

## RESSALVA

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS  
(BIOLOGIA VEGETAL)

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MECANISMOS DE TOLERÂNCIA AO AL<sup>3+</sup> EM PLANTAS COMPARANDO  
ESPÉCIE DE CERRADO (*STYRAX CAMPORUM*), LIMOEIRO 'CRAVO' (*CITRUS  
LIMONIA* CV. CRAVO) E TRIGO (*TRITICUM AESTIVUM*)

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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Ciências biológicas (Biologia Vegetal)

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**MECANISMOS DE TOLERÂNCIA AO AL<sup>3+</sup> EM PLANTAS COMPARANDO  
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(*CITRUS LIMONIA* CV. CRAVO) E TRIGO (*TRITICUM AESTIVUM*)**

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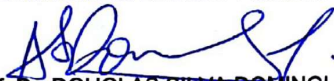
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**Mecanismos de tolerância ao Al<sup>3+</sup> em plantas comparando espécie de Cerrado (*Styrax camporum*), limoeiro ‘cravo’ (*Citrus limonia* cv. cravo) e trigo (*Triticum aestivum*)**

**Resumo**

Presente em solos ácidos, o alumínio principalmente encontrado na sua forma tóxica (Al<sup>3+</sup>) tem como principal efeito a diminuição do crescimento radicular. O Cerrado ocorre no centro-oeste do Brasil sob solos ácidos (pH <4) e com alto teor de alumínio (Al). *Citrus limonia* cv ‘Cravo’ Osbeck e *Styrax camporum* Pohl compartilham localização geográfica e características edáficas em que se estabelecem, no entanto exibem respectivamente sensibilidade e tolerância ao Al. O presente trabalho visa elucidar mecanismos envolvidos no estresse por Al<sup>3+</sup> a nível de expressão gênica radicular. Para as duas espécies foi feita uma análise de transcriptoma para avaliar os efeitos do Al por uma perspectiva global, e a partir destes resultados alguns genes foram selecionados para avaliação ao longo de experimentos de hidroponia mantido por 60 dias. São discutidas estratégias de tolerância e sensibilidade ao Al nas duas espécies, juntamente com análises de biometria, quantificação hormonal e anatomia que corroboram o comportamento sensível de *Citrus* e tolerante de *Styrax* por diversas vias. Além disso, o último capítulo trata da dependência do pH em um importante mecanismo de tolerância ao Al em trigo envolvendo exsudação de ácido orgânico.

Palavras-chave: Alumínio, Citros, Transcriptoma, qRT-PCR, Plantas lenhosas do Cerrado



**Al-tolerance mechanisms in plants comparing Cerrado species (*Styrax camporum*), 'Rangpur' lime (*Citrus limonia*) and wheat (*Triticum aestivum*)**

**Abstract**

Present in acid soils, aluminum mainly found in its toxic form ( $Al^{3+}$ ) has as main effect the decrease of root growth. Cerrado occurs in central-western Brazil under acid soils (pH <4) and high aluminum content (Al). *Citrus limonia* (Osbeck) and *Styrax camporum* (Pohl) share geographic location and edaphic characteristics in where they are settled, however exhibiting respectively Al-sensitivity and Al-tolerance. The present work aims to elucidate the mechanisms involved in  $Al^{3+}$  stress at root gene expression level. For both species, a transcriptome analysis was performed to evaluate the effects of Al in a global perspective, and from these results some genes were selected for evaluation in hydroponic experiments maintained for 60 days. Al tolerance and sensitivity strategies are discussed in both species, together with analyzes of biometrics, hormonal quantification and anatomy data that corroborate the Al-sensitive behavior of Citrus and Al-tolerance in *Styrax* by several routes. In addition, the last chapter deals with the dependence of pH on one important mechanism of Al tolerance in wheat involving organic acid exudation.

Key words: Aluminum, Citrus, Transcriptome, qRT-PCR, Cerrado woody plants



## Introdução geral

Alternativas para aumentar a produção de alimentos têm sido estudadas em diversas áreas, já que em decorrência do crescimento acelerado da população mundial, a demanda torna-se maior. Atividades agropastoris teriam que aumentar a produção paralelamente ao crescimento populacional, mas áreas com as características de clima e solo necessárias para agricultura são escassas (Phalan et al. 2013).

Quarenta por cento dos solos agriculturáveis são ácidos ( $\text{pH} < 5,0$ ), sendo que na América do sul 60% dos solos têm  $\text{pH}$  abaixo de 4,0 (Von Uexküll and Mutert 1995). Além de características naturais do solo, chuvas ácidas e o uso de fertilizantes amoniacais contribuem para a acidez do solo (Kochian et al. 2004; Zheng 2010). Devido à larga ocorrência de solos ácidos em grandes proporções de áreas agricultáveis no Brasil (solos do Cerrado e Centro sul), esforços biotecnológicos para tornar possível a atividade agrária nestes solos têm sido feitos (Ryan et al. 2011).

A acidez do solo pode diminuir em até 60% a produtividade, dependendo da cultura (Blair et al. 2009). Para minimizar a acidez do solo são usadas técnicas como aplicação de calcário ( $\text{CaCO}_3$  ou  $\text{MgCO}_3$  - calagem) e alternância com cultura de leguminosas, técnicas essas com eficácia duvidosa, já que o investimento em calagem ( $\pm$  US\$400/ha/ano – Ratter et al. 1997) não é refletido na neutralização do  $\text{pH}$  em todo o perfil do solo, mas apenas superficialmente (0-20 cm prof.) (Delhaize et al. 2009). Quando  $\text{pH}_{\text{solo}} < 5$ , aluminossilicatos são ionizados e o alumínio passa à forma ionizada ( $\text{Al}^{3+}$ ), que é tóxica à maioria das plantas (Ulrich 1986). Outro problema de solos ácidos é a indisponibilidade de fósforo (P) para as raízes, porque o P fixa-se em compostos com ferro ( $\text{Fe}^{3+}$ ) e/ou  $\text{Al}^{3+}$  (Kochian et al. 2004; Zheng 2010).

Altas concentrações de  $\text{Al}^{3+}$  no solo são percebidas (bioquimicamente) pelo ápice das raízes, que diminuem o crescimento radicular quase que instantaneamente (Delhaize et al. 2004; Sun et al. 2010). Esta diminuição do alongamento diminui a chance de absorção de água e nutrientes em sub-superfícies do solo. A presença de  $\text{Al}^{3+}$  no simplasto causa desordens fisiológicas celulares: aumento da concentração de cálcio ( $\text{Ca}^{2+}$ ) no citosol, que culminam em desorganização do citoesqueleto e deposição de calose (Horst et al. 2010). Na parte aérea da planta, a toxicidade do  $\text{Al}^{3+}$  está relacionada ao processo fotossintético (Konrad et al. 2005; Jiang et al. 2008), que tem baixo rendimento devido principalmente ao dano causado às membranas dos tilacóides por espécies reativas de oxigênio (Horst et al. 2010; Ryan et al. 2011).

A diminuição no alongamento da raiz é o sintoma de toxicidade de  $\text{Al}^{3+}$  mais estudado. O crescimento e desenvolvimento da raiz dependem fortemente da concentração e transporte (distribuição simétrica) de auxinas (Perilli et al. 2012) e etileno no tecido radicular, hormônios vegetais que atuam em sintonia neste órgão (Kang et al. 1971; Swarup et al. 2007). Em solos ácidos, a elevada concentração de  $\text{Al}^{3+}$  induz maior síntese de etileno; este hormônio faz com que a distribuição polar de auxina fique alterada e com isso, o alongamento celular não ocorre corretamente, deixando as raízes mais curtas (De Cnodder et al. 2005; Ponce et al. 2005; Sun et al. 2010; Basu et al. 2011) e tortas (Kopittke et al. 2008). Estes últimos autores demonstraram que o  $\text{Al}^{3+}$  causa rigidez da rizoderme, enquanto que células das camadas internas do órgão continuam crescendo, causando rupturas e raízes tortas. Isso sugere que se a parede celular tem sua composição alterada (pela perturbação direta de  $\text{Al}^{3+}$  e/ou competição por  $\text{Ca}^{2+}$ ), a extensibilidade celular é também alterada. De fato, o grau de metilação das pectinas, pela enzima pectina metil esterase e grupos carboxílicos da parede celular geram cargas negativas que normalmente são estabilizadas por  $\text{Ca}^{2+}$  (Carpita, N. C.; McCann 2000). O  $\text{Al}^{3+}$ , no entanto, se presente no apoplasto, pode competir por estas cargas negativas. O  $\text{Al}^{3+}$  ligado à parede celular impede sua expansão, deixando-a mais rígida (Kopittke et al. 2008) e limita a expansão por causar ainda comprometimentos enzimáticos na parede (Horst et al. 2010).

Frente a todos os tipos de dano que o  $\text{Al}^{3+}$  solúvel no solo pode causar às plantas, estas categorizam-se em três grupos: plantas que são sensíveis ao(s) (efeitos tóxicos do)  $\text{Al}^{3+}$ ; as que são resistentes, com mecanismos para exclusão de  $\text{Al}^{3+}$ ; e as que são tolerantes, com mecanismos para quelar e armazenar o  $\text{Al}^{3+}$  em formas menos tóxicas (Ryan et al. 2011). Os mecanismos de resistência e tolerância ao  $\text{Al}^{3+}$  podem ser determinados por um ou vários genes (Delhaize et al. 2012) dependendo da espécie, e pode ainda haver herança quantitativa, com fenótipos gradativos de resistência ou de tolerância (Kochian et al. 2004; Delhaize et al. 2012).

Por outro lado, o bioma Cerrado que originalmente ocorre (ou ocorria) no centro-oeste do Brasil (originalmente, 23% do território nacional), encontra(va-se) sobre solos ácidos ( $\text{pH} < 4$ ), com alta concentração de  $\text{Al}^{3+}$  e baixa disponibilidade de nutrientes, principalmente P,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  e  $\text{Zn}^{2+}$  (Haridasan 2008). Isso faz com que as espécies nativas desta vegetação sejam adaptadas a tais condições edáficas (Haridasan 2008). Assim, as espécies lenhosas do Cerrado são divididas em acumuladoras obrigatórias de  $\text{Al}^{3+}$  (não se desenvolvendo na ausência desse nutriente), acumuladoras facultativas e espécies não acumuladoras (Haridasan 1982; Haridasan et al. 1987).

Rawitscher (1948) já descrevera o sistema radicular profundo de espécies lenhosas do Cerrado. Franco (1998) e Hao et al. (2008) demonstraram indiretamente que estas espécies mostram sistema radicular profundo. Habermann and Bressan (2011) demonstraram por medidas diretas que espécies de savanas mostram raízes com 1m de comprimento após 100 dias da germinação. Assim, é possível que o  $Al^{3+}$  do solo do Cerrado esteja associado a alguma relação com a biossíntese, transporte e/ou ação de auxinas e/ou etileno, promovendo o crescimento em profundidade dessas raízes. Não que a presença de  $Al^{3+}$  seja essencial para esta característica. Mesmo porque para algumas plantas do Cerrado, o significativo crescimento de raízes não é plástico ao ambiente edáfico, mesmo se cultivadas em solos muito férteis e ausentes em  $Al^{3+}$  (Habermann and Bressan 2011). No entanto, espécies não-accumuladoras de  $Al^{3+}$  mostram crescimento de raízes significativamente maior quando cultivadas em  $740 \mu M$   $Al^{3+}$  do que se cultivadas na ausência desse nutriente (de Souza and Habermann 2012). Porém, até hoje, a ciência agrônômica não mostrou interesse em entender o metabolismo de  $Al^{3+}$  nas espécies do Cerrado (de Souza and Habermann 2012).

Por outro lado, nas regiões norte, noroeste e central do estado de São Paulo e sul de Minas Gerais, que englobam áreas originalmente ocupadas pela borda sul da vegetação de Cerrado, a citricultura é desenvolvida com sucesso desde os anos 50 (de Souza and Habermann 2012). Porém os *Citrus* sp, ao contrário da vegetação nativa do Cerrado, podem exibir alta sensibilidade ao  $Al^{3+}$  solúvel em solos ácidos (Pereira et al. 2000; Jiang et al. 2008).

Para resistência a altas concentrações de  $Al^{3+}$  no solo o principal mecanismo já demonstrado consiste em secretar ácidos orgânicos pelas raízes (Kochian et al. 2004); o ácido liga-se ao  $Al^{3+}$  e impede que se ligue à parede celular ou seja absorvido. O tipo de ácido orgânico secretado depende da espécie, e também da especificidade do substrato da enzima extrusora (Ryan et al. 2011), podendo a secreção ser constituída de um ou mais tipos de ácido. A parte aérea também deve ter uma função na resistência ao  $Al^{3+}$  (Wu et al. 2013), mas são necessários mais estudos nesta área.

Os principais ácidos orgânicos na função de resistência à toxicidade do  $Al^{3+}$  são malato, citrato e oxalacetato (Kochian et al. 2004). A exsudação de citrato ocorre mais frequentemente nas plantas, talvez porque tenha maior poder para quelar o íon alumínio (Pereira et al. 2010). O citrato é também importante na distribuição de ferro nos organismos vegetais (Magalhaes 2010; Delhaize et al. 2012).

Segundo Ryan et al. (2011), o fator limitante para a exsudação de ácidos orgânicos não é a síntese do composto (que é sintetizado continuamente), e sim a

extrusão deste. O  $Al^{3+}$  do solo é responsável por ativar a função destas proteínas de transporte (Delhaize et al. 2009) sendo a transdução deste sinal mediada por quinases (Kumari et al. 2008).

Para a exsudação de malato na raiz existe a proteína ALMT (do inglês, aluminium activated malate transporter), exclusiva no reino *Plantae*. Já o citrato é secretado por um canal não específico (pode liberar outros compostos) que ocorre em todos os domínios vivos, pertencente à família MATE (do inglês, multidrug and toxic compound exudation Family) (Magalhaes 2010). O mecanismo de exsudação de oxalacetato ainda não é conhecido.

No mecanismo de tolerância, algumas espécies são capazes de acumular grandes quantidades de  $Al^{3+}$  (Mukhopadyay et al. 2012; Negishi et al. 2012). Em plantas acumuladoras, o  $Al^{3+}$  é absorvido pela raiz e complexado ao oxalacetato, depois o  $Al^{3+}$  troca de acceptor, ligando-se ao citrato para transporte no xilema, chegando ao mesofilo, onde o acceptor volta a ser o oxalacetato (Kochian et al. 2004). Segundo Horst et al. (2010), o mecanismo de armazenar o  $Al^{3+}$  no simplasto em complexos menos reativos evita o contato do cátion com a parede e por isso anula o efeito de restrição no alongamento celular.

O efeito do  $Al^{3+}$  na expressão gênica tem sido estudado analisando-se genes de síntese de malato e/ou citrato (Horst et al. 2010; Wang et al. 2013), transporte ALMT (Sasaki et al. 2004, 2006; Guo et al. 2007; Houde and Diallo 2008; Pereira et al. 2010), transporte MATE (Liu et al. 2009; Magalhaes 2010), síntese de etileno (Chandran et al. 2008; Sun et al. 2010), distribuição de auxina (Li et al. 2006; Goodwin and Sutter 2009; Sun et al. 2010), homeostase de cálcio (Chandran et al. 2008), genes relacionados à parede celular (Kumari et al. 2008; Maron et al. 2008; Horst et al. 2010), genes reguladores da expressão de outros genes (Delhaize et al. 2012), e genes envolvidos no próprio acúmulo de  $Al^{3+}$  (Delhaize et al. 2012). Embora a diversidade de genes estudados seja relativamente alta, as espécies nas quais são feitos estes estudos resumem-se basicamente a trigo, *Arabidopsis*, milho e cevada.

Diante do exposto, fica claro que há espaço para entendimento do metabolismo do  $Al^{3+}$  em espécies cítricas e, sobretudo, em espécies em que o  $Al^{3+}$  não é tóxico, mas benéfico, como as espécies nativas do Cerrado.

## Conclusões

Diante das discussões baseadas em experimentos realizados com *Citrus limonia* e *Styrax camporum* frente à toxicidade do Al, bem como uma análise de funcionamento de um dos canais mais importante da tolerância ao Al (*TaALMT*) conclui-se que:

- i) Apesar de considerada uma espécie sensível, o transcriptoma de *C. limonia* exibe genes de tolerância ao Al como a indução de expressão no canal MATE (responsável pela exsudação de citrato);
- ii) Mecanismos de tolerância ao Al ocorrem de forma desorganizada com o passar dos dias de experimento. Além disso, a diminuição na concentração de etileno e o aumento nos níveis de auxina contribuem significativamente para redução no crescimento radicular de *C. limonia* a 1480  $\mu\text{M}$  Al;
- iii) O transcriptoma de *Styrax camporum* revelou indução de genes de tolerância ao Al em diversas vias, como por exemplo para exsudação de ácido orgânico (ALMT, efluxo de malato);
- iv) De acordo com o tempo de tratamento a resposta de tolerância ao Al dá-se preferencialmente em uma via. Além disso, de modo geral em *S. camporum* mostra-se tolerante ao Al no nível de 740  $\mu\text{M}$  Al enquanto que a 1480  $\mu\text{M}$  Al as respostas indicam suscetibilidade;
- v) O canal *TaALMT1* atua na exsudação de malato unicamente em condições ácidas e na presença de Al, amenizando o estresse causado pelo  $\text{Al}^{3+}$ ;

Assim, em determinadas concentrações o  $\text{Al}^{3+}$  pode beneficiar o desenvolvimento radicular das espécies do Cerrado, mas quando o teor de Al é mais elevado (1480  $\mu\text{M}$ ) o crescimento radicular é reduzido tanto em *C. limonia* quanto em *S. camporum*.



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