells (TERASHIMA et al., 2011). Thus, the tissues with typical low percentage of intercellular spaces show high resistance to O_2 diffusion.

The anatomical barriers promoted by specific tissues on *T. domingensis* are reported on literature; however, its role on O_2 diffusion was not discussed or considered by the suggested models to explain the ROL. One of these tissues was described by Kaul (1974) that identified the intercalary meristem on the leaf base. In addition, the root exodermis several aquatic plants constrain the O_2 diffusion from root, permitting its use on root respiration

(ARMSTRONG; ARMSTRONG, 1988; ARMSTRONG; ARMSTRONG; BECKETT, 1992; BRIX; SCHIERUP, 1990; CORRÊA et al., 2017). According to Pi et al., (2009) the gas diffusion occurs naturally under low resistance within one plant organ. The high percentage of the aerenchyma and the volume of air contained in this tissue provide the necessary conditions for gas distribution in the root and leaf of *T. domingensis*. However, the aerenchyma percentage of the rhizome is significantly low as compared to roots and leaves providing a limited diffusion capacity in this organ.

The multiseriate exodermis in the root and the suberin deposition in the rhizome/root intarface provide additional barrier effect to O_2 diffusion. The hydrophobic trait of the suberin (POLLARD et al., 2008; SCHREIBER, 2010; SONG; YE; NII, 2011) is another factor which limits (or blocks) the gas diffusion at the root/rhizome interface and the O_2 release by root external cortex and epidermis.

In addition to the anatomical barriers to the O_2 diffusion throughout the plant organs, tissues located on the leaf also promote resistances to air uptake to aerenchyma as well as for the diffusion between the consecutive chambers. The stomatal resistance is very well known (TERASHIMA et al., 2011) which is largely reduced by the opening of the stomatal pore. This is the way air enters the leaf providing CO_2 to photosynthesis. However, in *T. domingensis* leaf, stomata are located just above the palisade parenchyma which has very little (or not noticeable) intercellular spaces (FIG. 1) which promotes a second resistance to air diffusion to aerenchyma. The ground parenchyma located internally to the palisade parenchyma creates a third resistance. The sum of these resistances creates a strong barrier to fill the aerenchyma chambers with sufficient air to increase the pressure to the high level required to ROL (0.077 MPa).

The Figure 4 indicates the significant resistance locations on the *T. domingensis* structure. According to the main tissues and organ interfaces the total resistance to the O_2 in the plant body can be defined as follows: $R_{TO2} = R_l + R_t + R_{lri} + R_{riro} + R_{ee}$; were R_{TO2} = total resistance to O_2 diffusion; R_l = leaf resistance, R_t = trabeculae resistance; R_{lri} = resistance of the leaf-rhizome interface; R_{riro} = resistance of the rhizome-root interface; R_{ee} = resistance exodermis and epidermis of the roots (FIG. 4). Therefore, for the actual model to O_2 diffusion (COLMER, 2003a) the accumulate resistances may severely limit the process. Thus, the anatomy of aquatic macrophytes such as *T. domingensis* is adapted to store O_2 in the aerenchyma and to its diffusion within an organ but the diffusion between two organs is limited by their interfaces.

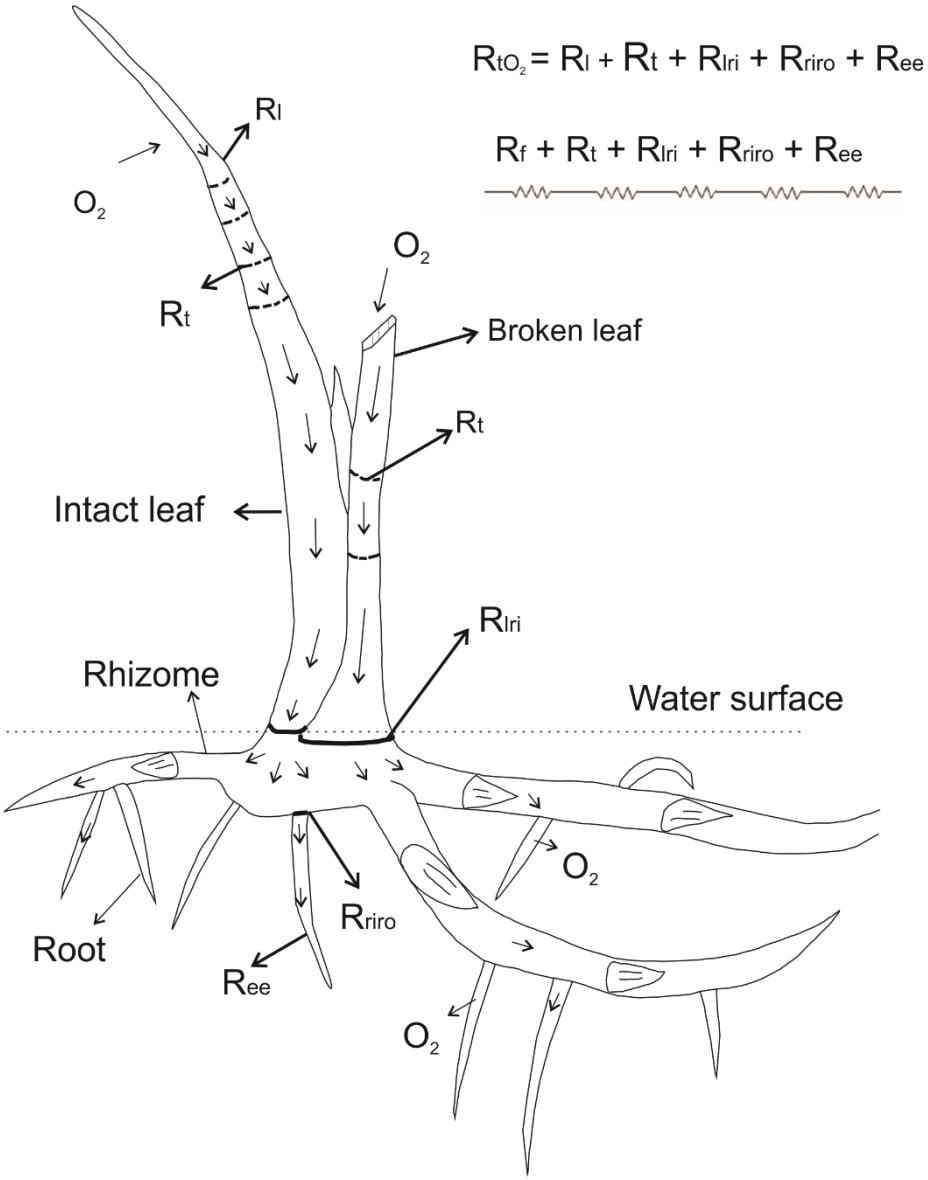

Figure 4. Scheme of the path to O_2 diffusion from the atmosphere to soil throughout the plant body, when considering anatomical resitances. R_{TO2} = total resistance to O_2 diffusion; R_l = leaf resistance, R_t = trabeculae resistance; R_{lri} = resistance of the leaf-rhizome interface; R_{riro} = resistance of the rhizome-root interface; R_{ee} = resistance exodermis and epidermis of the roots.

CAT activity is a source of O_2 for ROL

The CAT reaction consumes H_2O_2 as the substrate producing O_2 and H_2O (MØLLER, 2001). Its activity is related to the scavenging of the H_2O_2 as a reactive oxygen species (ROS), protecting plant cells against the oxidative stress (APEL; HIRT, 2004). Flooding causes O_2 deprivation which promotes the formation of ROS and the aerenchyma tissue evolved to store O_2 avoiding this effect (VOESENEK; BAILEY-SERRES, 2015). Therefore, aquatic macrophytes have permanent O_2 limitation, promoving the increase of root H_2O_2 which are detoxified by the CAT activity. This promotes a constant source of H_2O_2 to CAT

and this enzyme can operate almost at a steady state in the aquatic macrophytes roots such as *T. domingensis*.

The CAT activity is increased by several environmental factors in aquatic macrophytes such as heavy metals (PEREIRA et al., 2014), population density (CORRÊA et al., 2015), UV radiation (XU et al., 2014) and O_2 availability (XU et al., 2011). Thus, this enzyme is very responsive to environmental conditions and the CAT modifiers used in this experiment were efficient to change the activity of this enzyme. The changes promoted by the CAT modifiers on the activity of this enzyme were sufficient to change the ROL levels of the plants (TAB. 3). The increase of 81.9% on the ROL promoted by the CAT activator as well as the 49.5% reduction of this variable by the CAT inhibitor strongly supports the role of the enzyme on the ROL. In fact, the changes on the activity of this enzyme were proportionally related to ROL.

The results for the CAT activity and the H_2O_2 on *T. domingensis* support the CAT modifiers effect as well as the use of the substrate by this enzyme. The CAT activity was reduced by its inhibitor (AT) and, during this effect; the H_2O_2 was not consumed but accumulates on *T. domingensis* tissues. Passed the effect of the inhibitor, excess H_2O_2 present in the roots, increased the CAT activity. Therefore, the plants submitted to the inhibitor showed higher CAT activity due to the accumulation of the substrate for the enzyme, according to the Michaelis-Menten kinetics which state the concentration dependence to enzyme rates (REUVENI; URBAKH; KLAFTER, 2014).

Another question is the possibility of CAT to provide sufficient O_2 to fit the levels of measured ROL (1.15 mg L^{-1}). The average CAT activity for non-treated plants is average 0.08 $\mu molH_2O_2$ min^{-1} ug^{-1} protein (data not shown). The method to access CAT activity measure this variable by the consumption of H_2O_2, but, the analysis of the equilibrated equation shows that for each mol of this substrate 0.5 mol of H_2O_2 is produced (Moller, 2001). Thus, 0.04 μmol_{O2} min^{-1} ug^{-1} protein is produced by the activity of this enzyme on the *T. domingensis* roots. The average mass of the root system of *T. domingensis* plants at similar growth stage and size of the experimental plants is 12 g of fresh mass (data not shown). The protein proportion 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 ug^{-1}). Thus, the capacity for O_2 production of the *T. domingensis* root system was 38,4 μmol_{O2} min^{-1}, which is equivalent to 1.23 mgO_2 min^{-1} of enzyme activity. Thus, this enzyme has a very high capacity to produce O_2 and just one minute of its operation can provide sufficient O_2 to maintain the measured ROL of *T. domingensis* roots, in addition its enzyme activity rate is similar to previous

literature for *Typha* species as well as for other aquatic macrophyte species (BAH et al., 2011; CORRÊA et al., 2015; YANG; YE, 2015). It shows that, this enzyme can be a reliable source of O_2 to aquatic macrophyte roots by providing this gas to aerenchyma and root aerobic metabolism, in addition, it can support ROL rates on the aquatic macrophyte roots.

New model for aerenchyma O_2 filling and ROL

Because of the anatomical limitation, ROL can not be properly supplied of O_2 only by the transport from the shoots. Therefore, the Catalases acting as a root source for the O_2 is more likely way to replenish the lost gas. In addition, the root production of O_2 avoid all anatomical barriers except for the root exodermis (R_{ee} resistance) making far easier to O_2 follow its path to the soil. Thus, the new model can is shown in the Figure 5.

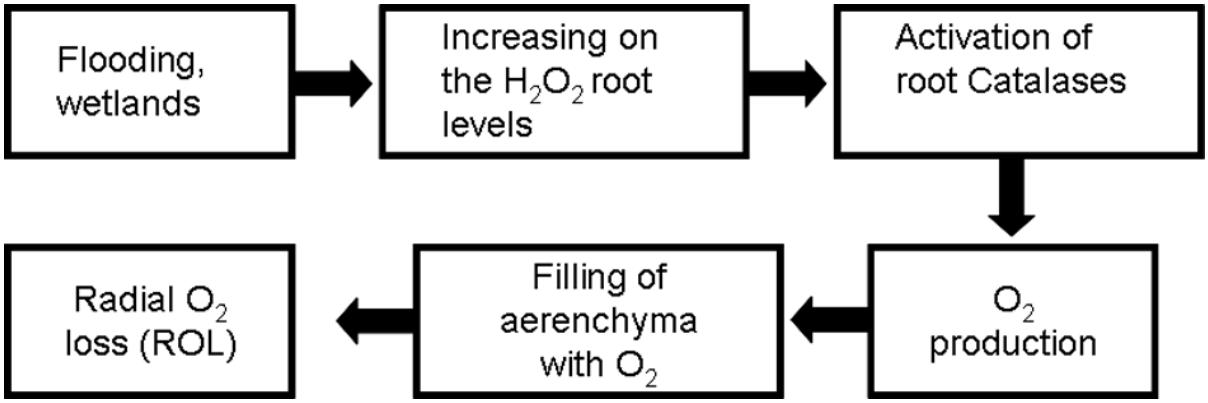

Figure 5. A new proposal for the origin of O_2 on the aerenchyma and to permit ROL in aquatic macrophytes such as *T. domingensis*.

5 CONCLUSSION

Anatomical barriers comprised of tissues poor in intercellular spaces constrain the O_2 pathway through shoots to roots in aquatic macrophytes. The root catalase has sufficient activity and substrate to provide necessary O_2 to fill aerenchyma chambers and to support the radial oxygen release. The O_2 released on wetlands by aquatic macrophyte has its origin in the catalase activity. *T. domingensis* roots were able to be independent of the aerial plant part for the O_2 production released in ROL .

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