DETERMINAÇÃO DE ÁCIDO ABSCÍSICO E RESPOSTAS METABÓLICAS EM ESPÉCIES CONGENÉRICAS DE SAVANA E MATA DO CERRADO PAULISTA

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“A mente que se abre a uma nova ideia
jamais voltará ao seu tamanho original”

Albert Einstein

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Determinação de ácido abscísico e respostas metabólicas em espécies congenéricas de savana e mata do Cerrado paulista

RESUMO

O ácido abscísico (ABA) é conhecido como hormônio do estresse hídrico, induzindo o fechamento estomático em episódios de seca. Visto que a concentração deste hormônio é muito baixa, primeiramente foi desenvolvido um método para quantificação foliar de ABA e outros cinco compostos de sua rota metabólica por cromatografia líquida em ionização '*electrospray*' com espectrometria de massas em tandem (LC-ESI-MS/MS). Espécies de *Styrax* (*S. Ferrugineus,* uma espécie de Cerrado *sensu stricto* e *S. pohlii,* uma espécie de mata ripária) foram analisadas quanto ao teor de ABA e seus metabólitos na estação seca e na estação chuvosa. Além disso, também medimos, na estação seca, a expressão do gene da enzima 9-cis-epóxi-carotenóide dioxigenase (NCED), chave na biossíntese de ABA. Medidas de trocas gasosas (assimilação de CO_2 e transpiração) e potencial da água na folha foram também realizadas nas estações chuvosa e seca. O método analítico desenvolvido mostrou-se eficiente para as quantificações de ABA, evidenciando como evitar o efeito da matriz nas análises. Em conjunto, os dados que obtivemos mostram que a espécie de mata, embora ocorra em hábitat com maior disponibilidade hídrica no solo, não se encontra tão bem adaptada à evento de seca prolongada quanto a espécie de Cerrado, o que pode explicar a distribuição espacial destas espécies.

ABSTRACT

Abscisic acid (ABA) is a plant hormone biosynthesized under water deficiency stress conditions, reducing the stomatal conductance (*gs*) and transpiration rates (*E*). Considering that it is detected at very low concentrations in the metabolism, we first developed an adapted method to quantify ABA and other five metabolites from its metabolic pathway, using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). *Styrax species* (*S. ferrugineus*, a savana species occurring in the Cerrado *sensu stricto*, and *S. pohlii*, a forest species from the riparian forest) had leaf concentrations of ABA and its metabolites analized in the wet (February) and dry (September) seasons. In addition, we have also measured the gene expression of the 9-cis epoxycarotenoid dioxygenase..(NCED) enzyme, which plays a key role on ABA biosynthesis. Leaf gas exchange and leaf water potential were also performed in both seasons. The analytical method proved to be highly efficient for ABA quantification, and it demonstrated how to avoid matrix effects when measuring ABA on leaves. Taken together, our data showed that although occurring in a habitat with great soil water availability, the forest species may not be considered as adapted to long seasonal droughts as compared to the savana species. These results may help explain their distinct distribution patterns in the Cerrado biome.

1. Introdução geral

O ácido abscisíco (ABA) é um hormônio vegetal da classe dos sesquiterpenos sintetizado nos plastídios. Atua em resposta ao estresse por deficiência hídrica no solo, levando ao fechamento dos poros estomáticos (Assmann, 2010).

Em condições de alto déficit de pressão de vapor (DPV) o ABA promove o fechamento dos poros estomáticos, diminuindo a transpiração (Assmann, 2010). Isso ocorre tipicamente ao meio-dia quando o DPV é máximo (Habermann & Rodrigues, 2009) e, mais acentuadamente, em estações secas, quando além dessas características atmosféricas, há sinais de estresse hídrico enviados pela raiz (Hu et al., 2012).

O Cerrado, conhecido com savana brasileira, ocorre na região Centro-Oeste do Brasil e apresenta espécies vegetais adaptadas à intensa restrição hídrica (Anjum et al., 2011), diferentemente de espécies de mata, onde o principal fator limitante não é a disponibilidade de água e sim a luminosidade (Hoffmann & Franco, 2003).

Considerando-se as adaptações das espécies do Cerrado às secas sazonais é cientificamente válido estudar-se como variam as concentrações de ABA nas folhas, que é sabidamente um fator de regulação estomática entre as estações mais contrastantes (seca e chuvosa). Do mesmo modo, nas espécies de mata, principalmente aquelas habitantes de matas úmidas e ripárias, é curioso saber se por habitarem ambiente rico em água, seriam menos dependentes de ácido abscísico para proteção contra a seca. No presente trabalho, foram comparadas as concentrações foliares de ABA, seus conjugados e derivados da rota de metabolismo em espécies de savana (*Styrax ferrugineus*) e de mata (*Styrax pohlii*), além de quantificar a expressão gênica em um passo chave na biossíntese de ABA também nestas duas espécies congenéricas. Concomitante à isso, utilizamos medidas de trocas gasosas foliares dessas mesmas espécies nas mesmas estações para melhor qualificar os significados fisiológicos das

diferentes concentrações de ABA e seus metabólitos nas folhas. Além disso, também testamos métodos analíticos para quantificação de ABA em tecidos foliares.

2. Revisão da Literatura

Sinalização do estresse hídrico

O déficit hídrico pode causar inúmeros danos tanto ao nível celular quanto à planta como um todo. Por isso, estratégias fisiológicas e moleculares para evitar/tolerar e mesmo entender este tipo de estresse são amplamente estudadas. A percepção do estresse ocorre quando um sinal físico é transformado em uma resposta bioquímica. Depois dessa primeira sinalização ocorre a transdução deste sinal (através de quinases) levando a uma cadeia de respostas (Bray, 1997). Respostas comuns ao aumento de ácido abscísico (ABA) dado pelo déficit hídrico incluem o acúmulo de solutos osmoticamente ativos como açúcares, glicerol, aminoácidos (prolina) e outros metabólitos de baixo peso molecular (Sanchez-Diaz et al., 2008) e o aumento da atividade de aquaporinas e da condutividade hidráulica na raiz (Wang et al., 2003).

Muitos dos genes induzidos pela seca são também induzidos por ABA, o que torna este hormônio essencial na percepção fisiológica da seca. No DNA há elementos que respondem ao ABA (ABRE, do inglês ABA response element) e os que respondem à desidratação (DRE, do inglês dehydration-responsive element). A transdução de sinal leva à transcrição de genes presentes nestes elementos, amplificando a resposta e tornando-a mais efetiva e rápida (Shinozaki & Yamaguchi-Shinozaki, 1997; Bray, 2002). Uma das principais respostas ao déficit hídrico é o aumento na síntese de ABA através da expressão do gene 9-cis-epoxi-carotenóide dioxigenase (NCED).

A biossíntese do ABA ocorre nos cloroplastos e outros plastídios. A via inicia-se com o isopentenildifosfato (IPP) e leva à síntese de xantofila C40 (violaxantina), síntese

esta catalisada pela zeaxantina epoxidase (ZEP). A violaxantina é convertida a um composto 9-cis-neoxantina, que é, então, clivado para formar o composto C15, xantoxal. Esta clivagem é então catalisada pela enzima NCED. A síntese de NCED é rapidamente induzida pelo estresse hídrico, sugerindo que a reação que esta enzima catalisa é uma etapa-chave de regulação da síntese do ABA (Iuchi et al., 2001). O xantoxal é convertido a ABA por meio de etapas oxidativas, envolvendo os intermediários ABA-aldeído e ABA (Figura 1), possivelmente com álcool abscísico e ácido xantóxico como metabolitos intermediários (Cheng et al., 2002).

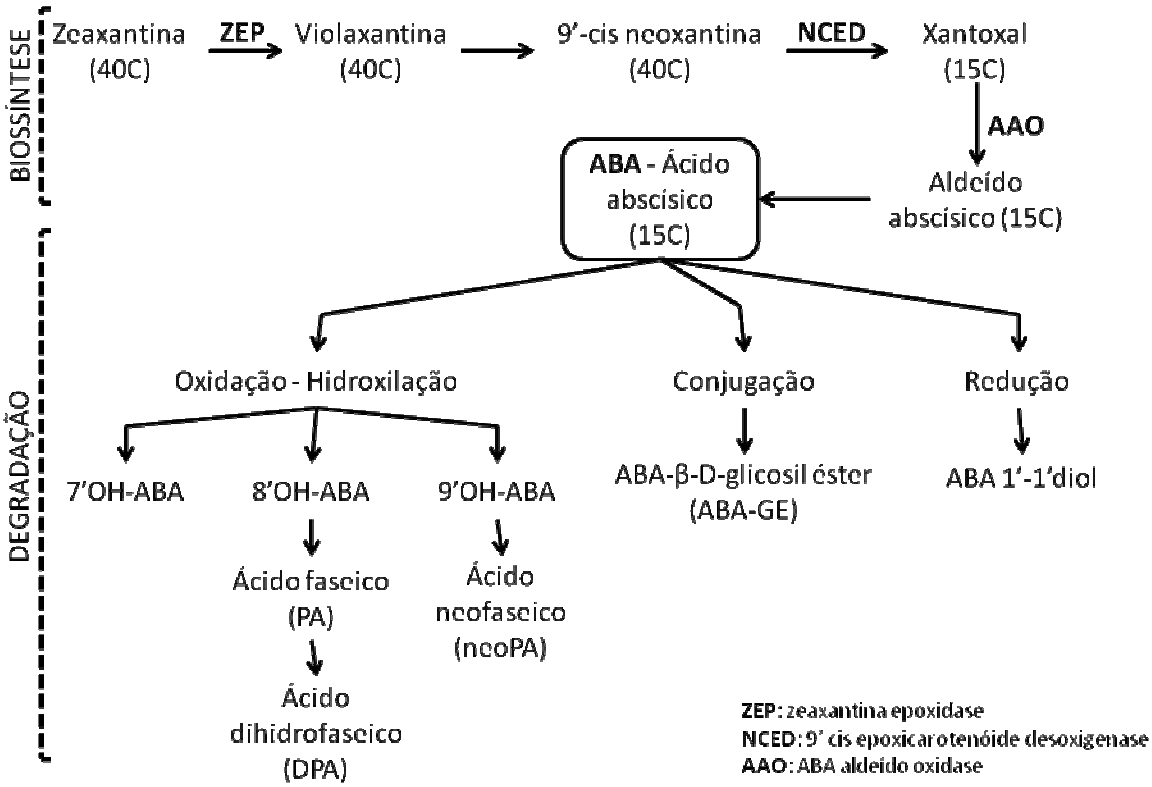

Figura 1 – Principais compostos nas rotas de biossíntese e degradação do ácido abscísico.

Metabolismo e quantificação de ácido abscísico

Em células vegetais, o ABA é sintetizado e degradado continuamente, porém o metabolismo de ABA pode ocorrer por diversas vias que frequentemente dependem de cada espécie, estágio de desenvolvimento ou tecido. O ABA pode ser metabolizado por três tipos de reação: oxidação, conjugação e redução (Figura 1). A principal rota oxidativa de ABA natural é através da 8'hidroxilação, formando 8'hidroxiABA, que espontaneamente se isomeriza em ácido faseico (PA) (Okamoto et al., 2009). O PA pode sofrer isomerização espontânea, formando com sua redução o ácido dihidrofaseico (DPA) como produto principal e ácido epihidrofaseico (epiDPA) em quantidades inferiores (Saika et al., 2007). A menor rota de oxidação inclui a formação de (+)7'hidroxiABA (7'OHABA), enquanto a menor rota redutiva produz ABA1'-1'diol instável. Uma nova rota, ABA 9'hidroxilação tem sido descrita, resultando em metabolitos 9'hidroxiABA (9'OHABA) e seu produto cíclico, o ácido neofaseico (neoPA) (Okamoto et al, 2011). Além disso, o ABA e seus metabólitos podem ser conjugados com glicose, formando ésteres glicosilados correspondentes a C1, ABA-β-D-glicosil éster (ABAGE) ou glicosídeos em C4 (Tureckova et al., 2009). Sabe-se que a enzima b-D-glucosidase libera ABA a partir de ABA-GE em algumas espécies de plantas. Também, a atividade de b-D-glucosidase é realçada pelo estresse salino.

Embora os conjugados de ABA possam ser considerados como fisiologicamente inativos e acumulados nos vacúolos durante o envelhecimento, tem-se proposto recentemente seu envolvimento no transporte a longas distâncias. As concentrações de ABA conjugado nem sempre variam em paralelo com a mudança na concentração de ABA, sugerindo que a conjugação é regulada em condições e em tecidos particulares (Lopez-Carbonell et al., 2009).

A concentração tanto da forma hormonal ativa (ABA) quanto de seus metabólitos é bastante baixa (da ordem de picogramas ou nanogramas por grama de folhas). Por isso, métodos analíticos com alta sensibilidade devem ser usados nesta quantificação. Os métodos mais comumente usados são cromatografia gasosa (com a necessidade de derivatização anterior) (Duffield & Netting, 2001; Birkemeyer et al., 2003), imunoensaios (Gokani et al., 1998) e cromatografia líquida (Chiwocha et al., 2003; Schwartz & Zeevaart, 2010; Susawawngsup et al., 2011) para separação dos compostos, seguidos de uma ferramenta de identificação, geralmente espectrometria de massas.

Ecofisiologia: Mata versus Cerrado

As pressões edafo-climáticas das savanas, citadas anteriormente, são coerentes com as adaptações morfo-fisiológicas observadas nas espécies. Tais adaptações relacionam-se principalmente com a resistência à deficiência hídrica sazonal, conferindo baixo crescimento relativo, baixa assimilação de carbono por unidade de área foliar, alta razão raiz: parte aérea (Habermann & Bressan, 2011) e baixos valores de área foliar específica (AFE), caracterizando as savanas como ambientes mais sujeitos à deficiência hídrica no solo e, consequentemente, mais "improdutivos" que florestas (Hoffmann & Franco, 2003).

Ambientes de mata também apresentam solos pobres (Haridasan, 2008), mas o dossel mais fechado em relação ao das savanas propicia menor penetração da irradiância e baixo DPV. A umidade do solo, embora seja dependente do regime de chuvas e da variação do nível do lençol freático, pode ser mais elevada que nas savanas. Assim, as espécies de mata acabam apresentando menor comprimento de raízes e mostrando

certos ajustes foliares para aproveitamento da luz escassa (Hoffmann & Franco, 2003; Habermann & Bressan, 2011; Habermann et al., 2011).

A manutenção da homeostase hídrica em espécies de savana indica a forte adaptação destas plantas à seca (sazonal). O tamponamento para mudanças rápidas no potencial da água na folha (Ψw) é evidente quando se considera o intervalo maior em espécies de savana comparado a espécies de mata (Prado et al., 2004; Hao et al., 2008). As espécies de mata, por outro lado, mostram adaptações significativas para o ("rápido") crescimento em altura para atingir o dossel, em busca da luz, sendo muitas vezes plásticas a este recurso (Scholz et al., 2008).

Assim, como as áreas de Cerrado *sensu lato*, onde ocorrem as vegetações savânicas em fisionomias típicas, como o Cerrado *sensu stricto* (*s. str.*), são entremeadas por matas de brejo, matas ripárias e de galeria, é também cientificamente importante a comparação entre espécies de mata e de savana. Tais comparações tornam-se mais ainda importantes quando se utilizam espécies congenéricas. Utilizando-se essas espécies, reduzem-se as diferenças filogenéticas normalmente presentes em indivíduos não aparentados.

Tanto para espécies savânicas como de mata, os ajustes fisiológicos podem se dar conforme a severidade da seca estacional. Há vários mecanismos fisiológicos para enfrentar a seca. Dentre eles, as mudanças no balanço hormonal endógeno parecem ser essencial (Lopez-Carbonell et al., 2009).

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CAPÍTULO I

The role of matrix effects on the quantification of abscisic acid and its metabolites in the leaves of *Bauhinia variegata* L. using liquid chromatography combined with tandem mass spectrometry

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Abstract

Phytohormone analysis using liquid chromatography combined with tandem mass spectrometry is a very important tool in studies involving plant metabolism; however, this analysis can be affected by matrix composition. Although it is necessary to understand this effect to produce reliable results, it has been widely neglected in analysis of plant hormones. Leaf extracts from *Bauhinia variegata* was obtained by solvent extraction followed by solid-phase cleanup. The extract was analyzed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in negative ionization mode using the multiple reaction monitoring mode with two or three transitions to enhance analytical quality control. Although deuterated standards were used as surrogates, pronounced matrix effects were detected for phaseic acid (PA), abscisic acid (ABA) -glycosyl ester (ABA-GE) and dihydrophaseic acid (DPA), whereas ABA, neoPA and 7′-hydroxy-ABA (7′OHABA) showed negligible matrix effects. The method was validated using spiked samples and was applied to monitoring ABA, PA, DPA, neoPA, 7´OHABA and ABA-GE on a daily basis. Analyte recoveries from spiked samples ranged from 67 to 87%. The highest leaf concentration of phytohormones was found at 14 and 17 h, which represent typical day times related to the decrease of stomatal conductance. Despite the use of deuterated phytohormones as internal standards, we showed that the use of a calibration curve constructed using a matrix extract is mandatory for reliable quantification of ABA and its metabolites, especially PA, ABA-GE and DPA. Daily variations in endogenous ABA leaf concentration were discussed.

Keywords: ABA-β-D-glucosyl ester, ABA daily variations, dihydrophaseic acid, LC-ESI-MS/MS, phaseic acid.

O papel do efeito matriz na quantificação de ácido abscísico e seus metabólitos em folhas de *Bauhinia variegata* L. usando cromatografia líquida acoplada a espectrometria de massas em tandem

Resumo

Análise de fitormônios usando cromatografia líquida acoplada a espectrometria de massas em modo tandem é uma ferramenta muito importante para estudos envolvendo metabolismo vegetal; no entanto, esta análise pode ser afetada pela composição da matriz. Embora seja necessário entender esse efeito para obtenção de dados confiáveis, tal análise tem sido negligenciada em estudos envolvendo hormônios vegetais. Extratos de folhas de *Bauhinia variegata* foram obtidos por extração com solvente seguida por extração em fase sólida. O extrato foi analisado usando cromatografia líquida em ionização '*electrospray*' com espectrometria de massas em tandem (LC-ESI-MS/MS) em modo de ionização negativa, usando monitoramento de reações múltiplas com duas ou três transições para melhorar o controle analítico. Mesmo com o uso de padrões deuterados, foram obtidos efeitos de matriz consideráveis para ácido faseico (PA), ácido abscísico (ABA) glicosilado (ABA-GE) e ácido dihidrofaseico (DPA), enquanto que ABA, neoPA e 7'hidroxi-ABA (7'OHABA) apresentaram pouco efeito de matriz. O método foi validado usando amostras enriquecidas e foi aplicado para monitorar a variação foliar diária de ABA, PA, DPA, neoPA, 7'OHABA e ABA-GE. A recuperação dos analitos foi de 67 a 87%. As maiores concentrações de fitormônios foram encontradas as 14 e 17h, horários com condutância estomática tipicamente baixos. Apesar de fitormônios deuterados serem usados como padrões internos, nós mostramos que o uso da curva de calibração construída para analisar o efeito de matriz é essencial

para quantificação confiável de ABA e seus metabolitos, especialmente PA, ABA-GE e DPA. Variações diárias nas concentrações de ABA endógeno são discutidas.

Palavras-chave: ABA glicosilado, variação diária de ABA, ácido dihidrofaseico, LC-ESI-MS/MS, ácido faseico.

INTRODUCTION

Plants exposed to daily increases in air temperature, typically peaking at midday, experience an increase in vapor pressure deficit (VPD) (Habermann and Rodrigues, 2009). Because stomatal conductance (*gs*) diminishes with increasing VPD, even well-hydrated plants of some species tend to transpire more under these conditions (Habermann et al., 2003). Plants can minimize such excessive transpiration rates (*E*) by reducing *gs*, which is regulated by abscisic acid (ABA), a plant hormone found in the apoplast of cells in the mesophyll (Assmann, 2010).

The biosynthesis of ABA occurs in chloroplasts and other plastids (Schwartz and Zeevaart, 2010). The metabolism of ABA differs among species and also with plant development stage and tissue type (Schwartz and Zeevaart, 2010). The catabolism of ABA is based on three reactions: oxidation, conjugation and reduction. The main oxidative route of natural ABA is 8′ hydroxylation, which results in the production of 8′-hydroxyABA, which isomerizes spontaneously to phaseic acid (PA), which may then be reduced to form dihydrophaseic acid (DPA), the main product, and lesser quantities of epidihydrophaseic acid (epiDPA) (Saika et al., 2007).

A second oxidative route involves oxidation of the 9-methyl group of ABA, resulting in a cyclic product called neophaseic acid (neoPA). The minor oxidative route results in the formation of (+)-7′-hydroxy-ABA (7′OHABA), whereas the minor reductive pathway produces the unstable 1′,4′-diol ABA. ABA and its metabolites can be also conjugated with glucose to form glycosylated esters corresponding to C1, ABA-β-D-glucosyl ester (ABA-GE) or glycosides at C-1'or C-4′ (Zaharia et al., 2005).

It is widely recognized that any deficiency in ABA biosynthesis can lead to plant desiccation (Schwartz and Zeevaart, 2010; Assmann, 2010). Therefore, analytical methods that are capable of determining variations in leaf ABA content with high sensitivity under varying conditions are of scientific interest. In many cases, satisfactory analytical results for ABA in plant tissues can be obtained using gas chromatography (Duffield and Netting, 2001; Birkemeyer et al., 2003), immunoassays or liquid chromatography (LC) as separation methods. These methods are always used in combination with an identification tool, typically mass spectrometry (MS). Derivatization of the compounds is a critical step that is essential for gas chromatography (GC) analyses (Pan and Wang, 2009). Because immunoassays generally display high error rates due to cross-reactivity, liquid chromatography is the preferred analytical separation method for hormonal analysis in plant tissues (Chiwocha et al., 2003; Schwartz and Zeevaart, 2010; Susawawngsup et al., 2011).

The complexity of the matrix and the small amounts of analytes present in the plant make the study of matrix effects an important step to enable the collection of reliable data (Thompson et al., 2002). When using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) for plant hormone analysis, co-extracted constituents in the matrix can interfere with the analysis and cause ionization enhancement or suppression (Fan et al., 2011). The type and magnitude of the matrix

effect may vary for different concentration ranges (Zrostlíková et al., 2001); thus, the use of surrogates (typically deuterated phytohormones) cannot effectively correct for interference in all cases. In general, specific surrogates are added to the samples at a single concentration; therefore, the nature and magnitude of the matrix effect must be determined for each compound to be quantified.

We developed an LC-ESI-MS/MS method for the rapid determination of the quantities of ABA and its metabolites present in *Bauhinia variegata* leaves sampled at different times over a 24-h period. We investigated the effects of the matrix on the analytical results to determine whether any corrective precautions were necessary to yield an accurate analysis of ABA and its metabolites. Based on the hypothesis that *Bauhinia variegata* leaves show higher levels of ABA in the afternoon compared with night and predawn periods, we evaluated variations in the concentrations of ABA and its metabolites over the course of the day.

MATERIALS AND METHODS

Plant material

To evaluate the ability of LC-ESI-MS/MS to discriminate between ABA and its metabolites (ABA-GE, DPA, PA, 7′-OHABA and neoPA) and to detect trace quantities of these compounds, we utilized leaf samples from three *Bauhinia variegata* plants harvested at 2, 5, 8, 11, 14, 17, 20 and 23 h during the dry season (August) of 2011. The plants were grown at the Universidade Estadual Paulista (UNESP) Rio Claro Campus (22°25' S, 47°33' W) in Brazil. The canopy of each plant was subdivided into quadrants (North, South, East and West), and leaves were randomly harvested from each quadrant. Following harvesting, the leaves were wrapped immediately in aluminum foil, frozen in liquid nitrogen and stored at -80°C until analysis.

Reagents and standards

Methanol and formic and acetic acids (all HPLC grade) were purchased from J.T.Baker (Xalostoc, Mexico). High-purity water was produced by a Millipore Milli-Q System (Billerica, MA, USA). Solid-phase extraction cartridges (Strata X, 200 mg, 6 mL) were purchased from Phenomenex (Torrance, CA, USA), and membrane filters (Nylon^{TM}, 0.22 µm porosity) were purchased from Sartorius (Goettingen, Germany).

Authentic ABA was purchased as a solid standard from Sigma-Aldrich (Oakville, Canada, 98.5% purity). The standards for DPA, ABA-GE, PA, 7′-OHABA and neoPA and the deuterated surrogates d_3-PA, d_4-7′-OHABA, d_5-ABA-GE, d_3-DPA, d_3-neoPA and d_6-ABA were purchased from the Plant Biotechnology Institute (Saskatoon, Saskatchewan, Canada) and were synthesized as necessary to at least 98% purity.

Extraction and purification

The method used was first proposed by Chiwocha et al 2003 and modified by Tureckova et al 2009. In our analysis, we used an adapted version of Tureckova's method. Leaves were ground using a porcelain mortar and pestle. The sample (50 mg) was then extracted using 750 µL of a methanol:water:acetic acid (10:89:1) solution for 1h at 4 °C. A solution containing each labeled standard (30 ng each of d_6-ABA, d_5-ABA-GE, d_3-DPA, d_3-PA, d_4-7′-OHABA and d_3-neoPA) was added as an internal standard and surrogate. The solvent was subsequently removed using a syringe, and the plant tissue was then extracted again for 30 min using the same conditions as in the initial extraction. Both extracts were combined [to avoid leaf particles in the extract, we filtered the solvent in a 0.45 µm x 47 mm glass microfiber filter (Sartorius AG,

Germany) membrane] and loaded onto a solid phase extraction (SPE) cartridge that had been conditioned with 2 mL methanol and equilibrated with 2 mL of a methanol:water:acetic acid (10:89:1) solution. After the sample was loaded, the cartridge was washed using 1 mL of the methanol:water:acetic acid (10:89:1) solution and eluted using 3 mL methanol:water:acetic acid (80:19:1). The eluate was dried under a gentle nitrogen flow and reconstituted using 300 µL of methanol:water (30:70) containing 0.1% formic acid, filtered through a 0.22-µm nylon membrane filter and analyzed. The extraction was performed in duplicate, and the LC-ESI-MS/MS analyses were performed in triplicate for each extract.

LC-ESI-MS/MS analysis

Abscisic acid and its metabolites were analyzed using a liquid chromatograph (Agilent 1200 series) coupled to an ESI-MS/MS system (3200 QTrap; Applied Biosystems/MDS Sciex) using a reversed-phase column (Agilent, C18, 150 mm × 4.6 × 5 µm). The mobile phase consisted of water (A) and methanol (B), both containing 0.1% formic acid in the gradient elution mode. The A:B elution began using 50% B and increased to 80% B over 7.5 min and then switched to 100% A over 2.5 min and remained at 100% A for 2.0 min. The re-equilibration time was 5.0 min. The flow rate was 500 µL min^{-1}, and the injection volume was 20 µL. To prevent contamination of the MS detector, a divert valve was used, which directed the column elution flow directly to waste before four min and after 12 min in each sample run. The instrument detection limit (the concentration that results in a signal:noise ratio above 1:3) was determined. The limit of quantification (LOQ) was defined as the lowest concentration in the linear response portion of the calibration curve (Eurachem, 1998).

Mass spectrometry operating parameters

To select suitable diagnostic precursor-to-product ion transitions, standard solutions containing 200 ng mL^{-1} of each compound in methanol:water (1:1) containing 0.1% formic acid were injected directly into a spectrometer (API 3200, Applied Biosystems/MDS Sciex) equipped with a TurboV™ ionization source operating in a negative ionization mode. All data were processed using the Analyst software (version 1.5.1). A Harvard infusion apparatus (Holliston, MA, USA) was used to optimize the MS conditions. A hybrid triple quadrupole/linear ion trap mass spectrometer with an electrospray (ESI) ion source was used.

The MS parameters were optimized by a flow injection analysis (FIA) of the solutions containing DPA, ABA-GE, PA, 7′-OH-ABA, neoPA, and ABA and their respective deuterated standards. The optimized ion source parameters were as follows: curtain gas (CUR), 10 psi; ion spray voltage (IS), -4,000 V; nebulizer gas (GS1), 45 psi; turboheater gas (GS2), 45 psi; and temperature (TEM), 450 °C. The CAD (collisionally activated dissociation) gas was set to medium pressure. In Table 1, we list the optimized MS parameters; the declustering potential (DP), the entrance potential (EP, Q_0 lens), the collision entrance potential (CEP), the collision energy (CE) and the cell exit potential (CXP) were optimized for each compound. N_2 (99.999% purity) was used as the collision gas. The transitions selected for the analysis are also described in detail in Table 1.

Method performance evaluation

Method performance assessment was conducted using spiked samples. Because all plant matrices contain these compounds, a sample extract was analyzed, and the area obtained for each compound was subtracted from the corresponding areas obtained for

the spiked samples. The spiking solution contained all of the compounds (natural and deuterated) at the same concentration.

Matrix effect: To evaluate the effect of the leaf compound matrix extracted with ABA and its metabolites, dry extracts were prepared that contained the same concentration of standard mixture as was used in the calibration curve prepared from neat reagent. The extracts were reconstituted in methanol:water (30:70) containing 0.1% formic acid. As noted by Thompson (2002), the matrix effect can influence the sensitivity of the analytical response either positively or negatively. Thus, the effect was calculated as the percentage difference between the slopes of the analytical curves that were constructed for the matrix and solvent. Using this approach, the effect can be considered important when it is greater than 10%.

The calibration curve in solvent was prepared using eight concentrations diluted in a methanol:water (30:70) solution containing 0.1% formic acid. The data were obtained by injecting 10 µL of each solution containing 1, 2.5, 5, 10, 25, 50, 100 or 250 ng mL^{-1} of the standard mixture. The response was calculated as the ratio between the natural standard peak area and the labeled standard peak area.

Recovery study: Labeled and unlabeled standards were added at four concentrations (0.3, 1.5, 15 or 75 ng) to 50 mg of ground plant tissue at the beginning of the extraction. In parallel, another portion of the plant tissue was extracted without spiking, and its final dry extract was reconstituted using a standard solution containing the compounds at same concentration as that used to spike the original samples. All procedures were performed in triplicate, and the extracts were also analyzed in triplicate. The recovery and the coefficient of variation were determined by comparing the peak areas of the analytical standard spiked before and after extraction.

Precision and accuracy: Precision was evaluated using the relative standard deviation determined from repeated extract injections and triplicate extractions. Accuracy was evaluated as the difference between the spiked analyte mass and the analyte mass obtained on analysis, i.e., the recovery.

To quantify ABA and its metabolites from the sample calibration curve, the calibration curve prepared using the matrix extract was used instead of the calibration curve prepared using the neat solvent when the calculated matrix effect was higher than 10% (Thompson et al., 2002). The method quantification limit was set as the lowest spiking concentration that yielded recoveries of between 70-120% and a CV below 20% (Brito et al., 2003).

Data analysis

A one-way analysis of variance (ANOVA) was performed to test the significance of the variations in the leaf contents of each ABA metabolite over the course of the day (at 2, 5, 8, 11, 14, 17, 20 and 23 h). For each ABA metabolite, Dunn's method ($\alpha = 0.05$) was used to perform post-hoc mean result comparisons.

RESULTS

The optimized chromatographic conditions avoided co-elution of the analytes, especially PA, neoPA and 7′OHABA, which have similar structures (Figure 1). A comparison between their retention times showed that the analytes were clearly differentiated in the chromatogram. However, in view of the similarities of the compounds, another form of identification, such as mass spectrometry, was necessary (Pan and Wang, 2009). Depending on the analyte, the method described here uses two

or three multiple reaction monitoring (MRM) transitions, which adds additional confirmatory information to our results (Table 1).

In this study, we established the ratios among transitions as the key to the reliable identification of these phytohormones. We determined these ratios using surrogate compounds in standard solutions (for all analytical curves) and using spiked samples. For the confirmation of analytes in plant samples, the chromatographic retention time of the sample plant hormones was not allowed vary by more than 2%, and the relative abundance of the monitored MRM transitions was required to be within ± 25% of both the calibration standards and the spiked samples (European community, 2002).

As recommended by EURACHEM (Eurachem, 1998), our quantification data are within the recovery limits for the given concentration zone (Table 2). An additional recovery study of a cluster spiked with 75 ng of a standard mixture, which yielded a coefficient of variation (CV) of approximately 40% (data not shown), indicating that this concentration zone was not validated.

The most significant matrix effects were observed for ABA-GE, DPA and PA, with matrix effects of 63, 29 and 27%, respectively (Table 3). On inspection of these relationships, if a solvent curve had instead been used for quantification in these cases, the results would have been grossly inaccurate.

Due to the presence of natural ABA and its metabolites in plant tissues, the detection limit cannot be used as a parameter to validate this method (Eurachem, 1998). Thus, we obtained limits of quantification of 4 ng g^{-1} for ABA, neoPA and 7′-OHABA and 20 ng g^{-1} for PA, DPA and ABA-GE.

As anticipated, the concentration of ABA in *B. variegata* leaves varied with the time of day. The highest leaf concentrations were found at 1400h and 1700h,

intermediate concentrations were observed at 800h, 2000h, 2300h and 200h, and the lowest concentrations were observed at 500h and 1100h (Figure 3A). There were high leaf concentrations of PA at 14 and 17 h (Figures 3C) and the concentrations of neoPA, 7′-OHABA, ABA-GE and DPA showed no significant variations with the time of day (Figures 3B, 3D, 3E and 3F).

DISCUSSION

Chromatography and mass spectrometry

One should consider that co-elution of substances with similar fragmentation profiles can result in false positives. Tandem analysis with mass spectrometry is widely used to identify ABA and its metabolites, with one (Turecková et al., 2009; Zhou et al., 2003; Ross et al., 2004; Lopez-Carbonell et al., 2009) or at most two transitions (Gómez-Cadenas et al., 2002; Vilaró et al., 2006) for each compound. However, even with different retention times, the use of only one MRM transition for each analyte may also be problematic.

Phaseic acid and neo-PA, under our optimized chromatographic conditions, showed good peak resolution but it has demonstrated to have MRM transitions with identical fragmentation patterns: PA, 1st transition 279 > 139; neo-PA, 3rd transition 279 > 139; PA, 2nd transition 279 > 205; neo-PA, 1st transition 279 > 205 (Figure 2). Thus, in this case, it is necessary to use at least two transitions along with retention time for reliable identification of these analytes.

When determining analytes (pollutants) in environmental water, it is recommended that one uses retention time plus at least two transitions, with a suitable ion-intensity ratio between them, for robust compound identification; in addition, the use of as many specific transitions as possible may help avoid false positives due to

common losses (e.g., H_2O or CO_2) (Pozo et al., 2006). Therefore, the same can be applied to plant tissues, because the achievement of reliable identification of compounds at low concentrations may be much more complex in plant matrices.

During the recovery study using spiked samples, we observed peaks that co-eluted with the phytohormones, chiefly with PA at *m/z* 279.0, neo-PA at *m/z* 279.0 and 7′-OH-ABA at *m/z* 279.0. These peaks may have been other plant constituents with similar structures. One of the most abundant chemical classes in plant tissues is the phenolics. Phenolic compounds show nonspecific losses of CO_2, CO and H_2O that are widely used for their identification in plant extracts (Ma et al., 1997; Vessecchi et al., 2011). However, using only these losses could lead to mistaken compound identifications.

Matrix effects

Given that co-eluting compounds can interfere with the ionization process, which is essential for quantification, the matrix effect is directly affected by the composition of the tissue being analyzed. Therefore, it is essential to perform a similar study using additional tissues from the same species or tissue from a different species.

In contrast to our results, Fan et al. (2011) reported no matrix effects on the analysis of several phytohormones (including ABA) in canola. Their results are in accord with our findings because ABA showed a 6.6% matrix effect (Table 3), allowing for its quantification by the solvent curve method (i.e., a matrix effect < 10%). This lack of a matrix effect may underlie the common practice of quantifying ABA without analyzing other compounds from the ABA metabolic route that are of similar importance for understanding water stress.

Considering quantification limits, our results are consistent with previously published values (Zhou et al., 2003; Turecková et al., 2009; Lopez-Carbonell et al., 2009). However, in several reports, the authors did not confirm whether the reported limit of quantification referred to the analytical system or to the entire method.
The matrix effect in most cases depends on the amount of sample used, because in more concentrated extracts co-extracted compounds will be, consequently, more concentrated, and increased effects on ionization processes will be also expected, generating unreliable results. On the other hand, in the present study the relationship between the amount of sample used and consequences for matrix effects was not the main objective.

Hormone quantification and physiological implications

Abscisic acid leaf content reported over the course of the day are rare for native plants, and for crop species ABA leaf contents are described following water deficient treatments, instead of over the course of the day. On the other hand, our findings fit the physiologically expected pattern of variation in the concentration of this plant hormone over the course of the day. As established in other studies, the stomatal conductance (*gs*) of leaves of many species diminishes when exposed to a high vapor pressure deficit, or VPD (Khairi and Hall, 1976; Levy, 1980; Sinclair and Allen, 1982; Grantz, 1990; Assmann, 2010). Citrus plants respond to elevated VPDs by lowering both transpiration rate and *gs* (Habermann et al., 2003). In this study, we did not measure *gs*, but the VPD was highest at midday and early afternoon and decreased in the evening in correlation with air temperature (data not shown). However, in maple plants has been suggested that ABA leaf content is linearly related to *gs* (Bauerle et al., 2006).

The leaf content of PA exhibited a daytime pattern similar to that of ABA (Figure 3C). Although both exert physiological effects, PA is considered to be the first compound to be degraded in a biochemical pathway that ends with the biosynthesis of the inactive DPA (Schwartz and Zeevaart, 2010). The inactive compounds (DPA, neoPA, 7′-OHABA and ABA-GE) measured in *B. variegata* leaves appeared to be maintained in high concentrations throughout the day (Figures 3B, 3D, 3E-F).

Because we did not measure these compounds in root samples or in xylem exudates, we were unable to determine whether the root system contributed to the changes in concentration of these compounds. However, ABA leaf concentration measured in maple plants was described as a better indicator of *gs* responses to soil water deficit, in comparison with xylem collected ABA (Bauerle et al., 2006). Although we were unable to determine the specific mechanisms underlying the fluctuations in the level of each measured compound, we performed an accurate quantitative analysis of the compounds in the abscisic acid biosynthetic pathway. Moreover, the endogenous concentrations of ABA and PA fit the physiologically expected daytime variations accurately.

In the prior literature, discussion of the matrix effect on phytohormone quantification has focused on ABA or other plant hormones exclusively (Fan et al., 2011). In our study, we included all the ABA metabolites. Plant tissue is a very complex matrix, and studies of matrix effects are critical for hormone quantification. ABA does not present a significant matrix effect; however, if these analyses were not performed, we would have obtained PA values smaller than the actual values. For ABA-GE and DPA, the concentrations obtained without accounting for the matrix effect would have been greater than the actual values. To our knowledge, no other study has analyzed daytime variation in ABA and its metabolites. We selected *Bauhinia variegata* as a

subject for the profiling of ABA hormones. Although used only as a confirmation of well known ABA daily leaf concentrations, since our investigation was mainly devoted to the ABA methodological concepts, including the matrix effects, our results corroborate the fact that the highest ABA and PA leaf concentrations were found at 1400h and 1700h, when VPD typically increases and stomatal conductance normally decreases, which is in accordance with classical ecophysiological responses in plants.

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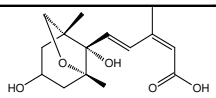
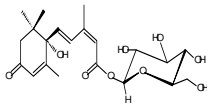
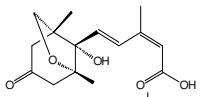
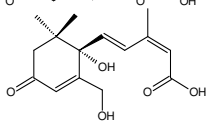
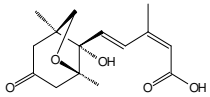
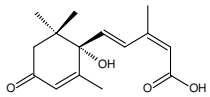
Tables:

Table 1. Specific ion ratios or area ratios collected by MS/MS for accurate confirmation.

Retention Time (min)	Analyte	Structures	MRM transitions		Calculated Ion Ratio* MRM1/MRM2 MRM1/MRM3
4,65	DPA		MRM1	281 > 237	1.35
			MRM2	281 > 171	
4,93	ABAGE		MRM1	425 > 261	1.02
			MRM2	425 > 148	
5,60	PA		MRM1	279 > 139	4.44
			MRM2	279 > 205	
			MRM3	279 > 168	4.35
7,08	7-OH-ABA		MRM1	279 > 151	1.50
			MRM2	279 > 217	
			MRM3	279 > 205	3.60
7,51	neo-PA		MRM1	279 > 205	1.55
			MRM2	279 > 122	
			MRM3	279 > 139	3.49
8,61	ABA 1		MRM1	263 > 153	2.30
			MRM2	263 > 219	

*n = 3 (spiked samples); Coefficient of variation ranged from 3 to 12%

Table 2. Recovery percentage and precision data obtained, using spiked samples in the recovery study.

Compound	Spiked samples[a]					
	----------6 ng g^{-1}----------		----------30 ng g^{-1}----------		----------300 ng g^{-1}----------	
	Recovery	CV	Recovery	CV	Recovery	CV
	----------%----------					
DPA	76	20	72	36	73	15
ABA-GE	68	31	74	13	75	21
PA	84	11	72	11	79	11
7′OH-ABA	75	18	67	12	75	18
neoPA	79	8	70	8	79	10
ABA	87	6	68	5	73	7

The method used for the recovery study is described in the text (Method Performance Evaluation Section, n=9).

[a] ng each compound per g plant tissue (dry mass)

Table 3. Matrix effect results showing curve equations in the solvent and in the matrix extracts, differences between angular coeficients and curve intervals

	Solvent		Matrix extract		Difference in angular coefficients (%)	Concentration range (ng mL^{-1})
	R^2	Curve equation	R^2	Curve equation		
DPA	0.9988	y = 1.6925x + 0.0394	0.9794	y = 1.1959x + 0.2083	29	5 - 250
PA	0.9474	y = 0.882x + 0.1074	0.9739	y = 1.1231x + 0.048	27	5 - 250
ABA-GE	0.9985	y = 1.2888x + 0.0135	0.8789	y = 0.4779x + 0.4151	63	5 - 250
neoPA	0.9981	y = 2.9893x + 0.0576	0.9968	y = 2.7x + 0.0598	9.7	1 - 250
7′OHABA	0.9992	y = 5.5002x + 0.0589	0.9993	y = 5.7308x – 0.1091	4.2	1 - 250
ABA	0.9989	y = 0.1484x + 0.0022	0.9998	y = 0.1386x + 0.0007	6.6	1 - 250

Figure Captions:

Figure 1.

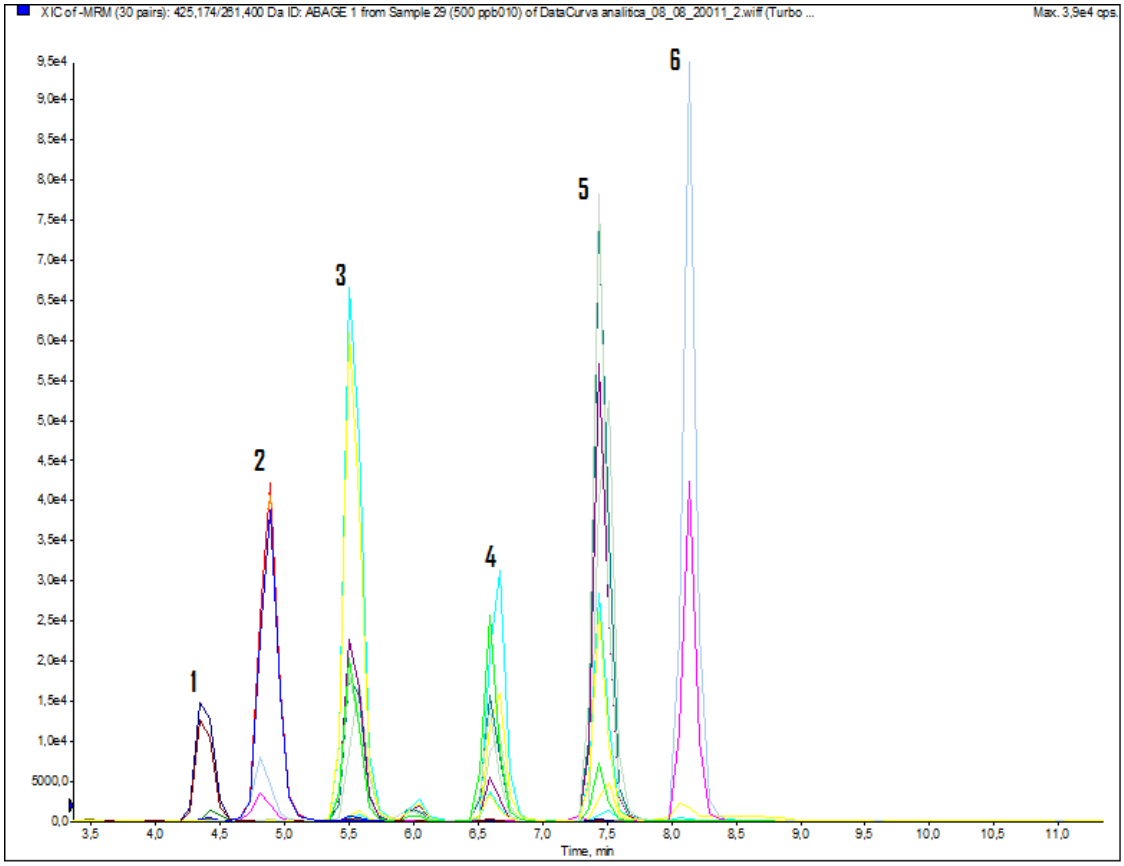
LC-ESI-MS/MS chromatogram for a standard solution of each compound (500 ng mL^{-1}). The different lines represent the transitions monitored for each substance. Peaks in elution order: 1, DPA; 2, ABA-GE; 3,PA; 4, 7´OH ABA; 5, neoPA; 6, ABA.

Figure 2.

Chromatogram of a sample - PA (t_r= 5.60 min) and neoPA (t_r= 7.51 min) have identical fragmentation patterns at 279 > 139 and at 279 > 205, which can lead to identification errors.

Figure 3.

Bauhinia variegata leaf contents of ABA (A), DPA (B), PA (C), neoPA (D), ABA-GE (E) and 7′-OHABA (F) over the course of a day. Similar lowercase letters or absence of letters indicates no significant differences ($p < 0.05$) among day times. Bars = SD


XIC of -MRM (30 pairs): 425,174/261,400 Da ID: ABAGE 1 from Sample 29 (500 ppb010) of DataCurva analitica_08_08_20011_2.wiff (Turbo ...
Max. 3,9e4 cps.
9,5e4
9,0e4
8,5e4
8,0e4
7,5e4
7,0e4
6,5e4
6,0e4
5,5e4
5,0e4
4,5e4
4,0e4
3,5e4
3,0e4
2,5e4
2,0e4
1,5e4
1,0e4
5000,0
0,0
1
2
3
4
5
6
3,5
4,0
4,5
5,0
5,5
6,0
6,5
7,0
7,5
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8,5
9,0
9,5
10,0
10,5
11,0
Time, min

Figure 1

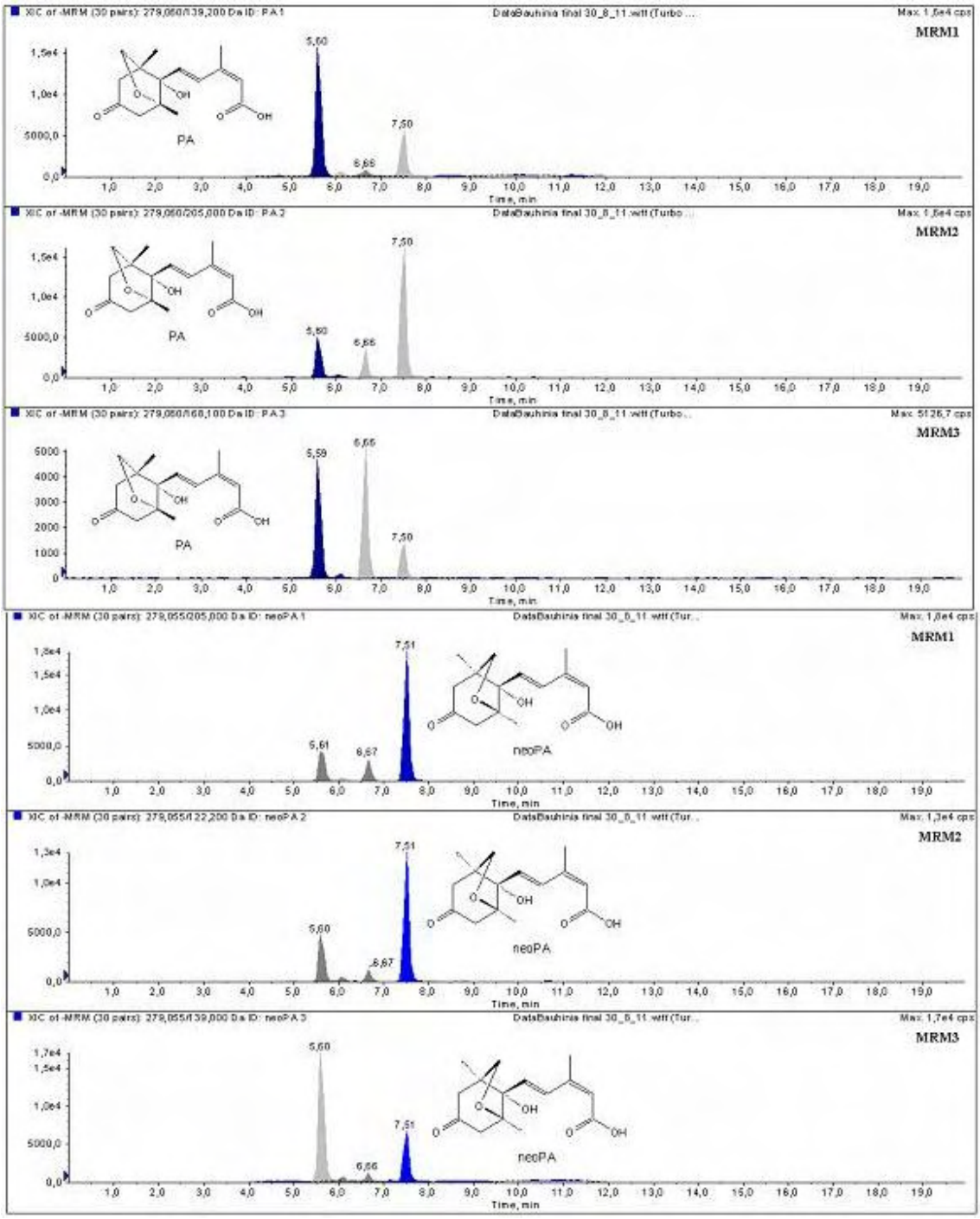
MRM1
PA
5,60
7,50
6,66
MRM2
PA
7,50
5,60
6,66
MRM3
PA
5,59
6,65
7,50
MRM1
neoPA
7,51
6,61
6,67
MRM2
neoPA
7,51
5,60
6,67
MRM3
neoPA
5,60
7,51
6,66
Time, min

Figure 2

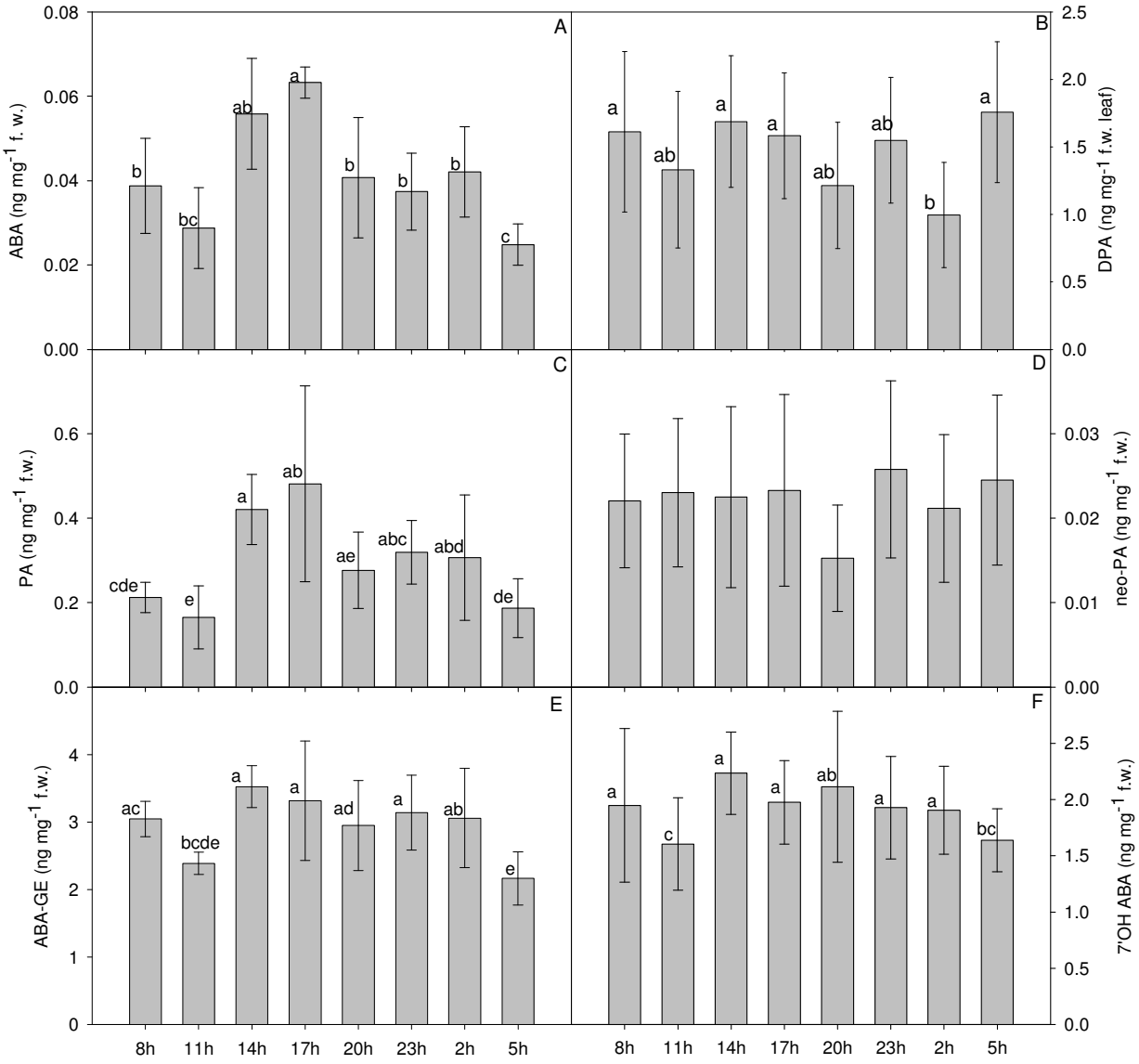
A
B
C
D
E
F
ABA (ng mg-1 f. w.)
DPA (ng mg-1 f.w. leaf)
PA (ng mg-1 f.w.)
neo-PA (ng mg-1 f.w.)
ABA-GE (ng mg-1 f.w.)
7'OH ABA (ng mg-1 f.w.)
8h
11h
14h
17h
20h
23h
2h
5h

Figure 3

CAPÍTULO II

Occurrences of congeneric species in the Cerrado areas in Brazil explained by abscisic acid metabolism in the wet and dry seasons

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Abstract

Abscisic acid (ABA) serves as an endogenous messenger in response to soil drought, attenuating stomatal conductance (*gs*) and transpiration rates (*E*). In the Brazilian savanna (Cerrado) there are savanna-type physiognomies, such as the Cerrado *sensu stricto* (*s. str.*), but within Cerrado areas, riparian forests with shallow water table and high soil water availability may occur. In such habitats, plants may respond differently to seasonal droughts. We assessed endogenous leaf concentrations of ABA and its metabolites, as well as *gs* and *E* in congeneric adult plants naturally occurring in a Cerrado *s. str.* and a riparian forest, in the wet (February) and dry (September) seasons. We also quantified the gene expression of the 9-cis epoxycarotenoid dioxygenase (NCED), a key enzyme to ABA biosynthesis. Even being very adapted to riparian forests, *Styrax pohlii* trees expressed the NCED gene in leaves and synthesized ABA, reducing *gs* in the dry season. This was contrary to our prediction, because this species experiences high soil water availability throughout the year. On the other hand, although water use efficiency (*A*/*E*) was lower in the dry as compared to the wet season, *E* and intrinsic water use efficiency (*A*/*gs*) were similar between seasons for *S. pohlii*. *S. ferrugineus*, a savanna species, showed NCED expression similar to *S. pohlii* during the dry season. Nevertheless, in *S. ferrugineus* the ABA-β-D-glucosyl ester (ABA-GE) seems to provide a pool of active ABA, which reduces *gs* and *E*, enabling this species to endure long and seasonal droughts, typical of the Cerrado *s. str*.

Key words: ABA, ABA-GE, Brazilian savanna, gas exchange, NCED, Styracaceae

Introduction

Abscisic acid (ABA) is a plant hormone involved in drought tolerance, acting mainly in the attenuation of stomatal conductance (*gs*) and transpiration rates, *E* (Chaves et al., 2002), with cascade reactions in the metabolism (Pinheiro and Chaves, 2011). Therefore, plants respond to drought by modulating endogenous ABA concentrations, which are attained by gene expression, increasing the concentration of ABA in the metabolism (Bray, 2002).

ABA biosynthesis occurs in chloroplasts and other plastids (Schwartz and Zeevaart, 2010), where carotenoid cleavage (between double bond 11 and 12) is facilitated by the 9-cis epoxycarotenoid oxygenase (NCED) enzyme, a rate-limiting step in this biosynthetic pathway (Tan et al, 2003).

Endogenous concentration of ABA and its metabolites differ among plant species and also depend on phenophases and plant tissues (Schwartz and Zeevaart, 2010). ABA catabolism is comprised of oxidation, conjugation and reduction. The main oxidative route of ABA is the 8′ hydroxylation, which produces the 8′-hydroxyABA, which spontaneously isomerizes to phaseic acid (PA), which finally may be reduced to dihydrophaseic acid (DPA) (Okamoto et al, 2009). A second oxidative route starts with the oxidation of the 9-methyl group of ABA, resulting in a cyclic compound, the neophaseic acid (neoPA). A minor existing oxidative route ends up with the (+)-7′-hydroxy-ABA (7′OHABA), whereas a minor reductive pathway produces the unstable 1′,4′-diol ABA. ABA and its metabolites may be also conjugated with glucose to form glycosylated esters corresponding to C1, ABA-β-D-glucosyl ester (ABA-GE) or glycosides at C-1’ or C-4′ (Zaharia et al., 2005).

To cope with the lengthy (up to five months) seasonal droughts that are typical of the Cerrado vegetation in Brazil, plants adjust *gs* (and *E*), as well as the leaf water potential, Ψw (Prado et al., 2004). Furthermore, drought tolerance of Cerrado woody plants can be achieved by leaf deciduousness and root deepness (Franco, 1998; Franco et al., 2005; Habermann and Bressan, 2011).

In the Cerrado vegetation, plants from savanna environments (the typical Cerrado *sensu stricto – s. str.*) present morphological adaptations, such as long and deep roots and low specific leaf area (Habermann and Bressan, 2011), whereas plants from humid forest environments, such as riparian and gallery forests, face low sunlight availability as the limiting factor. Plants from forests environments rely on high specific leaf area to improve light capture in order to grow and reach the forest canopy (Habermann and Bressan, 2011). On the other hand, stomatal adjustments as a result of ABA biosynthesis in response to soil drought is a universal response among plants (Wilkinson and Davies, 2010; Pinheiro and Chaves, 2011). Therefore, for both forest and savanna species physiological adjustments must occur according to the severity of seasonal droughts in each of these environments.

To better understand physiological seasonal adjustments, we assessed endogenous leaf concentrations of ABA and its metabolites as well as leaf gas exchange and the NCED gene expression in congeneric species naturally occurring in contrasting environments of the Cerrado. We used adult individuals of *Styrax ferrugineus* from a Cerrado *s. str.* fragment and of *S. pohlii* from a riparian forest remnant, measured in the rainy and dry seasons. We hypothesized that *S. pohlii*, typical of riparian forest, does not rely on great amounts of ABA during the dry season as compared to the wet season, and that it maintains high *gs* values in the dry season, as the high soil water availability

in the riparian forest would not require extensive drought-protective responses, as supposedly expected for *S. ferrugineus*, which is a savanna species.

Material and Methods

Site description and plant material

Five adult plants (replications) of *Styrax pohlii* A. DC. from a riparian forest remnant (32 ha; 24° 00' S, 47°32' W; 660 m of altitude), and of *S. ferrugineus* Ness & Mart. from a Cerrado *s. str.* fragment (260 ha; 22º13' S, 47º53' W; 730 m of altitude) were assessed in the wet (February) and dry (August) seasons 2011. These fragments were located in the respective municipalities of Ajapi and Itirapina, southern São Paulo state, Brazil.

The riparian forest remnant has a shallow water table, with high water availability (Kissmann et al., 2012) in its organic soil (Habermann and Bressan, 2011), which supports a closed-canopy forest comprised of trees with up to 20 m in height. The Cerrado *s. str.* fragment shows trees and shrubs scattered on sandy soil, which is covered with an herbaceous understory. In both locations, which are separated by 50 km, it was observed 247.2 mm of accumulated rainfall within the period of 30 days prior to measurements in the wet season (23.8 ± 1.1°C). In the dry season (19.8 ± 1.2°C), no rainfall was noted within the period of 30 days before evaluations.

The canopy of each plant was subdivided into quadrants (north, south, east and west), from which leaves were randomly harvested (for chemical and genetic expression analysis, and for leaf water potential) and measured (gas exchange). Since young trees of *S. pohlii* show thin stems, these trees were bent towards the ground so that their leaves could be assessed.

ABA analysis

After harvesting, the leaves were wrapped in aluminum foil, and immediately frozen in liquid nitrogen for subsequent storage at -80°C in an ultra-freezer.

To measure the concentrations of ABA, ABA-GE, PA, DPA, neoPA and 7'OHABA we used the method proposed by Silva et al. (2012). Leaf samples were ground, using a pestle, inside a porcelain mortar containing cold (4°C) solution of methanol:water:acetic acid (10:89:1) with 30 ng of each labelled standard of d_6-ABA, d_5-ABA-GE, d_3-DPA, d_3-PA, d_4-7′-OHABA and d_3-neoPA as surrogates. This organic solvent was subsequently loaded onto a solid phase extraction (SPE) and the cartridge was eluted using methanol:water:acetic acid (80:19:1). The eluate was dried and reconstituted using methanol:water (30:70) containing 0.1% formic acid. The extraction was performed in duplicate, and chromatographic analyses were performed in triplicate for each extract.

Abscisic acid and its metabolites were analyzed using a liquid chromatograph (Agilent 1200 series) coupled to an electrospray ionization-tandem mass spectrometer (ESI-MS/MS) system (3200 QTrap; Applied Biosystems/MDS Sciex) using a reversed-phase column (Agilent, C18, 150 mm × 4.6 × 5 µm). The mobile phase consisted of methanol (A) and water (B), both containing 0.1% formic acid in the gradient elution mode. The A:B elution was performed using 50% B and increased to 80% B over 7.5 min and then switched to 100% A over 2.5 min and remained at 100% A for 2.0 min. The re-equilibration time was 5.0 min. The flow rate was 500 µL min^{-1}, and the injection volume was 20 µL.

Gene expression analysis

Assays were performed using leaf samples collected in the dry season. Degenerate primers were designed according to conserved regions of mRNA sequences of the 9-cis epoxycarotenoid oxygenase (NCED) enzyme, as well as actin and elongation factor genes in other plant species present in the GenBank (actin: forward - ATGTAYGTTGCYATHCAGGC, reverse - AYCTGYTGRAAKGTGCTKAGG; elongation factor: forward - ATCTACAAGYTKGGWGGWAT, reverse - GGDAMCKRAGDGGYTTGTC; and NCED: forward - GGCGAGCTHCACGGCMA, and reverse - GCGTTCCAGAGGTRGA). Total DNA was extracted from leaf samples using DNeasy plant mini kit (Qiagen, Hilden, Germany) and amplified using the designed degenerate primers. The PCR products were sequenced and new internal primers were designed for qRT-PCR analysis of the *Styrax* genes. The internal primers were for actin: forward - AGCTGGAGACTGCAAAGAGC and reverse - TTCCATTCCAATCAATGACG, for elongation factor: forward - GCAACCACGCCAAAGTATTC and reverse - TGTTGTCACCCTCAAAACCA, and for NCED: forward - GAGCAACTCCACTCCACCA and reverse - GGTAAGGCTTTTGGATGACG.

Serial dilutions of genomic DNA from both species were amplified using the internal primers in order to access the amplification efficiency during qPCR analysis. The reagent used in these tests was LightCycler 480 SYBR Green I Master (Roche, Vilvoorde, Belgium) with the following procedural specifications: 95°C for 5 min (1 cycle), 95°C for 10 s, 58°C for 5 s, 72°C for 30 s (40 cycles) followed by a melting curve analysis (1% slope temperature; 60-95°C). Amplification efficiencies, respectively for actin, elongation factor and NCED were 112.1%, 116.4% and 109.6% for *S. ferrugineus* and 99.7%, 98.6% and 99.7% for *S. pohlii.*

For the quantitative analysis of NCED expression, total RNA was extracted from leaf samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Total RNA (2 µg) was treated with RNase-free TURBO DNase (Ambion, Carlsbad, USA) and reverse transcribed to cDNA using an oligo-dT primer and Super Script III according to the manufacturer's protocol (Life Technologies, Carlsbad, USA). The cDNAs were submitted to NCED gene expression analysis using actin and the elongation factor genes to normalize the cycle threshold (Ct) values. The reagent and cycles used for the qRT-PCR analysis were the same used for the amplification efficiency tests. In this protocol, every reaction was performed in triplicate.

Gas exchange rates

The CO_2 assimilation (*A*) and transpiration rates (*E*), and the stomatal conductance (*gs*) were measured with an open gas portable gas exchange system (LI-6400, Lincoln, NE, USA). The water use efficiency (*A*/*E*, WUE) and intrinsic water use efficiency (*A*/*gs*, IWUE) were also calculated. Leaves of adult plants were assessed between 9:00h and 10:30h (Feistler and Habermann, 2012), when *gs* values best reflect the leaf water status, potentially enabling gas exchange (Medrano et al., 2002).

The air temperature and vapor pressure deficit (VPD) within the leaf chamber (standard 2 x 3 cm, LI-COR) were allowed to vary with the external environment. Nevertheless, the Cerrado *s. str.* is an open physiognomy, in which the discontinuous canopy allows heavy irradiation load on the ground, whereas in the riparian forest, the evaporative demand is naturally lower than the Cerrado *s. str.* (see Habermann et al., 2011; Kissmann et al., 2012). Therefore, in each season leaves of both species underwent comparable VPD and RHs% during measurements, which were registered only under stable conditions (CV% ≈ 1%).

The photosynthetic photon flux density (PPFD) was provided by an artificial red (90%) and blue (10%) LED light source (6400-02B, LI-COR), and it was set to 1500 µmol photons m^{-2} s^{-1}, which was similar to the PPFD that canopies of adult plants of both species were exposed to on typical days of the wet and dry seasons.

Leaf water potential

The leaf water potential (Ψw) at predawn (Ψ_{pd}) and midday (Ψ_{md}) was measured by the pressure chamber method (Turner, 1981), using a DIK-7000 (Daiki Rika Kogyo, Tokyo, Japan) chamber.

Data analysis

ABA's and its metabolites' endogenous concentrations, gas exchange parameters (*A*, *gs*, *E*, WUE and IWUE), as well as Ψ_{pd} and Ψ_{md} and also the quantitative genetic expression were subjected to a one-way analysis of variance (Anova). The Mann Whitney test ($\propto = 0.05$) was used to perform post-hoc comparisons between data obtained in the wet and dry season for each of the two species. However, for NCED quantitative genetic expression, comparisons were performed between the two species.

Results

Abscisic acid (Fig. 1a) and PA (Fig. 1c) endogenous leaf concentrations were higher in the dry as compared to the wet season in both *S. ferrugineus* and *S. pohlii*. 7'OHABA, neoPA and DPA showed the opposite response pattern, in which values obtained in the dry season were lower than those obtained in the wet season (Figs. 1d, e, f). Although ABA-GE leaf concentrations significantly increased in both species in the

dry as compared to the wet season, in *S. ferrugineus* it increased by 75%, while in *S. pohlii* it increased by 30% (from 0.60 ± 0.02 to 0.91 ± 0.12) (Fig. 1b).

Gas exchange rates significantly decreased in the dry as compared to the wet season for both species (Figs. 2a, b), but transpiration rates (*E*) of *S. pohlii* remained the same between seasons (Fig. 2c). As the dry-season reduction of *A* and *gs* values for *S. ferrugineus* was lower when compared to such reductions in *S. pohlii* (Figs. 2a, b), *S. ferrugineus* maintained similar WUE between seasons, while *S. pohlii* showed lower WUE in the dry as compared to the wet season (Fig. 2d). Although higher in the dry as compared to the wet season for both species, the IWUE exhibited insignificant increases (Fig. 2e).

Both predawn and midday leaf water potentials were significantly lower in the dry as compared to the wet season (Fig. 3). However, in *S. ferrugineus* Ψ_{pd} was twice as low in the dry as compared to the wet season, whereas in *S. pohlii* such reduction was almost seven times greater (Fig. 3a).

The NCED gene expression measured in the dry season revealed no difference between *S. ferrugineus* and *S. pohlii* (Fig. 4). NCED expression levels were higher than those observed for the internal controls actin and elongation factor.

Discussion

The dry season caused significant changes in endogenous leaf concentrations of most ABA metabolites (Fig. 1). Abscisic acid leaf concentration increased by 42% in *S. ferrugineus* and by 33% in *S. pohlii* in the dry as compared to the wet season (Fig 1a), but these unequal increases did not represent differences ($P > 0.05$) when comparing ABA leaf concentrations between both species measured in the dry season. NCED gene expression was also similar between these species. One could interpret these results as

revealing some kind of (positive and direct) relationship between NCED gene expression and ABA concentrations in these species. However, these parameters were assessed only in the leaves. Therefore, our results did not demonstrate how much of the ABA found in the leaves came from roots. In root-to-shoot signaling, soil water deficit induces ABA biosynthesis in the root before leaves may reduce the water potential, and this ABA is transported by the xylem sap (Yang and Guo, 2007; Hu et al, 2012).

Although Ψ_{pd} and Ψ_{md} of both species significantly reduced in the dry season, in *S. pohlii* Ψ_{pd} strongly decreased (Fig. 3a). As Ψ_{pd} reflects the nocturnal plant rehydration capacity of Cerrado woody species (Franco, 1998; Habermann et al., 2011), then, it can be described that *S. pohlii* faced greater stress in the root system caused by the soil water deficiency, in relation to *S. ferrugineus* shrubs from the Cerrado *s. str.* Thus, even inhabiting an environment with 20 times more water in the soil than the Cerrado *s. str.* soil (Habermann et al., 2011; Kissmann et al., 2012), *S. pohlii* trees seems to be more sensitive to soil water deficits. One should also consider that *S. pohlii* trees from the riparian forest are taller and visually leafy than *S. ferrugineus* shrubs from the Cerrado *s. str.* In addition, *S. pohlii* exhibits greater specific leaf area (SLA) and shorter root length as compared to *S. ferrugineus* (Habermann and Bressan, 2011).

We could not confirm our hypothesis that *S. pohlii* keeps high *gs* values in the dry season. Both species had reduced *gs* (Fig. 2b) and *A* (Fig. 2a) values in the dry as compared to the wet season (Fig. 2b). However, *E* values of *S. pohlii* were maintained between seasons (Fig. 2c), which resulted in lower WUE of this species in the dry, as compared to the wet season (Fig. 2d). As VPD varies between these studied sites (Habermann et al., 2011; Kissmann et al., 2012), one could suspect that VPD or RHs% within the leaf chamber were not comparable when measuring both *S. ferrugineus'* and *S. pohlii's* leaves in their respective habitats during the wet season. But VPD and RHs%

were 1.83 ± 0.12 kPa and 59.9 ± 1.7% in the Cerrado *s. str.*, and 1.1 ± 0.11 kPa and 65.2 ± 0.65% in the riparian forest. Moreover, in a previous study performed in the wet season (Habermann et al., 2011), *S. pohlii* adult plants also showed lower *E* values as compared to *S. ferrugineus* shrubs from the Cerrado *s. str.*

Neither could we confirm the hypothesis that *S. pohlii* does not rely on great amounts of ABA during the dry as compared to the wet season. Both species increased ABA leaf concentrations (Fig. 1a) in the dry season. On the other hand, ABA-GE concentration in *S. ferrugineus* leaves conspicuously increased in such a manner that was not accompanied by *S. pohlii* (Fig. 1b). The glycosilade form of ABA (ABA-GE) is considered to be an important long-distance signaling (Sauter et al, 2002; Davies et al, 2005). It is stored in vacuoles when soil water deficiencies are imposed, and the enzyme β-glucosidase can release free ABA after physiological stimuli (Lee et al, 2006; Han et al, 2012). The concomitant increase in ABA-GE and ABA leaf concentrations was also observed by Thameur et al. (2011). López-Carbonell et al. (2009) demonstrated that in *Cistus albidus* the ABA-GE concentration peak occurs 30 days before the ABA peak. This suggests that the increase in ABA observed in both *Styrax* species, but mainly in *S. ferrugineus* (Fig. 1a), may have partially come from the breakdown of ABA-GE. Therefore, the conspicuous increase of ABA-GE in leaves of *S. ferrugineus* in the dry season provides a potential ABA pool, which can be used during the five-month seasonal drought (May - September), typical of Cerrado areas.

There are a considerable number of papers on ABA quantification, measured in leaves of different species, but rarely studies analyze other metabolites of this route. Comparing the dynamic of leaf concentrations of every ABA metabolite, one may note that, although occurring in Cerrado physiognomies with different water resources, *S. ferrugineus* and *S. pohlii* showed the same response patterns for almost every compound

analyzed between seasons (Fig. 1). Plant hormones are always in equilibrium between biosynthesis and catabolism, and under water deficit conditions this equilibrium tends to be displaced by ABA biosynthesis, but somehow other reactions occur to form ABA inactive compounds, and PA is the first metabolite to form from the main inactivation pathway, the 8' hydroxylation (Okamoto et al., 2009). Our data seemed to confirm this metabolic response, as PA leaf concentrations increased in both species in the dry season (Fig. 1c). In some cases this up-regulation of ABA over PA is so intense through the 8'hydroxylation that PA concentration exceeds the ABA's (Qin and Zeevaart, 2002).

Using the NCED degenerate primers we obtained a single amplification product for each *Styrax* species, but we did not obtain the complete sequence of these genes, therefore, we were not able to accurately describe which of the NCEDs genes in *Arabidopsis* our sequence corresponds to (Tan et al., 2003). The higher expression levels observed for NCED, when compared to the constitutively expressed internal controls actin and elongation factor, suggests that the NCED isoform obtained in this study is relevant for the control of ABA levels.

In conclusion, we demonstrated that even being very adapted to riparian forests, where water in the soil is largely available (Habermann et al., 2011; Kissmann et al., 2012), *S. pohlii* trees do express the NCED gene in leaves to synthesize ABA. This reduced *gs* but not *E* values in the dry season. Such NCED expression observed in *S. pohlii* was similarly performed in *S. ferrugineus*. However, in *S. ferrugineus* ABA-GE seems to provide a pool of active ABA, which reduces *gs* and *E*, enabling this species to endure long and seasonal droughts, typical of Cerrado areas.

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FIGURE CAPTIONS:

Fig. 1 Abscisic acid (a), ABA-GE (b), PA (c), 7'OHABA (d), neoPA (e) and DPA (f) in leaves of *Styrax ferrugineus* and *S. pohlii* measured in the wet (February) and dry (September) seasons. Asterisks indicate significant differences ($P < 0.05$) between seasons. Columns represent mean values (n = 5 plants) and bars are S.D.

Fig. 2 CO_2 assimilation (a), stomatal conductance (b), transpiration rates (c), water use efficiency (d) and intrinsic water use efficiency (e) in leaves of *Styrax ferrugineus* and *S. pohlii* measured in the wet (February) and dry (September) seasons. Asterisks indicate significant differences ($P < 0.05$) between seasons. Columns represent mean values (n = 5 plants) and bars are S.D.

Fig. 3 Predawn (Ψ_{pd}; a) and midday (Ψ_{md}; b) leaf water potentials in leaves of *Styrax ferrugineus* and *S. pohlii* measured in the wet (February) and dry (September) seasons. Asterisks indicate significant differences ($P < 0.05$) between seasons. Columns represent mean values (n = 5 plants) and bars are S.D.

Fig. 4 NCED gene expression in leaves of *Styrax ferrugineus* and *S. pohlii* measured in the dry season. ΔCt represent values normalized with actin and elongation factor as internal standards. Columns represent mean values (n = 5 plants) and bars are S.D. Absence of asterisk indicate a lack of significant difference between species.

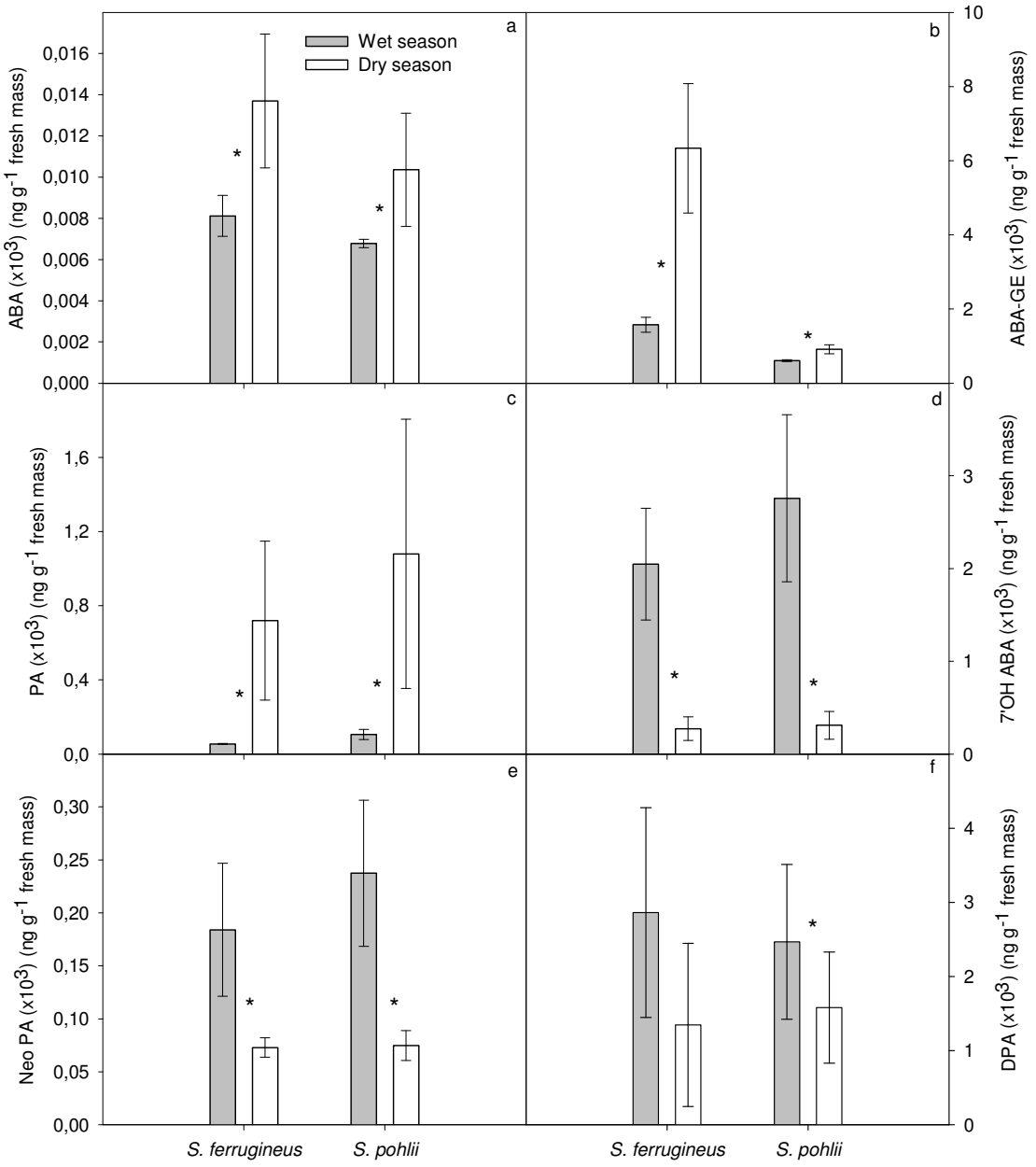
Wet season
Dry season
a
b
c
d
e
f
ABA (x10³) (ng g⁻¹ fresh mass)
ABA-GE (x10³) (ng g⁻¹ fresh mass)
PA (x10³) (ng g⁻¹ fresh mass)
7'OH ABA (x10³) (ng g⁻¹ fresh mass)
Neo PA (x10³) (ng g⁻¹ fresh mass)
DPA (x10³) (ng g⁻¹ fresh mass)
S. ferrugineus
S. pohlii
S. ferrugineus
S. pohlii

Fig. 1

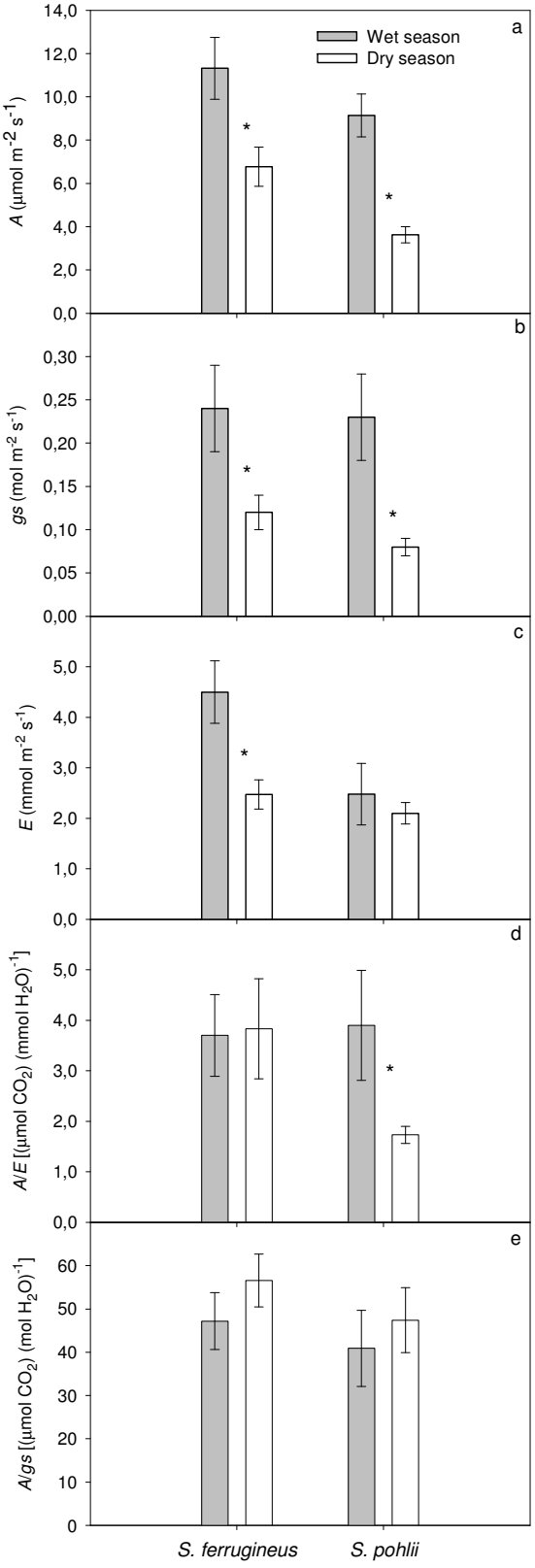
Wet season
Dry season
a
b
c
d
e
A (μmol m-2 s-1)
gs (mol m-2 s-1)
E (mmol m-2 s-1)
A/E [(μmol CO2) (mmol H2O)-1]
A/gs [(μmol CO2) (mol H2O)-1]
S. ferrugineus
S. pohlii

Fig. 2

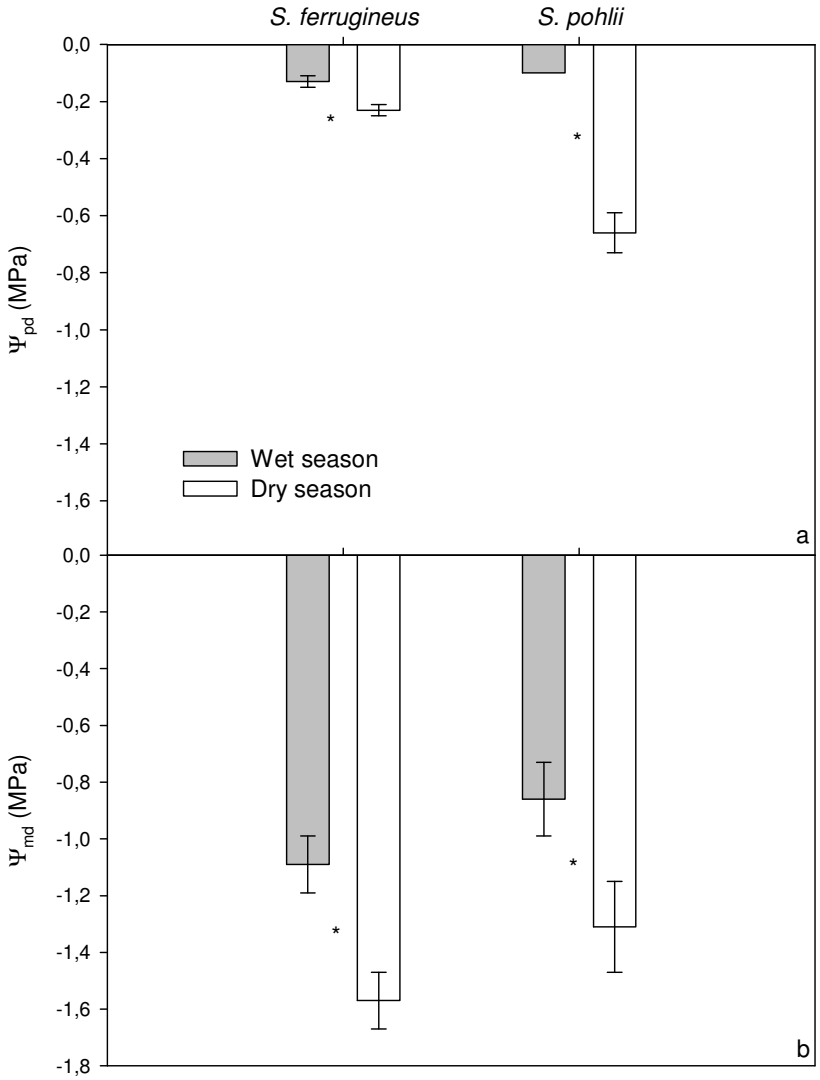
S. ferrugineus
S. pohlii
0,0
-0,2
-0,4
-0,6
-0,8
-1,0
-1,2
-1,4
-1,6
Ψpd (MPa)
Wet season
Dry season
a
0,0
-0,2
-0,4
-0,6
-0,8
-1,0
-1,2
-1,4
-1,6
-1,8
Ψmd (MPa)
b
*

Fig. 3

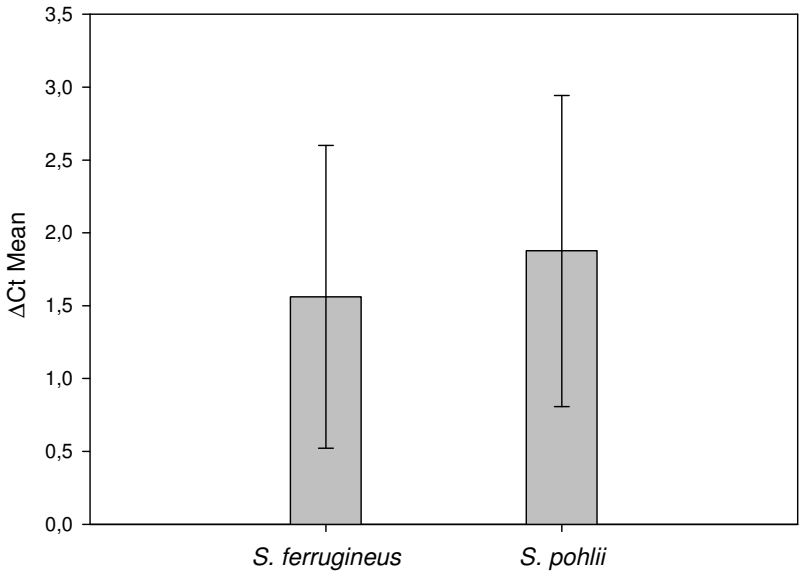

Fig. 4

Considerações finais

Considerando o papel fundamental do ácido abscísico frente ao estresse hídrico é possível inferir características ecofisiológicas ao quantificar este hormônio em tecidos vegetais. Analisando também vias de biossíntese (através da expressão gênica da enzima NCED) e degradação do ABA(pela quantificação de cinco de seus principais metabólitos) vemos a complexidade das respostas dadas pelas plantas em eventos de seca prolongada.

No âmbito analítico, a validação do método aliada ao uso de duas ou três transições MRM foram fundamentais para quantificar as substâncias com precisão, mesmo em uma matriz bastante complexa onde os analitos eram muito similares entre si estruturalmente.

A dinâmica do ABA (biossíntese e degradação) indica que em ambiente de mata, embora haja maior disponibilidade hídrica, as espécies (representadas por *Styrax pohlii*) são menos adaptadas à seca sazonal do que as espécies de savana (representadas por *Styrax ferrugineus*). Diferentemente de *S.pohlii*, na estação seca *S. ferrugineus* apresentou aumento duas vezes maior na concentração de ABA glicosilado (considerado um *pool* de ABA para secas futuras), maior eficiência do uso da água (pois perde menos água na transpiração) e melhor reidratação noturna (dada pelo potencial da água de madrugada). Tais características contribuem para que *S. ferrugineus* esteja adaptada a um ambiente de intenso estresse hídrico como é a savana brasileira.