ÍTALO DE OLIVEIRA BRAGA

CARACTERIZAÇÃO DA RESPOSTA DE *Gliricidia sepium* (JACQ.) STEUD. AO ESTRESSE SALINO MEDIANTE EMPREGO DA PROTEÔMICA E METABOLÔMICA

LAVRAS – MG
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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração Biotecnologia Vegetal, para a obtenção do título de Mestre.

Prof. Dr. Manoel Teixeira Souza Júnior
Orientador
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RESPONSE CHARACTERIZATION OF *Gliricidia sepium* (JACQ.) STEUD. TO SALINE STRESS THROUGH THE USE OF PROTEOMIC AND METABOLOMIC

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Dedico este a todos que, direta ou indiretamente, participaram de minha vida e trouxeram consigo confiança, apoio e auxílio.

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A todos citados, meu mais sincero, muito obrigado!

“Comece fazendo o que é necessário,
depois o que é possível e logo estará fazendo do
impossível!”
São Francisco de Assis

RESUMO GERAL

A população mundial está aumentando rapidamente ao longo dos anos e será necessário produzir aproximadamente 90% a mais de culturas alimentares do que estamos produzindo hoje, especialmente grãos, para suprir tal demanda até 2050 (REDDY et al., 2017). No entanto, o estresse abiótico, que inclui o sal, ameaça severamente a produção agrícola e causa perdas significativa de rendimento em grandes áreas (OSAKABE; OSAKABE, 2012). Desta forma, o objetivo geral do presente estudo foi aplicar estratégias de análise do metabolôma e do proteôma de folhas e raízes de plantas jovens de *Gliricidia sepium*, submetidas ou não a alto nível de estresse salino (>25 dS m^{-1}), visando adquirir conhecimentos sobre os mecanismos moleculares envolvidos na tolerância desta espécie à salinidade. Portanto, foram utilizados dados do banco de dados "Sal da Terra", pertencentes ao programa de PD&I de mesmo nome desenvolvido na Embrapa Agroenergia, que abriga dados de fenômica, ionômica, genômica, transcritômica (mRNA e microRNA), metabolômica e proteômica caracterizando a resposta de dendê (*Elaeis guineensis*), beldroega (*Portulaca oleracea*) e gliricídia (*Gliricidia sepium*) ao estresse salino. As amostras de metaboloma foram analisadas em um sistema UHPLC equipado com uma coluna de fase reversa. A espectrometria de massa de alta resolução (HRMS) foi realizada em um analisador Q-TOF usando fonte de eletrospray em ESI (+) - MS e ESI (-) - MS. Os dados adquiridos foram pré-processados usando o XCMS Online e posteriormente exportados para o MetaboAnalyst para análises estatísticas, anotação e observação das vias metabólicas. As amostras de proteoma foram preparadas e submetidas a análises LC – MS/MS pela empresa GenOne (Rio de Janeiro, RJ, Brasil), utilizou uma abordagem de quantificação "label-free", e os dados foram adquiridos usando o software Xcalibur e posteriormente exportados para o PatternLab para as análises quantitativas e qualitativas. Os resultados alcançados permitiram identificar proteínas e metabólitos, e vias metabólicas, responsivas a este estresse, tanto nas folhas quanto nas raízes.

Palavras-chave: Salinidade. Ômicas. Estresse abiótico.

GENERAL ABSTRACT

The world population is increasing rapidly over the years and it will be necessary to produce approximately 90% more food crops than we are producing today, especially grains, to meet this demand by 2050 (REDDY et al., 2017). However, abiotic stress, which includes salt, severely threatens agricultural production and causes significant yield losses in large areas (OSAKABE; OSAKABE, 2012). Thus, the general objective of the present study was applied to the analysis of the metabolome and proteome of leaves and roots of young plants of Gliricidia sepio, submitted or not to a high level of salt stress (> 25 dS m-1), before acquiring knowledge about the molecular mechanisms involved in this salinity tolerance. Therefore, data from the "Sal da Terra" database were used, belonging to the RD&I program of the same name developed at Embrapa Agroenergia, which houses data from physics, ionomics, genomics, transcriptomics (mRNA and microRNA), metabolomics and proteomics characterizing the response of oil palm (Elaeis guineensis), purslane (Portulaca oleracea) and gliricidia (Gliricidia sepium) to salt stress. How the metabolomes were analyzed on a UHPLC system equipped with a reversed phase column. High resolution mass spectrometry (HRMS) was performed on a Q-TOF analyzer using an electrospray source in ESI (+) - MS and ESI (-) - MS. The acquired data were pre-processed using XCMS Online and later exported to MetaboAnalyst for analysis, annotation and observation of metabolic pathways. Proteome Ame were prepared and submitted to LC - MS / MS analysis by the company GenOne (Rio de Janeiro, RJ, Brazil), using a "label-free" quantification approach, and the data were acquired using the Xcalibur software and subsequently exported to PatternLab for quantitative and qualitative analysis. The results achieved allowed to identify proteins and metabolites, and metabolic pathways, responsive to this stress, both in leaves and roots.

Keywords: Salinity. Omics. Abiotic stress.

SUMÁRIO

CAPÍTULO 1:
MEMÓRIA DE ESTRESSE DA PLANTA

1. INTRODUÇÃO GERAL

As plantas precisam lidar constantemente com diversas alterações ambientais durante seu ciclo de vida, sejam elas naturais ou causadas por estressores bióticos e/ou abióticos. Algumas dessas adversidades podem exigir forte resiliência das plantas para garantir sua sobrevivência (HILKER; SCHMÜLLING, 2019). Entretanto, as plantas desenvolveram numerosas adaptações em diferentes níveis moleculares, como no genoma, transcriptoma, proteoma, metabolôma e na epigenoma para perceber e responder efetivamente a essas variedades de tensões (LING et al., 2018). Estas adaptações são extremamente vantajosas quando o assunto é estresses que ocorrem regularmente. No entanto, muitos eventos estressantes acontecem de forma irregular e transitória (CRISP et al. 2016).

Considerando o impacto econômico que as alterações ambientais, principalmente estresse biótico e abiótico, acarretam ao agronegócio, melhorar a tolerância em plantas economicamente importantes é um dos principais objetivos atuais (SPILLANE et al. 2020). Estudos recentes indicam que as plantas apresentam memória de estresse, ou seja, que elas suportam e se adaptam ao estresse recorrente (VRIET et al. 2015, HILKER; et al. 2016). Do Amaral et al. (2020) traz um conceito de memória consolidado ao longo dos anos, onde memória é definido como uma modificação genética, fisiológica ou bioquímica de uma planta que acontece após a exposição a determinado estresse, tornando as respostas aos eventos estressantes subsequentes diferentes.

Portanto, a memória de plantas é uma característica fundamental do comportamento inteligente, podendo estar relacionada a diversos níveis de complexibilidade (NEVES et al. 2017). Estudos recentes mostram que estresses subsequentes podem atuar preparando a planta para futuras condições adversas, fenômeno conhecido com priming (CONRATH et al. 2006, AULER et al. 2017). Onde após um primeiro contato, e cessado o estresse, ocorre o armazenamento de informações referente àquele estímulo, acarretando numa resposta diferente (mais rápida e/ou mais forte) a um estresse recorrente, quando se compara a uma planta que não passou pelo evento de priming (DO AMARAL et al. 2020).

A técnica de priming é um dos diversos mecanismos epigenéticos relacionados a defesa/memória das plantas envolvendo a expressão gênica, mecanismos estes que estão intimamente relacionados ao estado dinâmico da cromatina, envolvendo a metilação do DNA e modificações de histonas (FRIEDRICH et al. 2019). Por definição, epigenética são mudanças mitóticas e/ou meióticas hereditárias que não alteram a sequência de DNA, permitindo que os organismos respondam e se adaptem. Contudo, modificações epigenéticas

são considerados mecanismos únicos, sendo críticos para a sobrevivência da planta em condições desfavoráveis (TURGUT-KARA et al. 2020).

Ao longo dos anos, tanto estudos genéticos quanto bioquímicos contribuíram para a melhor compreensão de vários processos epigenéticos que envolvem a metilação de DNA, modificação de histona e silenciamento genético mediado pelo RNA. E esses processos são correlacionados entre si, pois a metilação de DNA é necessária para modificações de cromatina e vice-versa, enquanto os mecanismos baseados em RNA interferente regulam ambos os processos (HIDETOSHI et al. 2012).

A *Gliricidia sepium* (Jacq.) Walp. é uma árvore com múltipla utilidade, cultivada para melhorar a fertilidade do solo; para fins medicinais; como madeira / lenha, como carvão; como ração animal, entre outros usos. É uma espécie vegetal conhecida por sua capacidade de se adaptar a uma ampla variedade de solos, desde solos ácidos erodidos, solos arenosos, argila pesada, calcário e solos alcalinos (ANDRADE *et al*., 2015). É uma arvore que pertence à família *Fabaceae* e subfamília *Faboideae (Papilionoideae*) (BAGGIO, 1984; SOUZA; LORENZI, 2012); e é uma espécie nativa da América Central (DRUMOND; DE CARVALHO FILHO, 1999).

Diante das informações levantadas até aqui referentes a memória ao estresse recorrente, os estudos realizados por Belo Silva (2019) e Carvalho da Silva et al. (2021) mostraram que plantas jovens de gliricidia submetidas a altas concentrações de NaCl apresentam uma resposta classificada como adaptação. Estas plantas, após perderem todas as folhas em até uma semana do início do estresse salino, começam a produzir novas folhas por volta do vigésimo dia de estresse. Carvalho Silva et al. (2021) caracterizou os perfis do transcritoma e do metaboloma das folhas de gliricidia, analisando os mesmos sob três cenários distintos: Efeito idade (plantas controle aos 2 e 45 dias sob estresse – DAT), estresse de curto prazo (plantas controle e estressadas aos 2 DAT) e estresse de longo prazo (plantas estressadas aos 2 e 45 DAT).

Carvalho da Silva e colegas ainda correlacionaram e diferenciaram grupos de plantas de *G. sepium* submetidas ao estresse salino, revelando genes / transcritos, metabólitos e vias responsivas a este estresse, e mostraram que a via de biossíntese dos fenilpropanóides desempenha um papel importante na resposta desta espécie ao estresse de curto prazo.

Portanto, o objetivo do presente trabalho foi ganhar conhecimento adicional sobre os possíveis mecanismos moleculares que conferem a esta espécie a capacidade de se adaptar às altas concentrações salinas. Para tanto, decidimos caracterizar os perfis do metaboloma e

proteôma das folhas e raízes de plantas jovens de gliricidia utilizando estratégias de análise individuais de proteômica e metabolômica.

2 REVISÃO DE LITERATURA

2.1 JUSTIFICATIVA

A adaptação ao estresse salino observado em gliricidia não é, a priori, um caso de priming – pois a mesma foi submetida ao estresse por salinidade e este se manteve ao longo de todo o experimento, mas há a possibilidade de podermos classifica-la como um mecanismo memória epigenética, como por exemplo, a planta passando por uma reorganização da cromatina para adaptação / sobrevivência em resposta ao estresse.

Porém, esse tipo de resposta, uma vez caracterizado o mecanismo responsável pela ocorrência da adaptação, apresenta um potencial de uso como priming não só nesta espécie, mas também em outras espécies perenes, mediante transferência horizontal da mesma.

Isso posto, decidimos por realizar uma revisão de literatura focada no tema “memória de plantas”, com o objetivo de abordar informações e aumentar os conhecimentos do nosso grupo de pesquisa sobre este tema e seu papel na resposta de plantas a estresses abióticos, principalmente, estresse por salinidade.

2.2 MEMÓRIA EPIGENÉTICA

O termo epigenética refere-se a mudanças hereditárias na atividade e expressão de genes, e também abrange alterações estáveis de longo prazo no potencial de transcrição de células que não são necessariamente hereditárias (SUDAN et al., 2018). Com isso, a epigenética descreve padrões hereditários de variações fenotípicas, ou seja, é uma transmissão estável de informações por meio da mitose ou meiose — que não são apenas atribuídos a diferenças na sequência de DNA (CRISP, 2016).

Os mecanismos epigenéticos desempenham um papel essencial na regulação da expressão genética em resposta às tensões ambientais nas plantas (SUDAN et al., 2018). Modificações epigenéticas são mecanismos únicos que respondem às mudanças nas condições em estados somáticos e transgeracionais, e são críticos para a sobrevivência da planta em resposta a condições desfavoráveis. Sendo que os organismos podem responder as alterações nos níveis de metilação do DNA, modificações das histonas e em RNAs não codificados. E essas modificações epigenéticas não alteram o código genético original, em vez disso, permitem que os organismos respondam e se adaptem (TURGUT-KARA et al., 2020).

Além disso, as modificações epigenéticas desempenham papéis cruciais na formação da memória do estresse, que pode ser herdada resultando em maior tolerância ao estresse

recorrente (LEPHATSI et al., 2021). Algumas dessas modificações podem persistir por mais tempo e são consideradas como "marcas de memória" transgeracionais, enquanto outras são modificações dinâmicas de curta duração que são rapidamente removidas, por exemplo, as marcas de cromatina (HILKER et al., 2016; KISHIMOTO et al., 2017, SCHWACHTJE et al., 2019).

A memória epigenética de uma célula define o conjunto de modificações que ocorrerá no DNA da célula, mas não altera a sequência de DNA. Essas modificações podem alterar a expressão genética e, consequentemente, as características e o comportamento da célula (TURGUT-KARA et al., 2020).

Dentre as modificações envolvidas na epigenética, a cromatina, é um complexo que contém DNA e proteínas nucleares, principalmente histonas, que desempenha um papel essencial na regulação da transcrição (JOHNSON E PATHUR, 2021). Uma vez que, DNA e histonas podem ser modificados pela adição de grupos químicos, sendo o grupo metil de longe o mais comum no caso de DNA e nas histonas temos as modificações químicas pós-traducionais (PTMs) (por exemplo, grupos metil, acetil, fosfato e ubiquitina sendo mais comum). A presença dessas marcas na cromatina ou suas combinações atuam na regulação da expressão de genes, modificando a acessibilidade ao DNA ou o recrutamento de proteínas específicas para a cromatina (ZENTNER E HENIKOFF, 2013).

Estudos recentes têm demonstrado a metilação do DNA como um dos principais componentes do estresse e da memória (HE et al., 2018; LAMKE, 2017; WIBOWO et al., 2018). Recentemente, Sun e colaboradores (2021), demonstraram os potenciais implicações da metilação do DNA na memória de estresse em plantas de *Boea hygrometrica* que passaram pelo tratamento a seca, e observaram que a seca induz a tolerância rápida à dessecação, e que essa tolerância pode ser mantida por pelo menos quatro semanas.

Além disso, alguns hormônios vegetais têm efeitos significativos na compactação da cromatina, que é conciliada pela metilação do DNA e modificações pós-traducionais nas histonas, o que significa que a ação epigenética e hormonal estão correlacionadas. Além disso, evidências sugerem que a biossíntese e o transporte de auxina são modulados por fatores epigenéticos como modificação de histona, remodelação de cromatina e metilação de DNA (YAMAMURO et al. 2016).

Desta forma, as respostas ao estresse resultam em mudanças em todo o genoma, na estrutura da cromatina e na transcrição do gene ou podem até mesmo ser associadas a modificações na sequência genômica (CHEN et al., 2019). Compreender os mecanismos que

regulam tais alterações, acarretará no desenvolvimento de estratégias para mitigar ou reverter essas mudanças (LEPHATSI et al., 2021).

2.3 PRIMING

A literatura traz que a exposição repetida de uma planta a determinado estresse pode prejudicar significativamente seu desenvolvimento, mas pesquisas tem demonstrado que a experiência de repetir o estresse capacita/treina a planta para lidar com o estresse subsequente, desta forma reduzindo a aptidão de perda de rendimento (HILKER et al., 2019), fenômeno denominado de priming. E este conceito implica que os genes responsivos ao estresse são treináveis, sendo essa memória transcricional responsável pela modulação da expressão diferencial em resposta ao estresse repetido (DE FREITAS GUEDES, 2018).

Ao longo da última década, uma série de excelentes revisões sobre priming relacionada a imunidade local e sistêmica das plantas, defesa contra pragas e tolerância ao estresse abiótico foram publicadas, como por exemplo, estudos realizados por CONRATH (2011 E 2015); GOZZO et al., (2013); KIM et al., (2013); PASTOR et al., (2013), JOHNSON et al., (2021) entre outros. E de forma geral, as etapas referentes ao priming implica: (1) numa primeira condição de estresse, que irá induzir resposta da planta ao estresse, além de acarretar na formação de uma memória molecular – melhorando a germinação de sementes; (2) seguido por cessar a condição de estresse e um período de tempo no qual a memória celular pode persistir – que pode variar de dias à semanas; (3) aplicação de um segundo estresse, com objetivo de ativar a memória gerando uma resposta aprimorada da planta. Além destas etapas, uma abordagem adicional envolve a perpetuação da memória para transferência às gerações futuras – processo chamado de memória inter/transgeracional (MOZGOVA et al., 2019).

Sendo esse conceito de memória inter/transgeracional relacionado a alterações no nível de expressão gênica (DING et al., 2014; Guedes et al. 2018), o critério para considerar a memória transcricional é que o nível de transcrição em respostas a estresses subsequentes, após o priming, deve ser significativamente diferente dos níveis de transcrição gerados após o primeiro estresse (DING et al., 2014). Desta forma, os genes de memória alteram expressão para o estresse subsequente, enquanto os genes “sem memória” respondem de forma semelhante a cada evento estressante (CADLE et al., 2015).

A fase de latência é a fase mais proeminente no priming, que inicia o evento de ativação precoce por meio da restauração da resposta ao estresse das atividades metabólicas, alcançada por meio da memória de estresse e permite um avanço. As sementes preparadas exibem uma diminuição no período de tempo da fase de latência durante a germinação,

também a taxa de reparo de DNA e ativação de enzimas, bem como o acúmulo de metabólitos necessários para a germinação seriam aumentados (LUTTS et al., 2016).

Diante disso, diversas técnicas tem sido utilizadas na tentativa de melhorar o crescimento e a produtividade das plantas abordando mecanismos genéticos, por exemplo, engenharia genética e melhoramento de plantas (JOHNSON et al., 2021). Mas estudos presentes na literatura, tem demonstrado que a técnica de priming de sementes melhora a produtividade da cultura (BANERJEE et al., 2018). E a sua popularidade é devido a fácil utilização, baixo custo, apresentar resultados eficazes e menor risco ambiental (GHOLAMI et al. 2015; IBRAHIM, 2016; AMOOAGHAIE e TABATABAIE 2017). Visto que o efeito benéfico do priming tem sido associado a vários eventos bioquímicos, celulares e moleculares que aumentam a atividade de enzimas antioxidantes, eleva o conteúdo de carboidratos solúveis, assim como conteúdo de prolina e diminui os danos as membranas (FARHOUDI et al., 2011).

Desta forma, a metodologia de memória mediada por priming consiste por mudanças na metilação do DNA, modificações de histonas e levam ao acumulo de proteínas sinalizadoras, como MAP (MAPK3 e MAPK6) quinases inativas e fatores de transcrição (TURGUT-KARA, 2020). Ling et al. (2018) recentemente realizou um estudo utilizando plantas de *Arabidopsis thaliana* para investigar o efeito do termocondicionamento na memória de estresse, e após analisar o transcriptoma e o splicing alternativo dessas plantas, eles concluíram que as plantas que foram termocondicionadas antes exibem uma memória de splicing.

Estudos realizados em 2013 por Sani e colaboradores, onde foi realizado o priming hiperosmótico também em *Arabidopsis* em comparação com plantas controles (sem a utilização do priming), foi observado que as plantas preparadas apresentaram crescimento e desenvolvimento idênticos ao das plantas controle, mas exibiram uma absorção reduzida ao sal e maior tolerância a seca após uma segunda exposição ao estresse. E tais respostas estão intimamente relacionadas a pequenas alterações nos níveis de modificação das histonas. Visto que, em resumo, foi observado que o tratamento com priming não acarretou numa grande remodelação dos perfis de modificação de histonas em todo genoma, mas sim, induziu pequenas mudanças no perfil das quatro histonas estudadas (H3K4me2, H3K4me3, H3K27me3 e H3K9me2).

O priming também facilita a replicação e o reparo precoce do DNA, aumenta o RNA e a síntese de proteínas e reduz o vazamento de metabólitos (JOHNSON et al., 2021). E muitos genes relacionados à germinação são regulados para cima durante o priming, que são

necessários para processos biofísicos e bioquímicos durante a germinação de sementes e podem acelerar a emergência de plântulas (PAPARELLA et al., 2015).

2.4 ESTRESSE SALINO E PRIMING

A exposição ao estresse abiótico, como sal, disponibilidade de água, temperatura ou a luz, acarreta em alterações globais nas modificações de histonas (FORESTAN et al., 2018) e alterações na metilação do DNA (WIBOWO et al., 2016; GANGULY et al., 2017) e isso está conectado a mudanças globais na expressão gênica (FORESTAN et al., 2016; WIBOWO et al., 2016), como mencionado anteriormente. Sendo que as alterações globais na estrutura da cromatina em resposta ao estresse se assemelham às alterações da cromatina durante as fases do desenvolvimento, sugerindo uma conexão funcional entre a reprogramação induzida pelo estresse e pelo desenvolvimento, propriamente dito (DONÀ E MITTELSTEN SCHEID, 2015).

Dentre os diversos tipos de estresses, que levam a perdas de bilhões de dólares anuais em todo mundo. O estresse salino se apresenta como um desses desafios, predominante em regiões áridas, semiáridas e sendo disseminado facilmente por terras irrigadas (MUNNS E TESTER, 2008). Visto que a salinidade é um estresse multidimensional com elevado níveis de minerais inorgânicos, gerando toxicidade osmótica e iônica, de forma a reduzir o potencial osmótico das células. É importante compreender os mecanismos de entrada de sal pelos quais os íons excessivos se acumulam na planta. Sendo isso, o principal fator limitante para o crescimento, desenvolvimento e produtividade das culturas (JOHNSON et al., 2021).

De acordo com Isayenkov e Maathuis (2019), a captação de íons pode ocorrer por via simplástica ou apoplástica. A via apoplástica é uma rota de desvio de fluxo, que apresenta uma conexão direta entre a raiz e o xilema, fator importante que contribui para a entrada de Na^+ no apoplasto. E o principal local de entrada do Na^+ é o citoplasma da raiz e, posteriormente, esse íon é transportado para o vacúolo por meio de transportadores, como o antiportador Na^+/H^+. Transportadores específicos estão envolvidos na absorção líquida através da via simplástica do $Na^+(Cl^–)$ nas raízes e são considerados catalisadores por um complexo específico de transportadores.

As plantas respondem ao estresse salino alterando dinamicamente seu comportamento fisiológico e modulando uma complexa rede de genes (TANI et al. 2018; SKORUPA et al. 2019). E estes efeitos da salinização ocasionam variações no fluxo de elétrons via cadeia transportadora de elétrons nas organelas e nas vias de redução de oxigênio, que são cruciais na superprodução de espécies reativas de oxigênio (ROS) (AHMAD *et al*, 2019). Uma vez

que, a superprodução de ROS causa desequilíbrios da homeostase no nível celular, assim como no nível subcelular, e pode finalmente causar apoptose (ZHELEV *et al*. 2016). No geral, o estresse é reconhecido pelos receptores transmembranas nas plantas e disparam uma cascata de sinalização até a ativação de genes específicos. Durante este processo, enzimas e receptores são ativados e/ou inibidos através de reações de fosforilação/desfosforilação ocasionado por proteínas quinases e fosfatases, gerando respostas ao estimulo (ARDITO *et al*., 2017).

Ainda nos estudos realizados por Isayenkov e Maathuis (2019), eles trazem que plantas expostas ao estresse por sal apresentam diferentes respostas, classificadas em duas fases. Inicialmente, a planta apresenta uma redução na taxa de crescimento, fator que é independente do acúmulo de íons - passando por eventos como inibição da expansão celular e fechamento estomático, que ocorre dentre minutos a dias. A segunda etapa inicia com o acúmulo de íons citotóxicos, seguido por uma regulação metabólica negativa, e consequentemente, acarretando na senescência precoce e morte celular, fase que acontece dentre dias a semanas. Uma vez que, o estresse por salinidade acarreta no acúmulo excessivo de íons tóxicos para as células, ou seja, os íons de sódio e cloreto, levam à inibição da absorção de água seguida por desequilíbrio nutricional e dano oxidativo devido a geração de radicais livres (ALAM et al., 2020).

Sendo os processos de tolerância determinado por mecanismos fisiológicos e moleculares, como: tolerância osmótica, iônica e tolerância do tecido (ROY et al., 2014). Huang et al. (2020) traz que, o estresse osmótico ocasionado pela salinidade ativa um mecanismo de sinalização da raiz ao caule, e posteriormente, um declínio simultâneo da condutância estomática, buscando a preservação de água. Já a entrada de sal no sistema radicular desencadeia a ativação de várias cascatas de sinalização que gera tolerância iônica por restringir o influxo e reduzir a translocação de Na^+ na raiz. E por fim, a tolerância tecidual é aumentada pela compartimentalização de íons tóxicos em vacúolos afim de evitar efeitos prejudiciais nos processos citoplasmáticos (ISAYENKOV E MAATHUIS 2019).

Devido ao papel negativo dos sais nas plantas, o primig de sementes com NaCl tem demonstrado melhora na germinação e no crescimento de mudas/culturas, aumentando a tolerância a este estresse, uma vez que, essa técnica reduz parcialmente os efeitos adversos do estresse salino (IBRAHIM et al., 2016; GEBREEGZIABHER e QUFA 2017). Tal melhora foi observada em tomate (*Solanum lycopersicum*), em milho (*Zea mays L*.), ervilha (*Pisum sativum L.)* e soja (*Glycine max L*.), entre outros, como citado no estudo de Do Amaral e colaboradores de 2021.

Do Amaral et al. (2020), observou que plantas que passaram pelo choque prévio de sal, apresentaram menor número de genes diferencialmente expressos (DEG) em relação as plantas que sofreram apenas no estágio reprodutivo, demonstrando o efeito de memória trasncricional. Resultados semelhantes foram encontrados por Guedes et al. (2018), em que duas cultivares de café apresentaram maior número de DEGs em plantas submetidas ao ciclo de seca e menor número de DEGs em plantas submetidas a três ciclos.

Diversos estudos trazem outros efeitos do priming com NaCl, Farhoudi et al. (2011) descobriram que o priming de NaCl em *Cucumis melo* melhorou o teor de antioxidantes, carboidratos solúveis e prolina. O priming de NaCl também aumentou o aparato fotossintético e o maquinário antioxidante em sementes de melão, pimenta (*Capsicum annuum L.*), alface (*Lactuca sativa L.*), *Triticum aestivum* (IQBAL E ASHRAF, 2007).

Já Hu et al. (2016) relataram que após eventos repetidos de estresse/recuperação, plantas de *Lolium perenne* pré-treinadas tiveram valores de vazamento de eletrólitos menores do que plantas não treinadas durante os 8 dias de estresse salino, indicando que o piming fortaleceu estabilidade da membrana celular. O estudo ainda traz a informação que o efeito do priming levou ao menor conteúdo de H_2O_2 e O_2.

Vale destacar também o efeito do priming em relação aos hormônios frente ao estresse salino, visto que o papel dos hormônios vegetais sob estresse salino é crítico na modulação das respostas fisiológicas que levarão à adaptação a um ambiente desfavorável (FEGHHENABI et al., 2016). Como exemplos, os osmólitos glicina betaína (GB) e prolina se acumulam rapidamente durante os estresses de sal e seca, sendo cruciais para a manutenção do equilíbrio osmótico (MANAF, 2016). Nakaune et al. (2012) observaram que o efeito do priming com NaCl na germinação de sementes de tomate (Solanum lycopersicum L.) é causado pelo aumento no conteúdo de giberelina via ativação do gene de biossíntese da giberelina.

O priming de colina melhorou a tolerância ao sal no trigo (*Triticum aestivum L.*), mantendo elementos benéficos (K^+, Ca^{2+}), minimizando elementos tóxicos (Na^+, Cl^-), aumentando o acúmulo de glicinabetaína e reduzindo o estresse oxidativo, que reflete na redução da perioxidação lipídica (SALAMA et al., 2011). Além disso, a colina manteve os lipídios, esteróis e fosfolipídios da membrana plasmática, que foram alterados pelo NaCl (SALAMA E MANSOUR, 2015).

A maioria dos estresses abióticos, incluindo seca, salinidade, temperatura extrema, luz alta e poluentes, como metais pesados ou pesticidas, resultam em estresse oxidativo, ou seja, um aumento nos níveis celulares de espécies reativas de oxigênio (KERCHEV et al., 2020), e

o sistema antioxidante é um dos principais mecanismos de defesa em sementes. O sistema de defesa antioxidante eficaz da planta inclui antioxidantes enzimáticos e não enzimáticos que auxiliam na desintoxicação por ROS (HASANUZZAMAN et al., 2020). E os tratamentos de priming aumentam as atividades de enzimas antioxidantes (como catalase, peroxidase e superóxido dismutase) (SEDGHI et al., 2014; SHARMA et al., 2014) e compostos antioxidantes (como ácido ascórbico e glutationa redutase).

No estudo realizado por Do Amaral et al., (2020), foi observado uma redução na concentração de O_2^- em plantas com genótipo tolerante que sofreram estresses recorrente, apesar da pouca alteração na atividade da enzima SOD, demonstrando a influência anterior do evento subsequente. Eles ainda trazem que em um genótipo sensível, o efeito do estresse recorrente foi positivo, mesmo não obtendo alteração no teor de superóxido, as plantas que receberam o estimulo ao estresse na fase reprodutiva apresentaram maior concentração de peróxido de hidrogênio e maior atividade das enzimas SOD e CAT.

Embora os mecanismos moleculares precisos pelos quais a tolerância se desenvolve permaneçam desconhecidos, suspeita-se que a tolerância induzida por priming entre tratamentos de salinidade, seca, metal pesado, calor e frio é devido a consequências comuns e a capacitação da planta está ligada à produção aumentada de ROS (O_2^-, H_2O_2), metil glioxilase (MG) e vias de sinalização oxidativa que operam na interface entre as redes de sinalização redox e hormonal são essenciais na regulação da expressão gênica responsiva ao estresse (HOSSAIN et al., 2016).

3 OBJETIVOS

O objetivo geral deste estudo é adquirir conhecimento sobre os mecanismos moleculares responsáveis pela tolerância a salinidade observada em *Gliricidia sepium* (Jacq.) Steud. através de estratégias de análise individual de proteômica e metabolômica.

3.1 ORGANIZAÇÃO DA DISSERTAÇÃO

A dissertação está organizada em quatro partes:

- Parte 1: Capítulo 1 - Revisão sobre o tema Memória de Estresse da Planta (MOI)
- Parte 2: Capítulo 2 – Artigo: “Deeper untargeted metabolomics analysis to further characterize the adaptation response of response of *Gliricidia sepium* (Jacq.) Walp. to very high salinity stress”
- Parte 3: Capítulo 3 – Artigo: “Proteomics analysis to further characterize the adaptation response of *Gliricidia sepium* (Jacq.) Walp. to very high salinity stress”
- Parte 4: Considerações finais

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CAPÍTULO 2

Deeper untargeted metabolomics analysis to further characterize the adaptation response of *gliricidia sepium* (jacq.) Walp. to very high salinity stress

A versão apresentada do presente artigo será submetida para publicação (a definir), sendo uma versão preliminar e o conselho editorial do periódico poderá sugerir alterações.

RESUMO

A *Gliricidia sepium* (Jacq.) Walp. é uma árvore polivalente e apresenta a capacidade de se adaptar a um nível muito alto de estresse salino (≥20 dS m-1), com a produção de novas folhas em torno de duas semanas após a perda de todas as folhas devido ao estresse salino severo. A integração dos perfis do metaboloma e do transcriptoma de gliricídia apontou para o papel central da via de biossíntese dos fenilpropanóides na resposta de curto prazo ao estresse salino. Neste estudo, uma análise metabólica não direcionada mais profunda das folhas e raízes de plantas jovens de gliricídia teve como objetivo caracterizar o(s) mecanismo(s) por trás dessa resposta de adaptação. As frações polares e lipídicas das amostras de folhas e raízes foram extraídas e analisadas no sistema UHPLC.ESI.Q-TOF.HRMS. Os dados adquiridos foram analisados nas plataformas XCMS Online e MetaboAnalyst, por meio de três estratégias distintas e complementares. Juntos, os primeiros resultados obtidos nos levaram a postular que essas plantas estão excluindo sal, que se adaptaram ao estresse de alta salinidade por meio de dois mecanismos de exclusão de sal, começando na parte aérea - desfolha severa - e concluindo nas raízes - entrada limitada de Na^+. Além disso, foi possível mostrar que a via de biossíntese dos fenilpropanóides desempenha um papel ao longo de toda a resposta de adaptação, começando no curto prazo e continuando no longo prazo. A análise do metaboloma das raízes revelou onze vias metabólicas distintas afetadas pelo estresse salino, e a análise inicial das duas mais afetadas - Biossíntese de esteróides e Biossíntese de lisina - nos levou também a postular que o acúmulo de lignina e alguns fitoesteróis, bem como a biossíntese de lisina - mas não a degradação, desempenham um papel na promoção da resposta de adaptação. No entanto, estudos adicionais são necessários para investigar essas hipóteses.

Palavras-chave: Quimiometria, Espectrometria de Massa de Alta Resolução, Estresse Abiótico, Fenilpropanóides, Fitoesteróis, Lisina, Lignina.

ABSTRACT

The multipurpose tree *Gliricidia sepium (Jacq.) Walp.* adapts to a very high level of salt stress (≥20 dS m^{-1}) and resumes the production of new leaves around two weeks after losing all leaves due to abrupt salinity stress. The integration of metabolome and transcriptome profiles from gliricidia leaves pointed to a central role of the phenylpropanoid biosynthesis pathway in the short-term response to salinity stress. In this study, a deeper untargeted metabolomics analysis of the leaves and roots of young gliricidia plants aimed to characterize the mechanism(s) behind this adaptation response. The polar and lipidic fractions from leaf and root samples were extracted and analyzed on a UHPLC.ESI.Q-TOF.HRMS system. Acquired data were analyzed using the XCMS Online, and MetaboAnalyst platforms, via three distinct and complementary strategies. Together, the results obtained first led us to postulate that these plants are salt-excluding plants, which adapted to high salinity stress via two salt-excluding mechanisms, starting in the canopy - severe defoliation - and concluding in the roots - limited entry of Na. Besides that, it was possible to show that the phenylpropanoid biosynthesis pathway plays a role throughout the entire adaptation response, starting in the short term and continuing in the long one. The roots metabolome analysis revealed eleven distinct metabolic pathways affected by salt stress, and the initial analysis of the two most affected ones - Steroid biosynthesis and Lysine biosynthesis – led us also to postulate that the accumulation of lignin and some phytosterols, as well as lysine biosynthesis - but not degradation, play a role in promoting the adaptation response. However, additional studies are necessary to investigate these hypotheses.

Keywords: Chemometrics, High Resolution Mass Spectrometry, Abiotic Stress, Phenylpropanoids, Phytosterols, Lysine, Lignin.

Deeper untargeted metabolomics analysis to further characterize the adaptation response of *Gliricidia sepium* (Jacq.) Walp. to very high salinity stress

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Keywords: Chemometrics, High Resolution Mass Spectrometry, Abiotic Stress, Phenylpropanoids, Phytosterols, Lysine, Lignin.

Introduction: The multipurpose tree *Gliricidia sepium (Jacq.) Walp.* adapts to a very high level of salt stress (≥20 dS m^{-1}) and resumes the production of new leaves around two weeks after losing all leaves due to abrupt salinity stress. The integration of metabolome and transcriptome profiles from gliricidia leaves pointed to a central role of the phenylpropanoid biosynthesis pathway in the short-term response to salinity stress.

Method: In this study, a deeper untargeted metabolomics analysis of the leaves and roots of young gliricidia plants aimed to characterize the mechanism(s) behind this adaptation response. The polar and lipidic fractions from leaf and root samples were extracted and analyzed on the UHPLC.ESI.Q-TOF.HRMS system. Acquired data were analyzed using the XCMS Online, and MetaboAnalyst platforms, via three distinct and complementary strategies.

Results: Together, the results obtained first led us to postulate that these plants are salt-excluding plants, which adapted to high salinity stress via two salt-excluding mechanisms, starting in the canopy - severe defoliation - and concluding in the roots - limited entry of Na. Besides that, it was possible to show that the phenylpropanoid biosynthesis pathway plays a role throughout the entire adaptation response, starting in the short term and continuing in the long one.

Conclusion: The roots metabolome analysis revealed eleven distinct metabolic pathways affected by salt stress, and the initial analysis of the two most affected ones - Steroid biosynthesis and Lysine biosynthesis – led us also to postulate that the accumulation of lignin and some phytosterols, as well as lysine biosynthesis - but not degradation, play a role in promoting the adaptation response. However, additional studies are necessary to investigate these hypotheses.

1. Introduction

Soil salinity is an environmental limiting factor for plant biomass production worldwide, with approximately 20% of all agricultural land in the world having either saline or sodic soils, and between 25% and 30% of the irrigated land area is affected by salt (Negrão et al., 2017; Shahid et al., 2018). The annual global cost of salt-induced land degradation in irrigated areas can reach US$ 27.3 billion due to a decrease in productivity (Pan et al., 2020). Salinity imposes adverse effects on plant growth by causing water imbalance, oxidative stress, and Na+ toxicity (Zarei et al., 2020). Besides that, it also compromises germinative processes, photosynthetic pigmentation, and photosynthesis; and at the cellular level, it induces osmotic and ionic stress (Parihar et al., 2015).

Metabolomics (study of modification in metabolites) is a comprehensive and quantitative analysis of all small molecules in a biological system (Belinato, 2019). It is one promising approach used to detect and quantify primary and secondary metabolites of low molecular weight, generally <1500 Da (Bueno and Lopes, 2020). Recent studies show that some metabolites are present in metabolic changes induced by salt stress, and they can act as effectors of osmotic readjustment or antioxidant response (Arbona, 2013). The presence of specific metabolites may be associated with tolerance to stress and serve as biomarkers for salt-tolerant genotypes selection in plant breeding programs (D'Amelia et al., 2018).

Gliricidia sepium (Jacq.) Walp., a medium-sized legume (10-15 m) from the Fabaceae family, is originally from Central America. It is one of the most well-known multipurpose trees, known for its ability to adapt very well to a wide range of soils, from eroded acidic soils, sandy soils, heavy clay, limestone, and alkaline soils (Rahman et al., 2019). As pointed out by Rahman et al. (2019), G. sepium salinity tolerance limits alongside the morphophysiological responses to salt stress are not yet well understood. Rahman and colleagues reported a study where seawater from the southern coastal area of Bangladesh induced salinity stress in one-month-old gliricidia seedlings for 90 days and showed that seawater-induced salinity negatively affected several growth-related attributes. They also showed enhanced accumulation of proline, the proteinogenic secondary amino acid that participates in metabolic signaling and is known to be metabolized by its own family of enzymes responding to stress (Phang et al., 2010), postulating that it might help adjust to water deficit conditions (Rahman et al., 2019).

In a previous study done by our group, we described two distinct responses of gliricidia plants - tolerance and adaptation - to salt stress, depending on the amount of NaCl used (Carvalho da Silva et al., in press). Additionally, when employing single and integrative

transcriptomic and metabolomic analysis approaches, it showed that the phenylpropanoid biosynthesis pathway was the most salt stress-affected pathway in the leaves of young gliricidia plants, with 15 metabolites and three genes differentially expressed; and that this pathway role was more evident at the beginning of the stress, not in the long-term.

Ho et al. (2020) characterized the transcriptomes, metabolomes, and lipidomes of domesticated and landrace barley plants with distinct seedling root growth responses under salt stress. The phenylpropanoid biosynthesis was the most statistically enriched biological pathway among all salinity responses observed, based on pathway over-representation of the differentially expressed genes and metabolites (Ho et al., 2020). Zhu et al. (2021) also performed a combined transcriptomic and metabolomic analysis of salt-stressed *Sophora alepecuroide* plants, a leguminous perennial herb found mainly in the desert and semi-desert areas of the China northwest region, and also showed that the differentially expressed genes and metabolites in the phenylpropanoid biosynthesis pathway significantly correlated under salt stress. A difference between the work done by Carvalho da Silva et al. (in press) and the ones by Ho et al. (2020) and Zhu et al. (2021) is that it used leaves and the later ones used roots.

The current study is a follow-up to our previous study (Carvalho da Silva et al., in press). Hence, the objective of this present study was to carry out a deeper metabolome analysis not only of the leaves but also the roots of *G. sepium* plants submitted to a very high salt stress (27 dS m-1 of electrical conductivity). For this purpose, gliricidia plants were under salinity stress, and leaves and roots samples were collected from control and stressed plants at two and 55 days after the onset of the stress for untargeted metabolomics (UM) analysis.

2. Materials & Methods:

2.1. Plant material, growth conditions, experimental design and saline stress

The accession of gliricidia [*Gliricidia sepium* (Jacq.) Steud.] used in this study belongs to the Gliricidia Collection at Embrapa Tabuleiros Costeiros (www.embrapa.br/en/tabuleiros-costeiros). After soaking the seeds in 2% sodium hypochlorite and Tween® 20 for 5 min under slow agitation, we washed them with sterile water and dried them on sterilized filter paper. Then they were placed in a Petri dish with filter paper moistened with sterilized water until the radicle emission. Subsequently, each germinated seed was transferred individually to a 5 L plastic pot containing 4 kg of substrate previously prepared by mixing sterile soil, vermiculite, and a commercial substrate (Bioplant®), in the ratio 2:1:1 (v:v:v); and kept in a greenhouse for three months.

Groups of five and a half-month-old gliricidia plants were kept under control conditions or subjected to saline stress (27 dS/m of electric conductivity) for 2 (short-term stress) or 55 (long-term stress) days. The experimental design was completely randomized with 12 replicates (plants) per treatment.

The NaCl was dissolved in deionized water to salinize the substrate. We replaced the water lost by evapotranspiration with deionized water on a daily basis, and the amount of deionized water used corresponded to the difference between the amount of water previously present in the substrate and the amount of water necessary for the substrate to reach field capacity. Applying the right amount of water – up to the substrate field capacity – was a means of ensuring no leakage of the solution out of the pot and no loss of Na^+ or Cl^- (Carvalho da Silva et al., in press). The electric conductivity and water potential in the substrate solution were monitored at zero and 25 days after imposing the stress treatment for all replicates.

2.2. Mineral analysis

Plant biomass was determined by harvesting the gliricidia plants at the end of the experiment, separating into their component parts, i.e., canopy and roots, which were weighed for fresh biomass determination. Then, they were dried in a forced-air oven at 65°C to constant weight for determination of dry biomass. Samples were collected for determination of mineral content; as well as samples of the substrate before and after plant growth. Dried samples were ground in a Wiley mill Tecnal Mod. TE 680 (Tecnal, Piracicaba, SP, Brazil), passed through a 1 mm (20 mesh) sieve and then subjected to extraction of minerals by the standard methods used in laboratory routine at Soloquímica (www.soloquimica.com.br).

Initially, mineral analysis data were examined for normality using the Shapiro-Wilk test. Then, parametric and non-parametric data were compared using the T-tests ($p<0.05$) and Mann-Whitney test ($p<0.05$), respectively; and biomass data were examined by linear regression analysis.

2.3. Metabolomics analysis

Leaves and roots from control and stressed plants – five replicates per treatment – were collected at 2 and 55 days after stress treatment (DAT), immediately immersed in liquid nitrogen, and then stored at -80 °C until extraction of metabolites.

2.3.1. Chemicals and metabolites extraction

Samples were grounded in liquid nitrogen before solvent extraction. The solvents methanol grade UHPLC, acetonitrile grade LC-MS, formic acid grade LC-MS and sodium

hydroxide ACS grade LC-MS were from Sigma-Aldrich (St. Louis, MO, USA); and the water treated in a Milli-Q system (Millipore, Bedford, MA, USA).

We employed a well known protocol (Vargas et al., 2016; Rodrigues-Neto et al., 2018) to extract the metabolites through a mixture of polar solvents. After transferring aliquots of 50 mg of grounded sample to 2 mL microtubes, 1 mL of a 1:3 (v:v) methanol: methyl tert-butyl ether mixture was added, and then left for homogenization at 4 °C on an orbital shaker for 10 min, followed by an ultrasound treatment in an ice bath for another 10 min. Next, 500 µL of a 1:3 (v:v) methanol: water mixture (1:3) was added to each microtube before centrifugation (12,000 rpm, 4 °C for 5 min). After centrifugation, three phases were obtained: a nonpolar phase for lipid analysis, a polar phase for secondary metabolism analysis and a pellet for protein analysis. The lipidic and polar fractions were transferred separately to 1.5 mL microtubes and vacuum dried overnight in room temperature in a Speed vac system (Centrivap, Labconco, Kansa, MO, USA). Polar and lipidic fractions samples were resuspended in a 500 µL of 1:3 (v:v) methanol: water mixture and transferred to vials prior to chemical analysis.

2.3.2. UHPLC-MS and UHPLC-MS/MS

A UHPLC chromatographic system was used (Nexera X2, Shimadzu Corporation, Japan) equipped with an Acquity UPLC HSS T3 (1.8 µm, 2.1 x 150 mm) reverse phase column (Waters Technologies, Milford, MA), maintained at 35 °C. A polar mobile phase was used, composed by water (solvent A) and acetonitrile / methanol (70/30, v/v) (solvent B), both in 0.1% formic acid. The gradient elution used, with a flow rate of 0.4 mL/min, was as follows: isocratic from 0 to 1 min (0% B), linear gradient from 1 to 3 min (5% B), from 3 to 10 min (50% B), and 10 to 13 min (100% B), isocratic from 13 to 15 min (100% B), followed by rebalancing in the initial conditions for 5 min.

A high-resolution mass spectrometry equipment (HRMS) (MaXis 4G Q-TOF MS, Bruker Daltonics, Germany), coupled to the UHPLC system, performed the chemical m/z detection using an electrospray source in positive (ESI (+) - MS) and negative (ESI (-) - MS) ionization modes. Final plate offset, 500 V; capillary voltage, 3800 V; nebulizer pressure, 4 bar; dry gas flow, 9 L/min, and dry temperature, 200 °C. The rate of acquisition spectra was 3.00 Hz, monitoring a mass range of 70 to 1200 m/z. A sodium formate solution (10 mM HCOONa solution in 50/50 v/v isopropanol/water containing 0.2% formic acid) was injected directly through a 6-way valve at the beginning of each chromatographic run for external calibration. Ampicillin ([M+H] + *m/z* 350.11867 and [M-H] - *m/z* 348.10288) was added to each sample as an internal standard for peak normalization.

Tandem mass spectrometry (MS/MS) parameters have been adjusted to improve mass fragmentation, with collision energy ranging from 20 to 50 eV, using a step method. Precursor ions were acquired using the 3.0 s cycle time. The general AutoMS settings were: mass range, *m/z* 70-1000 (polar fraction) and *m/z* 300-1600 (lipidic fraction); spectrum rate, 3 Hz; ionic, positive polarity; pre-pulse storage, 8 μs; funnel 1 RF, 250.0 Vpp. The UHPLC-MS and UHPLC-MS/MS data were acquired by HyStar Application version 3.2 (BrukerDaltonics, Germany).

2.3.3. Metabolomics data analysis

The raw data from UHPLC-MS were exported as mzMXL files, using DataAnalysis 4.2 software (Bruker Daltonics, Germany). During data pre-processing, peak detection, retention time correction and metabolites alignment were performed using XCMS Online (Gowda et al., 2014; Tautenhahn et al., 2012). Peak detection was performed using centWave peak detection (Δm/z = 10 ppm; minimum peak width, 5 s; maximum peak width, 20 s) and mzwid = 0.015, minfrac = 0.5, bw = 5 for the alignment of retention time. The unpaired parametric t-test (Welch t-test) was used for statistical analysis.

The processed data (csv file) were exported to MetaboAnalyst 5.0, and submitted to analysis in the Statistical Analysis module (Chong et al., 2019; Chong & Xia, 2020). Before the chemometric analysis, all data variables from the polar fraction were normalized by internal standard (ampicillin-rT = 7.9 min; [M+H], *m/z* = 350.1169509; sodium formate adduct-rT = 0.1 min, [M-H], *m/z* = 112.9854317); and, all data variables from the lipidic fraction were also normalized by internal standard (1,2-diheptadecanoyl-sn-glycero-3-phosphocholine = 4.85 min; [M+H] + *m/z* = 762.6002192). All three sets of data were scaled using the pareto method.

The differentially expressed peaks (DEP) were selected according to the following criteria: adjusted P-value (FDR) ≤ 0.050, of the Welch t-test. The selected DEPs were then submitted to analysis in the MS Peaks to Pathway module (Chong et al., 2019; Chong & Xia, 2020) and analyzed using the following parameters: molecular weight tolerance of 5 ppm; mixed ion mode; analysis using the mummichog algorithm (Li et al., 2013) with the default p-value cutoff, ranked by p-values, and the latest KEGG version of the *Arabidopsis thaliana* pathway library.

In the case of a DEP with two or more matched forms (isotopes) and later a matched compound with two or more DEPs, the criterion of metabolite selection applied was the mass difference comparing to the metabolite database – choosing the smallest one. Then, we used

the formula and exact mass data from KEGG to perform the putative annotation of the metabolites of interest, with just one candidate on each detected ion.

The KEGG IDs of the matched compounds were then submitted to pathway analysis (integrating enrichment analysis and pathway topology analysis) and visualization in the Pathway Analysis module (Chong et al., 2019; Chong & Xia, 2020) and analyzed using the Scatter plot (testing significant features) as visualization method, the Hypergeometric Test as the enrichment method, the Relative-betweeness Centrality as topology analysis, and the latest KEGG version of the *A. thaliana* pathway library as reference metabolome (from October 2019).

2.3.4. Metabolomics downstream analysis

Initially, correlation analyses were done between pairs of differentially expressed metabolites – a pairwise combination of the different scenarios evaluated – using homemade python scripts. The input data for the correlation analysis was the Log_2(FC).

Then, the Omics Fusion web platform (Brink et al., 2016) was used to verify which pathways the metabolites selected in the correlation analysis belonged to. The input data on the platform were the KEGG IDs and the Log_2(FC) of the metabolites and an enrichment analysis was initially carried out to discover the metabolic pathways to which they belong. The "KEGG feature distribution" module was used, which showed the metabolic pathways and the amount of metabolites differentially expressed in each pathway. To verify which were these metabolites, for each pathway, the module "Map data on the KEGG pathway" was then used.

3. Results:

3.1. *Gliricidia sepium* morphophysiological responses to salt stress

The evapotranspiration rates of the control (Figure 1A) were higher than salt-stressed treatment (Figure 1B) throughout the experiment. However, the shape of the curve was similar in both treatments. In addition to the temporal fluctuation in transpiration rates, it was observed that as the plants got older, more water was demanded.

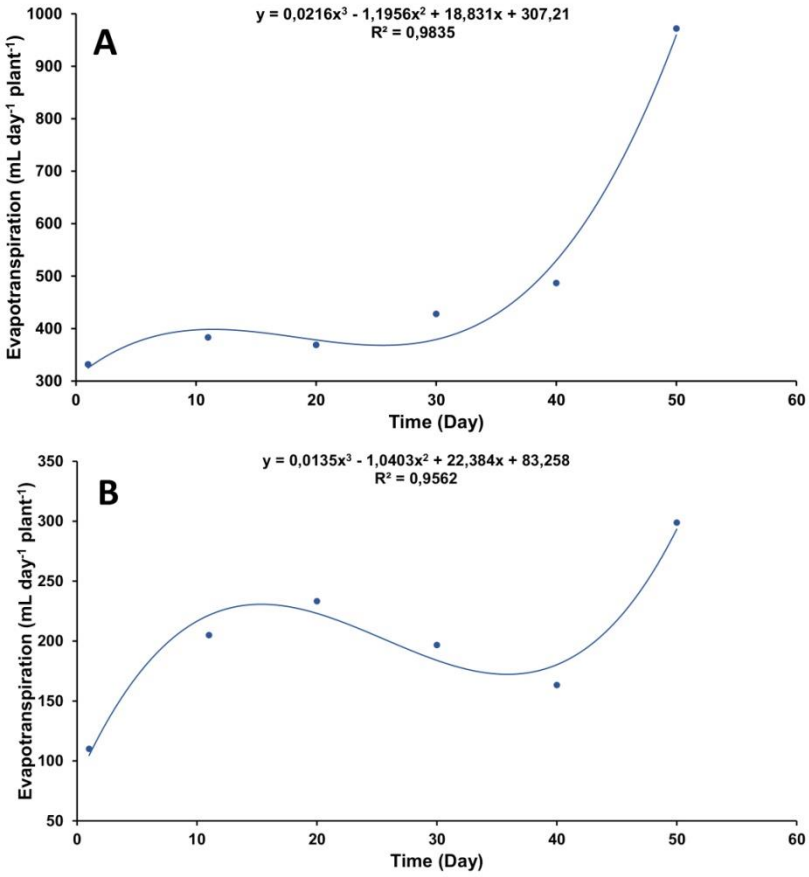


Figure 1. Daily evapotranspiration rate in gliricidia plants at 0.0 (A) and 0.8 (B) g of NaCl per 100 g of the substrate. The values represent the average of eleven replicates, in a timeline starting from one day up to 50 days of treatment.

The young gliricidia plants submitted to a very high level of salinity in this study showed the adaptation response previously reported by Carvalho da Silva et al. (in press). Immediately after adding salt to the substrate, the gliricidia plants were fully leafy, with completely green leaves (Figure 2A), but went on to lose all leaves before the end of the first week under salt stress (Figure 2B). Approximately three weeks after the onset of stress, new leaves started to emerge and continued to emerge and grow in the following weeks (Figure 2C and D).

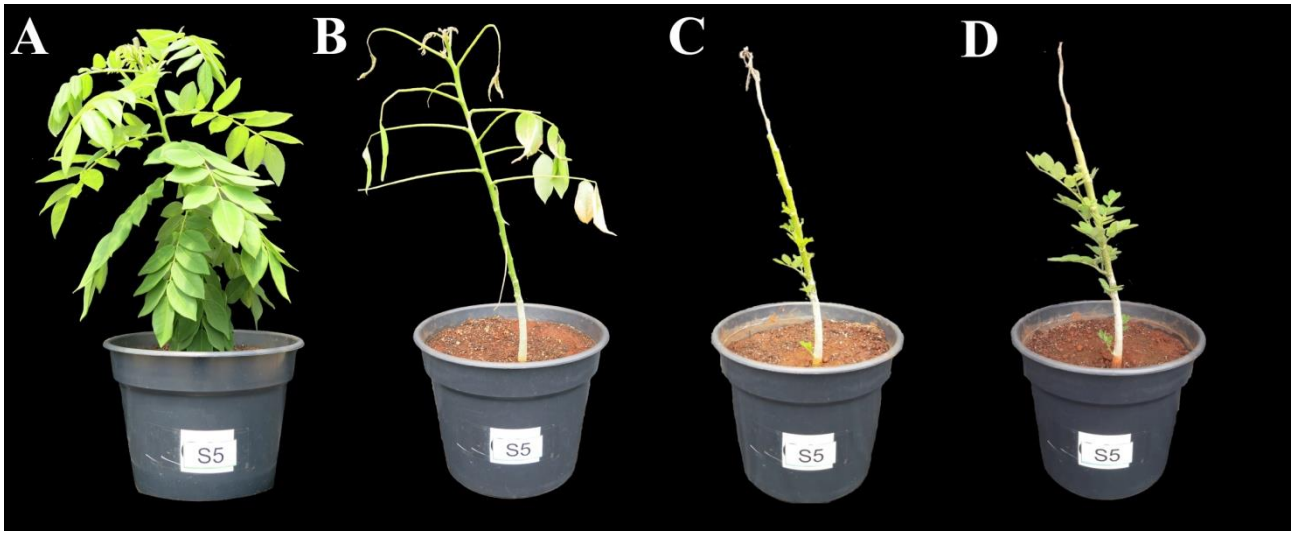


Figure 2. Timeline of the adaptation response in gliricidia plant under salinity stress. Plants at 0.8 g of NaCl per 100 g of the substrate. After 1 (A), 5 (B), 23 (C), and 34 (D) days of stress.

The addition of NaCl to the substrate led to a significant increase in the concentration of exchangeable Na^+, Mg^{2+}, and K^+. As a consequence, there was an increase in the sum of bases, cation exchange capacity, base saturation, and sodium saturation index. Additionally, a significant increase in organic matter and C was observed (Supplementary Table 1). The plants under salt stress accumulated significantly more Na^+ and Cl^- than control in both the canopy and the roots. P, Cu, and Zn ions were significantly increased in the canopy under salt stress, while Fe was significantly increased in the roots.

3.2. Gliricidia metabolome under salinity stress

Before submitting the data for analysis in the statistics module of MetaboAnalyst 5.0, they got separated into leaf and root data and organized as follows: all in treatments data set (control and stressed plants at 2 and 55 DAT); age effect – AE (samples from the control plants at 2 and 55 days after treatment - DAT); short-term stress – STS (the control and the stressed plants at 2 DAT); long-term stress 1 – LTS1 (control and stressed plants at 55 DAT); and long-term stress 2 – LTS2 (the stressed plants at 2 and 55 DAT). Each data set had five biological replicates per treatment.

3.2.1. Leaves

PLS-DA (partial least squares discriminant analysis) permutations tests were performed using the leaves all in treatments data set to validate the model, applying permutation number = 2,000. When evaluated by group separation distance, the probability

that the model was created by chance was 0.06 for the lipidic-positive fraction and less than 0.0005 for the other two fractions (Supplementary Figure 1).

The ANOVA analysis using the leaves all treatments data set generated 4,464 peaks with a p-value cutoff of 8.0E-5 that were submitted to functional interpretation via analysis in the MS Peaks to Pathway module. After applying the initial criteria of metabolite selection, 367 peaks with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in 13 pathways with a Raw p-value ≤ 0.05; they were: Ubiquinone and other terpenoid-quinone biosynthesis; Arginine biosynthesis; Lysine biosynthesis; Phenylpropanoid biosynthesis; Monobactam biosynthesis; Steroid biosynthesis; Carotenoid biosynthesis; Flavone and flavonol biosynthesis; Tyrosine metabolism; Valine, leucine and isoleucine biosynthesis; Porphyrin and chlorophyll metabolism; and Indole alkaloid biosynthesis. These pathways came out with 19, 11, 7, 20, 6, 19, 18, 6, 8, 10, 18, and 3 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

The changes in the metabolic profile due to the age effect, once there is a 53-day gap between the two assessments, were measured using the AE data set. The AE data set from leaves contained 816, 550, and 4,010 peaks, respectively, in the polar-positive, polar-negative, and lipidic-positive fractions; and a total of 3,168 (58.93%) peaks out of the 5,376 seen in the three fractions were differentially expressed - a differentially expressed peak (DEP) is a peak with an adjusted P-value (FDR) ≤ 0.05, and Log_2 (FC) > 0 (up-regulated) or Log_2 (FC) < 0 (down-regulated) (Table 1).

The short-term stress data set from leaves was employed to evaluate how distinct are the metabolome profiles of the control and stressed plants at 2 DAT, just one day before the leaves started to wilt. The samples applied to evaluate the STS scenario in the leaves contained 829, 589, and 4,010 peaks, respectively, and a total of 1,550 (28.56%) peaks out of the 5,428 seen in the three fractions were differentially expressed (Table 1).

The LTS1 data set from leaves was employed to evaluate how distinct are the metabolome profiles of the control and stressed plants at 55 DAT. The samples applied contained 788, 580, and 4,001 peaks, respectively, and a total of 1,285 (23.93%) peaks out of the 5,369 seen in the three fractions were differentially expressed (Table 1). The LTS2 data set from leaves was employed to evaluate how distinct are the metabolome profiles of the stressed plants at two and 55 DAT. The samples applied contained 827, 573, and 4,010 peaks, respectively, and a total of 2,615 (48.34%) peaks out of the 5,410 seen in the three fractions were differentially expressed (Table 1).

All 1,550 peaks differentially expressed in the STS were submitted to functional interpretation via analysis in the MS Peaks to Pathway module, with a p-value cutoff of 1.0E-5. After applying the initial criteria of metabolite selection, 197 DEPs with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in 16 pathways with a Raw p-value ≤ 0.05; they were: Valine, leucine, and isoleucine biosynthesis; Phenylpropanoid biosynthesis; Arginine biosynthesis; Monobactam biosynthesis; Flavone and flavonol biosynthesis; Indole alkaloid biosynthesis; Pyruvate metabolism; Lysine biosynthesis; Glycine, serine and threonine metabolism; beta-Alanine metabolism; Pantothenate and CoA biosynthesis; Glyoxylate and dicarboxylate metabolism; C5-Branched dibasic acid metabolism; Fructose and mannose metabolism, Anthocyanin biosynthesis, and Tyrosine metabolism (Figure 3A). These pathways came out with 10, 15, 8, 5, 5, 3, 7, 4, 9, 6, 7, 8, 3, 6, 4, and 5 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

The 2,615 peaks differentially expressed in the LTS2 were also submitted to functional interpretation via analysis in the MS Peaks to Pathway module, with a p-value cutoff of 1.0E-5. After applying the initial criteria of metabolite selection, 258 DEPs with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in nine pathways with a Raw p-value ≤ 0.05; they were: Ubiquinone and other terpenoid-quinone biosynthesis; Steroid biosynthesis; Monobactam biosynthesis; Lysine biosynthesis; Phenylpropanoid biosynthesis; Porphyrin and chlorophyll metabolism; Tyrosine metabolism; Arginine biosynthesis; and Brassinosteroid biosynthesis (Figure 3B). These pathways came out with 17, 18, 6, 6, 15, 15, 7, 7, and 9 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

3.2.3. Roots

PLS-DA permutations tests were performed using the roots all treatments data set (control and stressed plants at 2 and 55 DAT) to validate the model, applying permutation number = 2,000. When evaluated by group separation distance, the probability that the model was created by chance was 0.011 (polar-positive), 0.0005 (polar-negative), and 0.0415 (lipidic-positive) (Supplementary Figure 1).

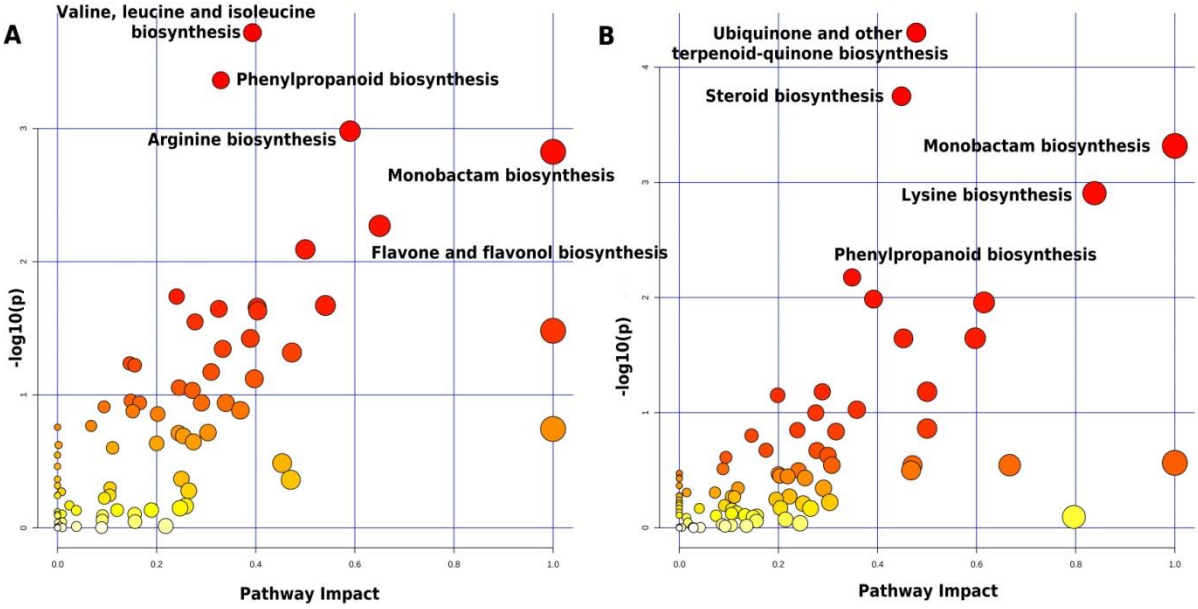


Figure 3 – Summary of Pathway Analysis of the data from leaves – STS (A) and LTS2 (B) – using the Pathway Topology Analysis modules of MetaboAnalyst 5.0. The metabolome view resulted from the analysis in the Pathway Topology Analysis module using the Hypergeometric test, the relative betweenness centrality node importance measure, and the latest KEGG version of the *A. thaliana* pathway library. STS = Short-term stress (the control and the stressed plants at 2 DAT), and LTS2 = Long-term stress 2 (the stressed plants at 2 and 55 DAT).

The ANOVA analysis using the roots all in treatments data set generated 3,600 peaks with a p-value ≤ 0.05, submitted to functional interpretation via analysis in the MS Peaks to Pathway module, with a p-value cutoff of 1.0E-5. After applying the initial criteria of metabolite selection, 326 peaks with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in 10 pathways with a Raw p-value ≤ 0.05; they were: Steroid biosynthesis; Lysine biosynthesis, Ubiquinone, and other terpenoid-quinone biosynthesis; Arginine biosynthesis; Monobactam biosynthesis; Carotenoid biosynthesis; Valine, leucine and isoleucine biosynthesis; Tyrosine metabolism; Phenylpropanoid biosynthesis; and Porphyrin and chlorophyll metabolism. These pathways came out with 20, 7, 17, 10, 6, 18, 10, 8, 16, and 16 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

The age effect data set from roots was employed to evaluate how distinct are the metabolome profiles of the control plants at 2 and 55 DAT, a 53-day gap between the two assessments. The AE data set contained 636, 392, and 3,976 peaks, respectively, in the polar-positive, polar-negative, and lipidic-positive fractions; and a total of 2,588 (51.72%) peaks out of the 5,004 seen in the three fractions were differentially expressed (Table 1).

The STS data set from roots was employed to evaluate how distinct are the metabolome profiles of the control and stressed plants at 2 DAT, just one day before the leaves started to wilt. The samples applied to evaluate the STS scenario in the leaves contained 666, 429, and 3,993 peaks, respectively, and a total of 390 (7.67%) peaks out of the 5,088 seen in the three fractions were differentially expressed (Table 1).

The LTS1 data set from roots was employed to evaluate how distinct are the metabolome profiles of the control and stressed plants at 55 DAT. The samples applied contained 561, 380, and 3,748 peaks, respectively, and a total of 160 (3.41%) peaks out of the 4,689 seen in the three fractions were differentially expressed (Table 1). A principal component analysis (PCA), an unsupervised method commonly used to identify patterns between multivariate samples, was applied to detect any inherent patterns within the data in the LTS1, and it was not able to completely separate the groups between the control and the stressed samples in the polar positive and lipidic positive fractions (Figure 4). The LTS2 data set from roots was employed to evaluate differences in the profiles from the stressed plants at two and 55 DAT. The samples applied contained 671, 421, and 3,990 peaks, respectively, and a total of 3,055 (60.11%) peaks out of the 5,082 seen in the three fractions were differentially expressed (Table 1).

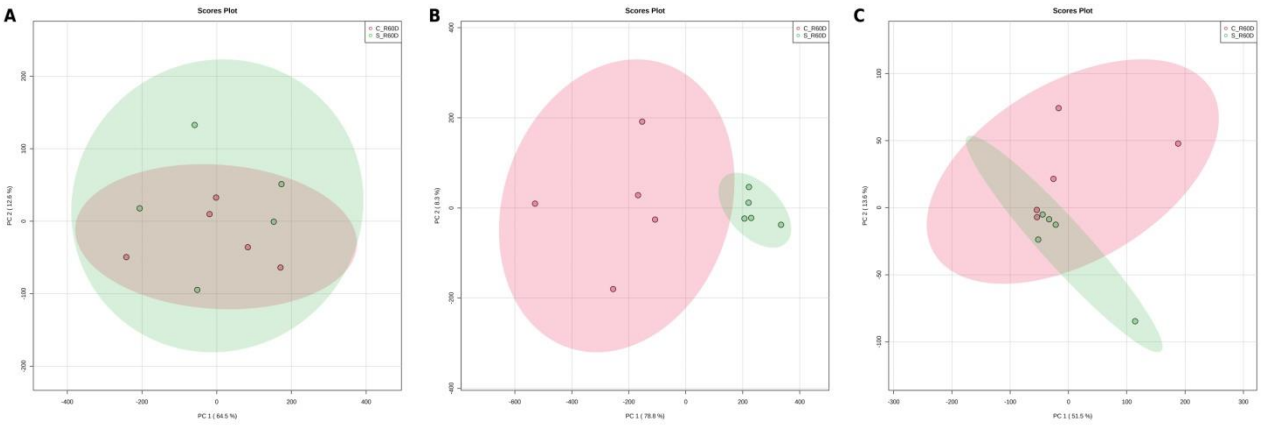


Figure 4. PCA score plots comparing roots samples from control and stressed plants at 55 DAT - LTS1 (Long-term stress 1). Polar positive (A), polar negative (B) and lipidic positive (C) fractions.

All 390 peaks differentially expressed in the STS were submitted to functional interpretation via analysis in the MS Peaks to Pathway module, with a p-value cutoff of 1.0E-5. After applying the initial criteria of metabolite selection, 83 DEPs with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in eight pathways with a Raw p-value ≤ 0.05. The indicated pathways were: Galactose metabolism; Valine, leucine, and isoleucine biosynthesis; Glucosinolate biosynthesis; Glycine, serine, and threonine metabolism; Pantothenate and CoA biosynthesis; Arginine and proline metabolism; C5-Branched dibasic acid metabolism; and Biosynthesis of secondary metabolites - other antibiotics. These pathways came out with 6, 5, 9, 5, 6, 4, 5, 2, and 2 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

All 160 peaks differentially expressed in the LTS1 were submitted to functional interpretation via analysis in the MS Peaks to Pathway module, with a p-value cutoff of 1.0E-5. After applying the initial criteria of metabolite selection, 39 DEPs with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in seven pathways with a Raw p-value ≤ 0.05; they were: Glyoxylate and dicarboxylate metabolism; Biosynthesis of secondary metabolites - other antibiotics; beta-Alanine metabolism; Citrate cycle (TCA cycle); Monobactam biosynthesis; Alanine, aspartate and glutamate metabolism; and Pantothenate and CoA biosynthesis. These pathways came out with 5, 2, 3, 3, 2, 3, and 3 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

At last, all 3,055 peaks differentially expressed in the LTS2 were submitted to functional interpretation via analysis in the MS Peaks to Pathway module, with a p-value cutoff of 1.0E-5. After applying the initial criteria of metabolite selection, 262 DEPs with a hit to just one known compound were submitted to the pathway topology analysis module, resulting in 10 pathways with a Raw p-value ≤ 0.05; they were: Steroid biosynthesis; Lysine biosynthesis; Carotenoid biosynthesis; Valine, leucine and isoleucine biosynthesis; Ubiquinone and other terpenoid-quinone biosynthesis; Monobactam biosynthesis; Arginine biosynthesis; Phenylpropanoid biosynthesis; Porphyrin and chlorophyll metabolism; and

Glyoxylate and dicarboxylate metabolism. These pathways came out with 20, 7, 16, 10, 14, 5, 8, 15, 14, and 9 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

3.3. Salt effect in metabolites differentially expressed contributing to the adaptation response

As the goal of this study was to look for metabolites which behavior could give insights into the salt resistance mechanisms behind the adaptation response, both in the leaves and the roots, a series of filters were applied to select peaks differentially expressed before undergoing analysis in the MS Peaks to Pathway module of the MetaboAnalyst 5.0.

First, the 5,354 peaks in the leaf samples common to the AE, STS, and LTS2 scenarios were selected. After that, the combined effect of the salt in the short and long-term stress - denominated LTS_Final - was calculated using the following equation: FC_LTS_Final = (FC_STS * FC_LTS2 * (1/FC_AE)); the log2(FC_LTS_Final) was then subsequently calculated. After removing those peaks non-differentially expressed in the AE and the LTS_Final scenarios, the remaining 4,126 ones underwent analysis in the MS Peaks to Pathway module. A total of 353 differentially expressed peaks with a single matched compound were submitted to the Pathway Topology Analysis module (Supplementary Table 2), resulting in 12 pathways with a Raw p-value ≤ 0.05; they were: Ubiquinone and other terpenoid-quinone biosynthesis; Phenylpropanoid biosynthesis; Arginine biosynthesis; Lysine biosynthesis; Monobactam biosynthesis; Steroid biosynthesis; Flavone and flavonol biosynthesis; Tyrosine metabolism; Valine, leucine and isoleucine biosynthesis; Porphyrin and chlorophyll metabolism; Indole alkaloid biosynthesis; and Pyruvate metabolism. These pathways came out with 19, 21, 11, 7, 6, 19, 6, 8, 10, 17, 3, and 9 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

At last, there were 4,954 peaks in the roots samples that were common to the AE, STS, and LTS2 scenarios. After submitting them to the same treatment mentioned above, a group of 3,557 peaks was left for analysis in the MS Peaks to Pathway module. A total of 309 differentially expressed peaks with a single matched compound were submitted to the Pathway Topology Analysis module (Supplementary Table 2), resulting in 11 pathways with a Raw p-value ≤ 0.05; they were: Steroid biosynthesis; Lysine biosynthesis; Monobactam biosynthesis; Ubiquinone and other terpenoid-quinone biosynthesis; Arginine biosynthesis; Valine, leucine and isoleucine biosynthesis; Carotenoid biosynthesis; Phenylpropanoid biosynthesis; Alanine, aspartate and glutamate metabolism; Porphyrin and chlorophyll

metabolism; and Tyrosine metabolism. These pathways came out with 20, 7, 6, 16, 9, 10, 16, 16, 9, 16, and 7 differentially expressed metabolites with the highest level of significance within the set of matched metabolites submitted to analysis, respectively (data not shown).

4. Discussion

One of the manners by which salt affects plants is through the osmotic effect. Salt reduces the osmotic potential and, consequently, diminish the soil water potential, making it difficult for plants to absorb water (Sánchez-Blanco et al., 2004; Franco et al., 2011; Hanin et al., 2016). Under such conditions, there is a reduction in the stomatal opening (Acosta-Motos et al., 2017). As a result, the plants transpire less, and therefore less water is lost by evaporation, as occurred with the salt-stressed gliricidia plants. However, irrespective of salt stress, weather variables such as radiation, temperature, and relative humidity continue to exert their effects on evapotranspiration. For this, the shape of both evapotranspiration curves, stressed or unstressed by salt, was the same (Figure 1).

The gliricidia control plants exerted their maximum transpiration capacity, as there was no obstacle in the soil limiting water availability. They had full foliage and high rates of evapotranspiration (Figure 1A and Figure 2A). In the salt-stressed gliricidia plants, on the other hand, the osmotic shock caused by the abrupt addition of salt to the substrate led to severe defoliation (Figure 2B). This fact is a drastic artifice used by plants to reduce the transpiring surface and maintain the water status (Alarcon et al., 2006), although it could also be a mechanism of salt exclusion (Acosta-Motos et al., 2017). The fact is that, after metabolic adjustment to that stressful situation, gliricidia plants developed new leaves and continued their development (Figure 2C and 2D).

The availability of exchangeable Na in the substrate represented 36% of the base saturation and was at least six times higher than K in the salt-stressed treatments. Even under these conditions, the gliricidia plants only absorbed a tiny part of the Na, while they absorbed Mg, Ca, and K just like the control ones (Supplementary Table 1), which suggests that it limited the entry of Na by the roots (Hanin et al., 2016) and maintain the nutritional balance. Therefore, it is a typical salt-excluding plant (Acosta -Motos et al., 2017). The accumulation of P, Cu, and Zn in the aerial part of stressed plants are compatible with the energy demand and enzymatic antioxidant activities during the adjustment process when new leaves were emerging.

The heatmaps generated out of the metabolome from the roots of young gliricidia plants submitted to salinity stress, using the all treatments data sets, revealed much closer proximity between treatments within the short-term scenario, as well as within the long-term

one, but only for the polar positive and lipidic positive fractions evaluated (Figure 5). To a certain extent, the same was true for the metabolome from the leaves (Figure 5). That led us to postulate that, once adapted to high salinity stress, most likely via a salt-excluding mechanism that limited the entry of Na in the roots, gliricidia plants resumed growth and went back to have almost the same metabolome profile as the control plants at the end of the experiment.

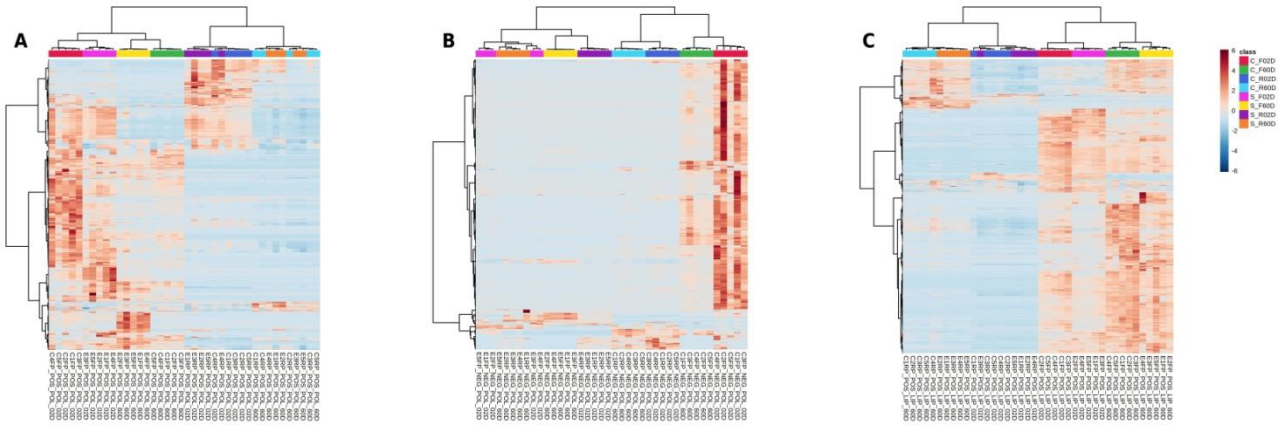


Figure 5. Heatmap analysis of the all treatments data sets (control and stressed plants at 2 and 55 DAT). Blue color indicates low intensity and red color indicates high intensity. The upper row represent sample groups (C – Control, S – Stressed, F – Leaves, and R – Roots). Polar positive (A), polar negative (B) and lipidic positive (C) fractions.

In the leaves, the Phenylpropanoid biosynthesis pathway always appeared as one of the most affected pathways, independent of the strategy used to analyze the different data sets, with 21 metabolites differently expressed. When further evaluating the changes experienced by these metabolites in the short-term and long-term stress, we found that 14 did downregulate and seven upregulate at 55 DAT in the stressed plants, in comparison with the control plants also at 55 DAT. Besides that, 12 were downregulated in the short-term but not long-term, two differentially regulated at short-term and long-term, and seven differentially regulated only at long-term (Table 2).

Taken together, the results that are shown in this study corroborate the previous work done by Carvalho da Silva et al. (in press), who showed that this pathway is highly affected in the leaves of young gliricidia plants by salt stress, but also that most of the salt effect occurs in the short-term. However, it goes forward and shows that most of those changes remained in the long-term, and also that some metabolites change in expression only during the process

of adaptation to high salinity experienced by these plants. In summary, this pathway plays a role in the response of young gliricidia plants to high salinity throughout the entire adaptation response, starting in the short term and continuing in the long one.

Zhu et al. (2021) also applied an integrated transcriptomic and metabolomic analysis to show that the Phenylpropanoid biosynthesis pathway had genes and metabolites that show a significant correlation in the roots of salt-stressed *Sophora alopecuroides*, which is a leguminous perennial herb that is an excellent sand-fixing pioneer plant distributed mainly in the desert and semi-desert areas of Northwest China.

Phenylpropanoids, a class of secondary metabolites showing indispensable roles in plant survival, are synthesized from phenylalanine (or tyrosine) through a series of enzymatic reactions (Deng; Lu, 2017). Their biosynthesis includes a collection of the first two or three steps of the phenylpropanoid pathway, which redirects carbon flow from primary metabolism to phenylpropanoid metabolism; and after the generation of intermediates in the initial steps, the carbon flow is channeled into specific branch pathways to produce flavonoids, stilbenes, monolignols, phenolic acids, and coumarins (Deng; Lu, 2017).

In the present study, L-Phenylalanine (C00079) is one of the three metabolites from this pathway that only differentiated in the long-term stress, experiencing an increase of 3.56X in its peak intensity due exclusively to the salt effect. The other two differentiated metabolites were Sinapic acid (C00482) and N1, N5, N10-Tricaffeoyl spermidine (C18070), which experienced a decrease of 96 and 54%, respectively, also due exclusively to the salt effect.

Ferulic acid (C01494) and Coniferyl aldehyde (C02666) were the two metabolites that experienced the most increase in expression level in the long-term - due exclusively to the salt effect - among those 21 metabolites differentially expressed in this pathway, experiencing 9.11X and 20.45X increase in intensity, respectively (Figure 6). Ferulic acid belongs to the hydroxycinnamic acid group, which is one of the two major subgroups of phenolic acids, likely produced by the oxidation of coniferaldehyde under the catalysis by coniferyl-aldehyde dehydrogenase. Many steps of the phenolic acid pathway are unknown, and it is still under revision (Deng; Lu, 2017). Recently, Linic et al. (2021) showed that exogenous application of a 10.0 µM solution of ferulic acid attenuated effects on salt-stressed Chinese cabbages plants, causing a decrease in proline and salicylic acid.

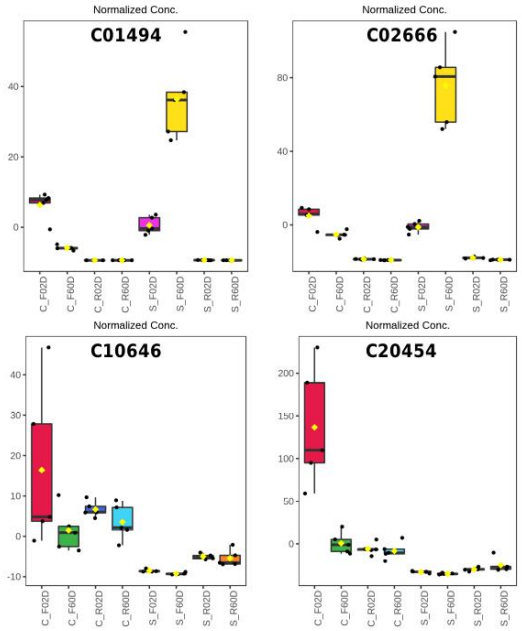


Figure 6. Box plot showing the normalized concentration of metabolites in the leaves and roots of young gliricidia plants submitted to salt stress. The expression profiles of Ferulic acid (C01494), Coniferyl aldehyde (C02666), (+)-Lariciresinol (C10646), and (-)-Lariciresinol (C20454) are shown in all treatments (C – Control, S – Stressed, F – Leaves, R – Roots, 02D – short-term stress, and 60D – long-term stress).

Besides Ferulic acid and Coniferyl aldehyde, three other metabolites upregulated at 55 DAT in the stressed plants, in comparison with the control plants also at 55 DAT; they were: 1-O-Sinapoyl-beta-D-glucose (C01175), Cinnamaldehyde (C00903), and 4-Coumaroylshikimate (C02947), which experienced an increase of 3.32X, 1.46X, and 2.22X, respectively. Accordingly to Chun et al. (2019), enzymes such as phenylalanine ammonia-lyase (PAL), cinnamoyl CoA reductase (EC 1.2.1.44), ferulate 5-hydroxylase (F5H), caffeate 3-O-methyltransferase (EC 2.1.1.68), and cinnamyl alcohol dehydrogenase (EC 1.1.1.195) - to mention a few - take part in the synthesis of monolignols. Peroxidases and laccases polymerize monolignols to yield lignin as a final product. At least four out of the seven metabolites that the expression level in the long-term increased due exclusively to the salt effect in this present study - L-Phenylalanine, Ferulic acid, Coniferyl aldehyde, and Cinnamaldehyde – are directly linked to these five enzymes.

When silencing the *CcoAOMT1* gene in *A. thaliana* and demonstrating that the mutants became phenotypically hypersensitive to salt stress, Chu and colleagues provided molecular and genetic evidence indicating the importance of enhanced lignin accumulation in the cell wall of this plant species during the responses to salt stress (Chu et al., 2019). The

lignin accumulation and the strong expression of lignin biosynthetic genes are factors key to acquiring salt tolerance in plants, and, based on the results from our present study, we postulate that this phenomenon is playing a role in the adaptation of young gliricidia plants to high levels of salt stress. Additional studies are necessary to investigate this hypothesis.

Only 3.4% of the peaks show differential expression in the LTS1 scenario, in contrast to the AE (51.7%) and LTS2 (60.1%) ones, in the roots of gliricidia plants. The principal component analysis (PCA) did not completely separate the control and stressed samples in the polar positive and the lipidic positive fraction (Figure 4), and all 160 differentially expressed peaks in the subsequent analysis came from the negative one. Together, these results show that the young gliricidia plants did experience a metabolic adjustment that led their metabolic status in the roots to pretty much the same one of the control plants; and that this adjustment most likely took place after the loss of the leaves.

Only 39 DEPs with a hit to just one known compound were found in LTS1 and submitted to the pathway topology analysis module, resulting in seven pathways with a Raw p-value ≤ 0.05. However, only the Biosynthesis of secondary metabolites - other antibiotics one suffered the utmost impact seen, with two lignans downregulated in the short-term and kept at a low level in the long-term stress, (+)-Lariciresinol (C10646) and (-)-Lariciresinol (C20454) (Figure 6). This finding opposes Xiao et al. (2020), who showed that the upregulation of lariciresinol biosynthesis in *Isatis indigotica*, particularly in tetraploids compared to diploids, improved root development, and enhanced salt and drought stress tolerance.

In the roots, the Steroid biosynthesis is the most affected pathway in the long-term stress, with 20 differently expressed metabolites. Fourteen of them are from the Phytosterol biosynthesis module (M00917 - squalene 2,3-epoxide => campesterol/sitosterol). When evaluating the changes experienced by these 20 metabolites in the short-term and long-term stress, none of them expressed differentially at 02 DAT (Table 2). Lathosterol (C01189) had the utmost change in the expression level due exclusively to salt stress, with a 4.14X increase after the short-term. Cycloeucalenone (C22121), with 1.95X increase, and Squalene 2,3-epoxide (C01054) and 24-Methylidenecycloartanol (C08830), both with approximately 1.7X.

Over 250 different plant sterols are known, among which campesterol, sitosterol, and stigmasterol are the most abundant ones in most plants (Moreau et al., 2018). An overall summary of the enzymes involved in the biosynthesis of phytosterols is lacking, and very little research on the metabolism and regulation of phytosterols biosynthesis has been reported (Zang et al., 2020). According to Zang et al. (2020), sterols profiles vary greatly among plant

species and seem to be accumulated more in plants with higher tolerance to salinity; these changes of phytosterols accumulation can indicate plant adaptability to stresses to a certain extent. Phytosterols may help plants adapt to environmental stresses by adjusting membrane structure and characteristics, including the recruitment of specific proteins attached to the plasma membrane to transduce signals (Zang et al., 2020). Maintenance of membrane homeostasis represents one of the principal functions of sterols in plant cells (Rogowska & Szakiel, 2020), and phytosterols and their corresponding esters have a pivotal role in imparting tolerance to abiotic stresses by maintaining cell membrane integrity (Kumar et al., 2018).

In the roots of salt-stressed gliricidia plants, the Lysine biosynthesis appeared as the second most affected pathway in the long-term, with seven metabolites expressed differently; being all of them from the DAP aminotransferase pathway module (Hudson et al., 2006). When further evaluating the changes experienced by these seven metabolites in the short-term and long-term stress, four of them were downregulated (C00441, C00666, C00680, and C03972) and three upregulated (C00049, C03082, and C20258) in the long-term, due exclusively to salt stress. None of them expressed differentially at 02 DAT, short-term (Table 2).

In higher plants, the synthesis of Lysine (Lys) occurs in plastids via the diaminopimelate (DAP) pathway, and no evidence exists for its cytosolic synthesis (Hudson et al., 2006; Kishor et al., 2020). When accumulated in high concentrations, Lys may be toxic to the plants and must then undergo degradation via the cadaverine, the saccharopine, or the NHP pathway (Kishor et al., 2020; Arruda and Barreto, 2020). The biosynthesis of lysine or its accumulation profoundly affects the cellular energy status, leading to an energy limitation (Ali et al., 2019). Lysine catabolism, consequently, is stimulated to create additional energy (Ali et al., 2019). In the present study, Lys was not among the metabolites expressed differentially in the roots of gliricidia plants due to salt stress; so, it is not possible to say whether these plants are experiencing energy limitations or not. However, as six metabolites (C00026, C00042, C00164, C00408, C00449, and C04076) from the Lys degradation pathways were found in the roots of gliricidia plants after 55 days under stress, and all of them at levels similar to the ones in the control plants (data not shown), one can postulate that Lys degradation did not play a role in promoting the adaptation response.

The (2S,4S)-4-Hydroxy-2,3,4,5-tetrahydrodipicolinate - HTPA (C20258) metabolite experienced, by far, the utmost increase (11.47X) in expression level in the long-term after removing the age effect; and the LL-2,6-Diaminopimelic acid (C00666) the utmost decrease

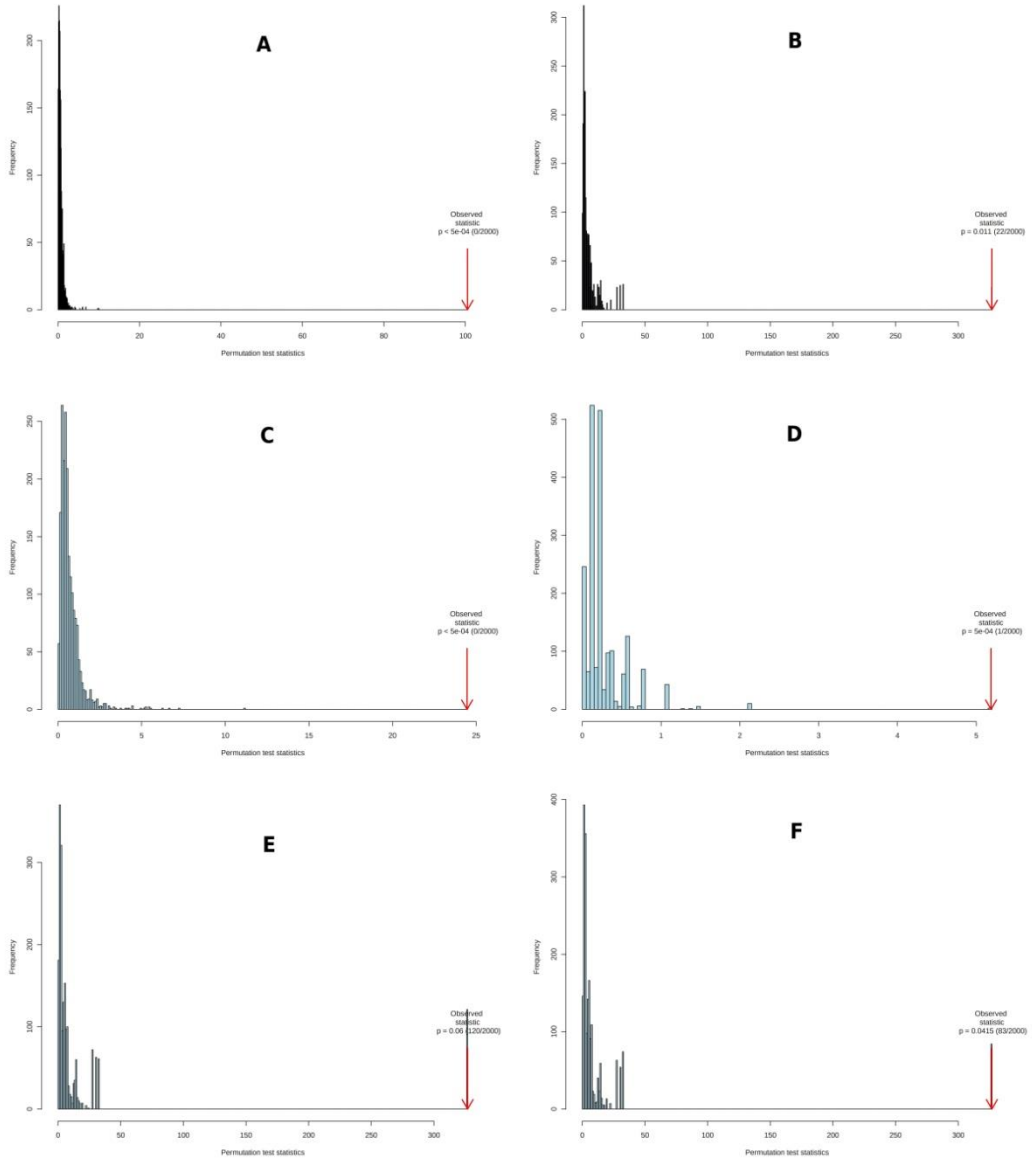
(94%). Since HTPA expression upregulates due to salt stress, one can infer that the protein expressed by a putative homolog of the *dapA* gene in *G. sepium* is Lys-insensitive or that the amount of Lys is not enough to trigger a feedback inhibition (Galili, 2002). The 4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7), which catalyzes the production of HTPA, is coded by the *dapA* gene (Soares da Costa et al., 2021).

5. Conclusion

In the salt-stressed gliricidia plants, the osmotic shock caused by the abrupt addition of salt to the substrate led to severe defoliation. After a metabolic adjustment to that stressful situation, those plants released new leaves and continued their development. These salt-adapted gliricidia plants only absorbed a tiny part of the Na present in the substrate while absorbing Mg, Ca, and K just like the control ones. The limited entry of Na and the maintenance of the nutritional balance in the roots, plus the fact that the roots metabolome profiles of the control and adapted plants were very similar, led us to postulate that these plants are salt-excluding plants that adapted to high salinity stress via two salt-excluding mechanisms, starting in the canopy - severe defoliation - and concluding in the roots - limited entry of Na.

This present study not only corroborates our previous study that indicated that the phenylpropanoid biosynthesis pathway has a role in the response of gliricidia plants to a very high level of salinity (Carvalho da Silva et al., in press) but went forward and also showed that most of the changes experienced by this pathway in the leaves during the short-term stress, remained in the long-term. However, some metabolites from this pathway play a role only in the long-term response to this stress. In summary, this pathway plays a role throughout the entire adaptation process, starting in the short term and continuing in the long.

Based on the results from our present study - from leaves and roots - we can also postulate that the accumulation of lignin and some phytosterols, as well as lysine biosynthesis - but not degradation, play a role in promoting the adaptation response of gliricidia plants to a very high level of salinity. However, additional studies are necessary to investigate this hypothesis.



Supplementary Figure 1. PLS-DA permutation validation evaluated by group separation distance, applying permutation number = 2,000. From the all treatments leaves (A, C, and E) and roots (B, D, and F) data sets (control and stressed plants at 2 and 55 DAT). Polar-positive (A, B), polar-negative (C, D), and lipidic-positive (E, F) fractions.

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Conflicts of Interest

The authors declare no conflicts of interest.

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List of Figures

List of Tables

Table 1 - Differentially expressed peaks and features in the leaves and roots of gliricidia plants submitted to salinity stress in four distinct scenarios: age effect - AE (control plants at 2 and 55 days under salinity stress – DAT); short-term stress – STS (control and the stress plants at 2 DAT); long-term stress 1 – LTS1 (control and stressed plants at 55 DAT); and long-term stress 2 – LTS2 (stressed plants at 2 and 55 DAT). The differentially expressed peaks are those with an adjusted P-value (FDR) ≤ 0.05, of the Welch t-test; and Log_2 (Fold Change) ≠ 1.

Table 2 - Differentially expressed peaks and features from the Phenylpropanoid Biosynthesis pathway (leaves), Steroid Biosynthesis (roots), and Lysine Biosynthesis (roots), in gliricidia plants submitted to salinity stress in three distinct scenarios: age effect - AE (control plants at 2 and 55 days under salinity stress – DAT), short-term stress – STS (control and the stress plants at 2 DAT); and long-term stress 2 – LTS2 (stressed plants at 2 and 55 DAT). The differentially expressed peaks are those with an adjusted P-value (FDR) ≤ 0.05, of the Welch t-test; and Log_2 (Fold Change) ≠ 1. LTS_Final = Effect of salt stress in the long-term discounted the age effect. Down = Downregulated, Up = Upregulated, Non-DE = Non-Differentially Expressed. If False Discovery Rate (FDR) ≤ 0.05, then Profile was called Non-DE, and FC (Fold Change) became 1.0.

Supplementary Table 1. Physicochemical properties of the substrate and the mineral composition of canopy and roots of gliricidia plants at the end of the experiment, accordingly to the NaCl level (g of NaCl per 100 g of substrate) applied. The values represent an average of five samples, followed by the standard deviation of the mean. The asterisks indicate a significant difference between the two groups (t-test or Mann-Whitney test).* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. pH H_2O: pH of the soil in water; P: Phosphorus; Ca^{2+}: Calcium; Mg^{2+}: Magnesium; K^{+}: Potassium; Na^{+}: Sodium; Al^{3+}: Aluminum; CTC: Cation exchange capacity; V: Base saturation; m: Saturation by aluminum; ISNa: Sodium Saturation Index; OM: Organic Matter; B: Boron; Cu: Copper; Fe: Iron; Mn: Manganese; Zn: Zinc; S: Sulfur; N: Nitrogen; and Cl-: Chlorine. ND – Not Determined.

Supplementary Table 2. List of differentially expressed peaks (m.z) resulted from the Pathway Analysis using the MS Peaks to Pathway module of MetaboAnalyst 5.0, by organ – Leaf or Roots. Data set showing the m.z, the mode – positive or negative, the KEGG id of the matched compound, matched form, mass difference, fold change - FC, Log_2(FC), and profile, in each one of the three scenarios evaluated: age effect - AE (control plants at 2 and 55 days

under salinity stress – DAT), short-term stress – STS (control and the stress plants at 2 DAT); and long-term stress 2 – LTS2 (stressed plants at 2 and 55 DAT). The differentially expressed peaks are those with an adjusted P-value (FDR) ≤ 0.05, of the Welch t-test; and Log_2 (Fold Change) ≠ 1. LTS_Final = Effect of salt stress in the long-term discounted the age effect. Down = Downregulated, Up = Upregulated, Non-DE = Non-Differentially Expressed. If False Discovery Rate (FDR) ≤ 0.05, then Profile was called Non-DE, and FC (Fold Change) became 1.0.

CAPÍTULO 3

Proteomics analysis to further characterize the adaptation response of *gliricidia sepium* (jacq.) Walp. to very high salinity stress

A versão apresentada do presente artigo será submetida para publicação (a definir), sendo uma versão preliminar e o conselho editorial do periódico poderá sugerir alterações

RESUMO

A *Gliricidia sepium* (Jacq.) Walp é uma árvore polivalente e pode se adaptar a um nível muito alto de estresse salino (≥20 dS m-1). Estudos iniciais sobre a integração de perfis de metaboloma e transcriptoma de folhas de gliricídia mostraram que a via de biossíntese de fenilpropanóides tem um papel central na resposta de curto prazo desta espécie ao estresse salino. Um estudo subsequente sobre a metabolômica não direcionada de folhas e raízes de plantas de gliricídia sob estresse salino não apenas corroborou esse estudo inicial, mas avançou e também mostrou que a maioria das mudanças experimentadas por essa via nas folhas durante o estresse de curto prazo permaneceu no longo prazo; e também mostrou que alguns metabólitos dessa via desempenham um papel apenas na resposta de longo prazo. O objetivo do presente estudo foi realizar uma análise proteômica usando as mesmas amostras de folhas e raízes usadas no estudo metabolômico anterior para obter informações adicionais sobre os mecanismos moleculares por trás da resposta de adaptação. Após os extratos protéicos totais para análise LC-MS, os dados brutos gerados no curto prazo (STS) e longo prazo (LTS) foram quantificados e a caracterização qualitativa realizada pelo software PatternLab for Proteômic V nos cenários distintos. Os números de proteínas encontradas no STS foram muito menores do que no LTS, em ambos os órgãos. A análise de expressão diferencial revelou três grupos de proteínas, aquelas presentes apenas nas plantas controle, aquelas presentes apenas nas estressadas e aquelas presentes em ambas. A análise de anotação funcional revelou a prevalência de proteínas dos grupos de Processamento de Informação Genética e Metabolismo de Carboidratos em ambos os tecidos de plantas estressadas por sal; e em ambos os cenários.

Palavras-chave: Proteômica, Estresse abiótico, PatternLab for Proteômica V, Processamento de Informação Genética, Metabolismo de carboidratos.

ABSTRACT

The multipurpose tree *Gliricidia sepium (Jacq.) Walp* can adapt to a very high level of salinity stress (≥20 dS m-1). Initial studies on the integration of metabolome and transcriptome profiles from gliricidia leaves did show that the phenylpropanoid biosynthesis pathway does have a central role in the short-term response of this species to salinity stress. A subsequent study on untargeted metabolomics of leaves and roots of gliricidia plants under salt stress not only corroborated that initial study but went forward and also showed that most of the changes experienced by this pathway in the leaves during the short-term stress remained in the long-term; and also showed that some metabolites from this pathway play a role only in the long-term response. The objective of this present study was to carry out a proteome analysis using the same leaves and roots samples used in that previous metabolomics study to gain additional insights into the molecular mechanisms behind the adaptation response. After submitting the total protein extracts to LC-MS/MS analysis, the raw data generated underwent quantitative and qualitative characterization in the PatternLab for Proteomics V software in two distinct scenarios - short-term (STS) and long-term stress (LTS). The numbers of proteins found in the STS were much lower than in the LTS, in both organs. The differential expression analysis revealed three groups of proteins, those present only in the control plants, those present only in the stressed, and those present in both. The functional annotation analysis revealed a prevalence of proteins from Genetic Information Processing and Carbohydrate metabolism groups in both tissues of salt-stressed plants; and in both scenarios.

Keywords: Proteomics, Abiotic stress, PatternLab for Proteomics V, Genetic Information Processing, Carbohydrate metabolism.

Proteomics analysis to further characterize the adaptation response of *gliricidia sepium* (jacq.) Walp. to very high salinity stress

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1 Introduction

Climate change generates plant stresses that negatively impact plant development and, consequently, agriculture production. Salinity stress is an example of primary stress with a broad distribution worldwide which is increasing in intensity due to climate change, mainly due to anthropogenic activities, including deforestation and inadequate irrigation practices (FRUKH; AHMAD; SIDDIQI, 2019; CHE-OTHMAN; MILLAR; TAYLOR, 2017).

Gliricidia sepium (Jacq.) Walp. is a medium-sized legume belonging to the Fabaceae family. It presents rapid growth and easy propagation, is nitrogen-fixing species, and is used for various purposes, such as fences, shade, firewood, animal feed, and others (TARIQ; ZAFAR; NAZLI, 2019). At an economic level, gliricidia can improve water infiltration and increase the soil's water retention capacity, enabling the reduction of erosion and restoring and improving soil quality (DIOUF et al., 2017). Some studies have shown that gliricidia is highly tolerant to salinity stress (RAHMAN et al., 2019; CARVALHO DA SILVA et al., 2021; BRAGA et al., unpublished).

In previous studies done by our group, we reported that young gliricidia plants undergo severe defoliation due to an osmotic shock caused by the abrupt addition of salt to the substrate, but can perform a metabolic adjustment to that stressful situation, adapting to the stress and resuming growth (CARVALHO DA SILVA et al., 2021; BRAGA et al., unpublished). Salt-adapted young gliricidia plants are most likely salt-excluding plants adapted to high salinity stress via two salt-excluding mechanisms, severe defoliation, and limited entry of Na through the roots (BRAGA et al., unpublished). When employing single and integrative transcriptomic and metabolomic analysis approaches, the studies above-mentioned did show that the phenylpropanoid biosynthesis pathway plays a role throughout the entire adaptation process, starting in the short term and continuing in the long (BRAGA et al., unpublished). The accumulation of lignin, lysine, and some phytosterols seems to also play a role in promoting the adaptation response of gliricidia plants to a very high level of salinity (BRAGA et al., unpublished).

A proteome is a set of proteins produced in an organism, system, or biological context, and proteomics is the large-scale study of proteomes. The proteome is not constant; it differs from cell to cell and changes over time. The proteome reflects the underlying transcriptome; however, many other factors modulate its expression besides the expression level of the

relevant gene (J V JORRIN NOVO, 2021). To unveil the mechanisms behind plant responses to stress, as part of a multi-omics/integratomics approach to understanding/explaining a tolerance to that stress, it is also essential to study the role of proteins. Therefore, the study of proteomics has evolved exponentially to become one of the best "omics" tools for discovering proteins and pathways associated with physiological processes in response to stress (AHMAD et al., 2016).

The current study is a follow-up to our previous studies (CARVALHO DA SILVA et al., 2021; BRAGA et al., unpublished). Hence, the objective of this present study was to carry out a proteome analysis of *G. sepium* plants submitted to a very high salt stress (27 dS m^{-1} of electrical conductivity). For this purpose, gliricidia plants were under salinity stress, and leaves and roots samples were collected from control and stressed plants at two and 55 days after the onset of the stress for proteomics analysis.

2 Materials & Methods

2.1 Plant material, growth conditions, experimental design and saline stress

The accession of gliricidia [*Gliricidia sepium* (Jacq.) Steud.] used in this study belongs to the Gliricidia Collection at Embrapa Tabuleiros Costeiros (www.embrapa.br/en/tabuleiros-costeiros). After soaking the seeds in 2% sodium hypochlorite and Tween® 20 for 5 min under slow agitation, we washed them with sterile water and dried them on sterilized filter paper. Then they were placed in a Petri dish with filter paper moistened with sterilized water until the radicle emission. Subsequently, each germinated seed was transferred individually to a 5 L plastic pot containing 4 kg of substrate previously prepared by mixing sterile soil, vermiculite, and a commercial substrate (Bioplant®), in the ratio 2:1:1 (v:v:v); and kept in a greenhouse for three months.

Groups of five and a half-month-old gliricidia plants were kept under control conditions or subjected to saline stress (27 dS m^{-1} of electric conductivity) for two (Short-term Stress, STS) or 55 (Long-term Stress, LTS) days. The experimental design was completely randomized with 12 replicates (plants) per treatment.

The NaCl was dissolved in deionized water to salinize the substrate. We replaced the water lost by evapotranspiration with deionized water on a daily basis, and the amount of deionized water used corresponded to the difference between the amount of water previously present in the substrate and the amount of water necessary for the substrate to reach field capacity. Applying the right amount of water – up to the substrate field capacity – was a means of ensuring no leakage of the solution out of the pot and no loss of Na^+ or Cl^-

(Carvalho da Silva et al., 2021). The electric conductivity and water potential in the substrate solution were monitored at zero and 25 days after imposing the stress treatment for all replicates.

2.2 Total Protein Extraction and LC-MS/MS Analysis

Leaves and roots for proteomics analysis were collected from all replicates at two and 55 days after imposing the treatments (DAT), immediately immersed in liquid nitrogen, and then stored at -80 ℃ until extraction of proteins.

The samples were ground in liquid nitrogen and added 0.02 g/g of PVPP (polyvinylpolypyrrolidone) to remove interfering polyphenolic compounds. Approximately 5.0 g of ground tissue were weighed and mixed with 3.0 mL of buffer (50 mM Tris HCl + 14 mM β-mercaptoethanol, pH 7.5) and 30 µL of protease inhibitor per sample. The suspension was gently stirred on ice for 10 minutes and then centrifugated at 10.000G at 4.0°C for 15 minutes. Soon after, 1.0 ml of the supernatant was transferred to 2.0 ml microtubes, mixed with 1.0 ml of 10% TCA (trichloroacetic acid) solution in acetone, and incubated for 2 hours at -20°C for protein precipitation. The samples were then centrifuged at 10,000G at 4.0°C for 15 minutes. After washing the protein pellet with ice-cold 80% acetone, it was centrifugated again under the same conditions as above; this final step was repeated twice. After drying the pellet, it was stored at -80°C until protein quantification by the method of (BRADFORD, 1976) and visualization in an SDS-PAGE Gel.

Samples were subjected to protein preparation and LC-MS/MS analysis at the GenOne company (Rio de Janeiro, RJ, Brazil). Firstly, the samples were treated with 10mM DTT at 56˚C for 30 min followed by 40 mM iodoacetamide (IDA) at room temperature in the dark for 30 min. The sample was then digested with Trypsin 1:50 and incubated for 20h at 37˚C in Thermomixer at 800 rpm. Tryptic peptides extraction was then performed by adding in 50 µl of 95% acetonitrile and 5% TFA, stirring at 1000 rpm for 15 min for 3x. The resulting sample was vacuum dried and dissolved in 20µl of 0.1% formic acid in water.

A label-free quantitation approach using spectral counting by LC-MS/MS was adopted to a global proteomic analysis. The samples were passed through a nano high performance liquid chromatography (EASY 1000; Thermo Fisher, US) coupled to Orbitrap Q Exactive Plus (Thermo Scientific, US) mass spectrometer. The MS scan spectra ranged from 375 to 2000 m/z and was acquired in the Orbitrap with a resolution of 70,000. Data were acquired in biological triplicates using the Xcalibur software (version 2.0.7).

2.3 Proteomics data analysis and annotation

Raw files were processed using the PatternLab for Proteomics V software (http://www.patternlabforproteomics.org) (Carvalho et al. 2016). The peptide sequence matching (PSM) was performed using the Comet algorithm (Eng et al. 2013) against the *Glycine max* protein database, downloaded in Uniprot proteome (Proteome ID: UP000008827). We employed a target-reverse strategy to increase confidence in protein identifications (Elias et al., 2007). The search performed considered semi-specific candidates, and a maximum of two missed cleavages were allowed. Cysteine carbamidomethylation and methionine oxidation were considered fixed and variable modifications, respectively. The Comet search engine did consider a precursor mass tolerance of 40 ppm and a fragment compartment tolerance of 0.02.

The validity of the peptide spectrum matches was evaluated using the Search Engine Processor (SEPro) module of PatternLab (Carvalho et al., 2012). Identifications were grouped by enzymatic specificity (semi-specific), resulting in two distinct subgroups. For each outcome, we used XCorr, DeltaCN, Spectral Counting Score, and Peaks Matched values to generate a Bayesian discriminator. SEPro then automatically established a cutoff score to accept a 1% false discovery rate (FDR) based on the number of decoys performed independently on each subset of data, resulting in a false positive rate independent of the triptych status. In addition, a minimum sequence length of 6 amino acid residues was required. Similar proteins that represent an identical sequence and consist of a fragment of another sequence were discarded. Thus, only PSMs with less than 5 ppm were considered to compose a final list of mapped proteins.

The results obtained through PatternLab V underwent a functional classification for both groups, leaves, and roots, in both treatments, STS and LTS. Distinct multiFASTA files generated for each organ and treatment - proteins present only in the control group, only in the stressed group, and differentially expressed when comparing the stressed and control groups - were submitted to the functional classification in the GhostKOALA platform (https://kegg.jp/ghostkoala).

3 Results

The protein quantification reproducibility was determined according to the normalized spectral abundance factors established by the PatternLab V for Proteomics software. The raw files obtained through MS are available upon reasonable request to use in several other proteomic analysis software.

3.1. Leaf proteome at Short-term Stress

The changes at the protein profiles in the leaves of young gliricidia plants submitted to short-term stress (STS) led to identifying a total of 817 and 828 peptides in control and stressed samples, respectively; which infers up to 1,189 protein entries from the database under both conditions (Supplementary Table 1A). Out of the 1,048 peptides identified, 34 were present in at least 10 proteins (Supplementary Table 1B). Approximately 11% (136) had at least one unique peptide observed, and about 16% of the proteins (194) inferred from more than four peptides (Supplementary Table 1C). A simplified list of 409 proteins, according to the maximum parsimony criterion, is available in Supplementary Table 1D.

The control and stressed samples presented 197 proteins, with 10 and 10 detected exclusively in control and stressed samples, respectively, when the minimum number of replicates applied was two (Figure 1A, Supplementary Table 2A and 2B). Out of the 177 proteins present in the leaf control and stressed, and differently expressed according to abundance were reported by the PatternLab's TFold module, only three showed statistically significant differences in their abundance in the short-term stress (Figure 2A – Blue dots – and Supplementary Table 2C). All three proteins were significantly down-regulated in the salt-stressed group compared to the control plants.

Regarding the functional classification, 17 and 14 protein groups were identified in the leaves of both control and stressed plants, respectively, and, in the case of the DE proteins, two distinct groups appeared (Figure 3). The carbohydrate metabolism (10 proteins), the genetic information process (9), and the energy, and the amino acid metabolism, tied with six

proteins each, were the main functional groups in the control treatment (Figure 3A). In the stressed plants, we identified the genetic information processing groups with 16 proteins, followed by carbohydrate metabolism (15) and metabolism-related proteins (6) (Figure 3B). At last, the proteins that had a significant difference belong to carbohydrate metabolism (2 proteins) and the cellular process (1 protein) (Figure 3C).

3.2. Leaf proteome at Long-term Stress

The changes at the protein profiles in the leaves of young gliricidia plants submitted to long-term stress (LTS) led to identifying 5,610 and 4,607 peptides respectively in control and stressed samples, which infers up to 3,944 protein entries from the database in both conditions (Supplementary Table 3A). Out of the 6,712 peptides identified, 81 were present in at least 10 proteins (Supplementary Table 3B). Approximately 8% (332) had at least one unique peptide observed, and about 28% of the proteins (1,109) inferred from more than four peptides (Supplementary Table 3C). A simplified list of 1,778 proteins, according to the maximum parsimony criterion, is available in Supplementary Table 3D.

The control and stressed samples presented 789 proteins, with 99 and 26 detected exclusively in control and stressed samples, respectively, when the minimum number of replicates applied was two (Figure 1B, Supplementary Table 3A and 3B). Out of the 664 proteins present in the leaf control and stressed, and differently expressed according to abundance were reported by the PatternLab's TFold module, 44 showed statistically significant differences in their abundance in the long-term stress (Figure 2B – Blue dots – and Supplementary Table 4C). Thirty proteins were significantly up-regulated in the salt-stressed group compared to the control plants, and 14 down.

In the functional classification, 19 protein groups were identified in both the leaves of control and stressed plants, and concerning DE proteins (forty-four proteins) 10 groups were observed (Figure 4). Analyzing the plants belonging to the control, we have 57 proteins

involved in the processing of genetic information, 27 related to carbohydrate metabolism, and 20 families of proteins involved in the processing of genetic information (Figure 4A); in stressed plants we identified the genetic information processing groups with 21 proteins, followed by carbohydrate metabolism with 17 and a family of proteins involved in the genetic information process with 13 (Figure 4B); and finally, the proteins that had a significant difference belong to the metabolism of carbohydrates (11 proteins), to the processing of cellular genetic information (8) and to the metabolism of amino acids (4) (Figure 4C).

3.3. Roots proteome at Short-term Stress

The changes at the protein profiles in the roots of young gliricidia plants submitted to short-term stress (STS) led to identifying a total of 1,599 and 2,047 peptides in control and stressed samples, respectively; which infers up to 2,110 protein entries from the database under both conditions (Supplementary Table 5A). Out of the 2,562 peptides identified, 100 were present in at least 10 proteins (Supplementary Table 5B). Approximately 15% (313) had at least one unique peptide observed, and about 28% of the proteins (585) inferred from more than four peptides (Supplementary Table 5C). A simplified list of 742 proteins, according to the maximum parsimony criterion, is available in Supplementary Table 5D.

The control and stressed samples presented 358 proteins, with 14 and 47 detected exclusively in control and stressed samples, respectively (Figure 1C, Supplementary Table 6A and 6B). Out of the 297 proteins present in the root control and stressed, and differently expressed according to abundance were reported by the PatternLab's TFold module, 17 showed statistically significant differences in their abundance in the short-term stress (Figure 2C – Blue dots – and Supplementary Table 6C). Seven proteins were significantly up-regulated in the salt-stressed group compared to the control plants, and 10 down.

Regarding the functional classification, 18 and 18 protein groups were identified in the roots of both control and stressed plants, respectively, and, in the case of the DE proteins, six

distinct groups appeared (Figure 5). The genetic information process (18 proteins) and carbohydrate metabolism (9) were the main functional groups in the control treatment (Figure 5A). In the stressed plants, we identified the carbohydrate metabolism (27), genetic information process (22), and amino acid metabolism (21) (Figure 5B). At last, the proteins that had a significant difference belong to carbohydrate metabolism (7) and genetic information process (3) (Figure 5C).

3.4. Roots proteome at Long-term Stress

The changes at the protein profiles in the roots of young gliricidia plants submitted to long-term stress (LTS) led to identifying a total of 6,714 and 6,977 peptides in control and stressed samples, respectively; which infers up to 4,563 protein entries from the database under both conditions (Supplementary Table 7A). Out of the 8,343 peptides identified, 137 were present in at least 10 proteins (Supplementary Table 7B). Approximately 26% (1,204) had at least one unique peptide observed, and about 32% of the proteins (1,482) inferred from more than four peptides (Supplementary Table 7C). A simplified list of 2,030 proteins, according to the maximum parsimony criterion, is available in Supplementary Table 7D.

The control and stressed samples presented 1,248 proteins, with 75 and 61 detected exclusively in control and stressed samples, respectively (Figure 1D, Supplementary Table 8A and 8B). Out of the 1,112 proteins present in the root control and stressed, and differently expressed according to abundance were reported by the PatternLab's TFold module, 32 showed statistically significant differences in their abundance in the long-term stress (Figure 2D – Blue dots – and Supplementary Table 8C). Seventeen proteins were significantly up-regulated in the salt-stressed group compared to the control plants, and 15 down.

Regarding the functional classification, 19 and 20 protein groups were identified in the roots of both control and stressed plants, respectively, and, in the case of the DE proteins, 10 distinct groups appeared (Figure 6). The genetic information process (23 proteins), protein

families: genetic information processing (21), and carbohydrate metabolism (15) were the main functional groups in the control treatment (Figure 6A). In the stressed plants, we identified the carbohydrate metabolism (22), protein families: genetic information processing (15), and amino acid metabolism (15) (Figure 6B). At last, the proteins that had a significant difference belong to carbohydrate metabolism (7), genetic information process (4), and lipid metabolism (4) (Figure 6C).

4. Discussion

Salinity alters the integrity and functionality of chloroplasts, consequently affecting cell function as a whole (AREFIAN et al., 2019). The plant response to salinity is a complex process that requires changes in gene and protein expression profiles (SOBHANIAN et al., 2010). Our data have revealed significant differences in protein expression patterns in the leaves and roots of young gliricidia plants submitted to saline stress compared to the control plants.

The proteomics data obtained underwent a comparative analysis of the protein profiles in two distinct scenarios - short-term (STS) and long-term stress (LTS), both in the leaves and roots of young gliricidia plants. The numbers of proteins found in the STS were much lower than in the LTS in both organs (Figure 1). Furthermore, the numbers of proteins found in the roots were much higher than in the leaves in both scenarios.

The software used to perform the differential expression analysis - PatternLab V (CARVALHO et al., 2016) - revealed three groups of proteins, those present only in the control plants, those present only in the stressed plants, and those present in both (supplementary tables 2, 4, 6, and 8). In the case of those present only in one treatment, only the proteins present in two or more samples (replicates) per treatment are reported in Figure 1.

After being submitted to differential expression analysis, those proteins present in both treatments came out separated into four distinct groups. The first group, represented by the

blue dots in Figure 2, contains proteins that satisfy the automatic fold and the statistical criteria. The second group, represented by the orange dots, contains proteins filtered out by the L-stringency and that deserve further experimentation to verify if they indeed are differentially expressed. Proteins that do not satisfy the L-stringency cutoff have low quantitative values (CARVALHO et al., 2016). The third group, represented by the green dots, contains proteins that did satisfy the automatic fold criteria but did not satisfy the statistical one and, for this reason, should not be considered for further analysis. At last, the group represented by the red dots contains proteins that did not satisfy either one of the criteria.

Only a short percentage of the proteins present in both treatments satisfied both the automatic fold and the statistical criteria (Figure 2). In the STS scenario, 1.7% (three out of 177 proteins) and 5.7% (17 out of 297) did so in the leaves and roots, respectively. In the case of the LTS scenario, 6.6% (44 out of 663) and 2.9% (32 out of 1,112) did satisfy both criteria, respectively. These small numbers of "blue dots" proteins will for sure reduce the efficacy of any attempt to perform Multi-Omics Integration (MOI) (CAVIL et al., 2016; JAMIL et al., 2020).

Salt stress induces several processes related to protein phosphorylation, post-translational modifications, and signaling in plants, which can occur on a time scale of minutes to seconds (ZHAO et al., 2019). High-throughput proteomics analysis reveals plant responses to salt stress at the protein level. Proteins play a role in the stress response and modulate physiological characteristics necessary to develop different phenotypes. In the present study, those proteins present only in one treatment, and those differentially expressed proteins present in both underwent functional annotation (Figures 3, 4, 5, and 6).

Those proteins found only in the tissues of the salt-stressed plants are likely the most important to help understand the adaptation response seen in young gliricidia plants under

very high salinity stress, together with those differentially expressed in both treatments - control and stressed (Carvalho da Silva et al., 2021). As the amount of those proteins found only in the tissues of the salt-stressed plants, in at least two replicates, was too small (Figure 1), we decided to include those proteins present in just one replicate as well to perform the functional annotation analysis.

Our results show a prevalence of proteins from Genetic Information Processing and Carbohydrate metabolism in both tissues of salt-stressed plants; and in both scenarios (Figures 3, 4, 5, and 6). In the case of those proteins differentially expressed and present in both treatments, the same results also prevailed. As the software used does not quantify the expression level of those proteins present in just one treatment, not much infer from these results.

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Conflicts of Interest

The authors declare no conflicts of interest.

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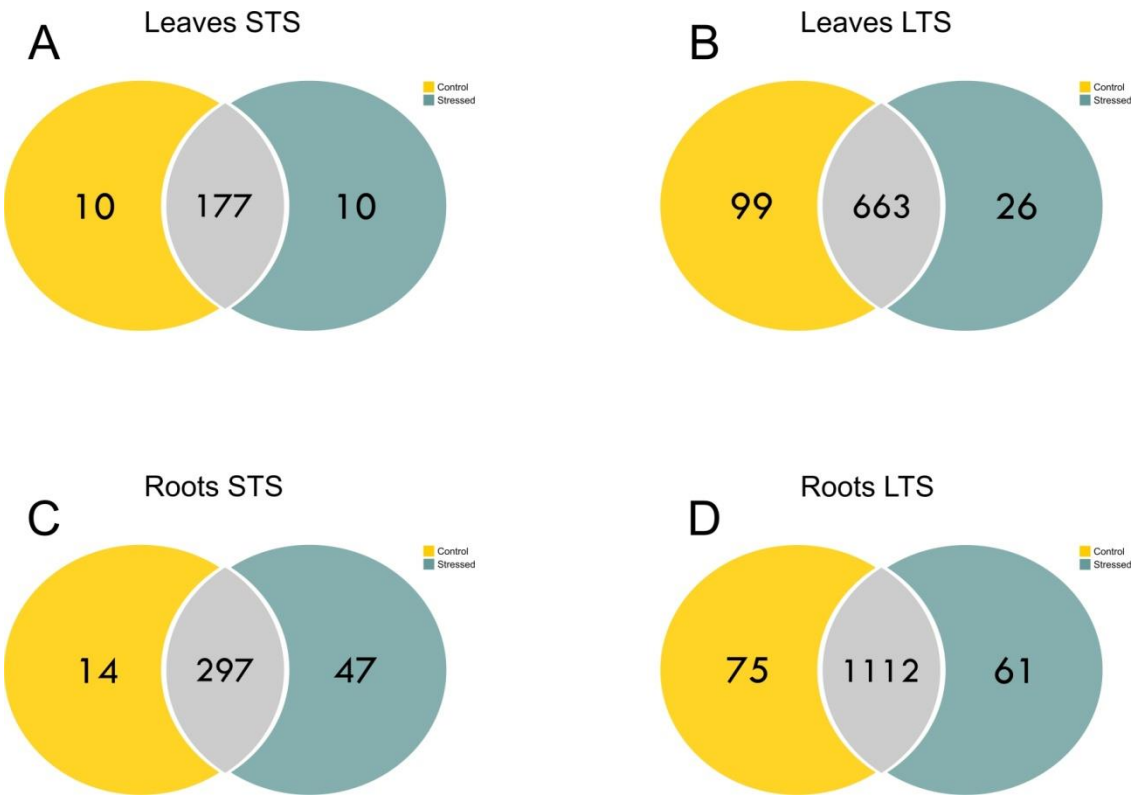

Figure 1. Venn diagram showing the number of proteins present only in the control (yellow) and only in the salt-stressed young gliricidia plants (green), and those present in both (gray). In the case of those present only in one treatment, only the proteins present in two or more samples (replicates) per treatment are reported. Short-term stress (STS) and Long-term Stress (LTS).

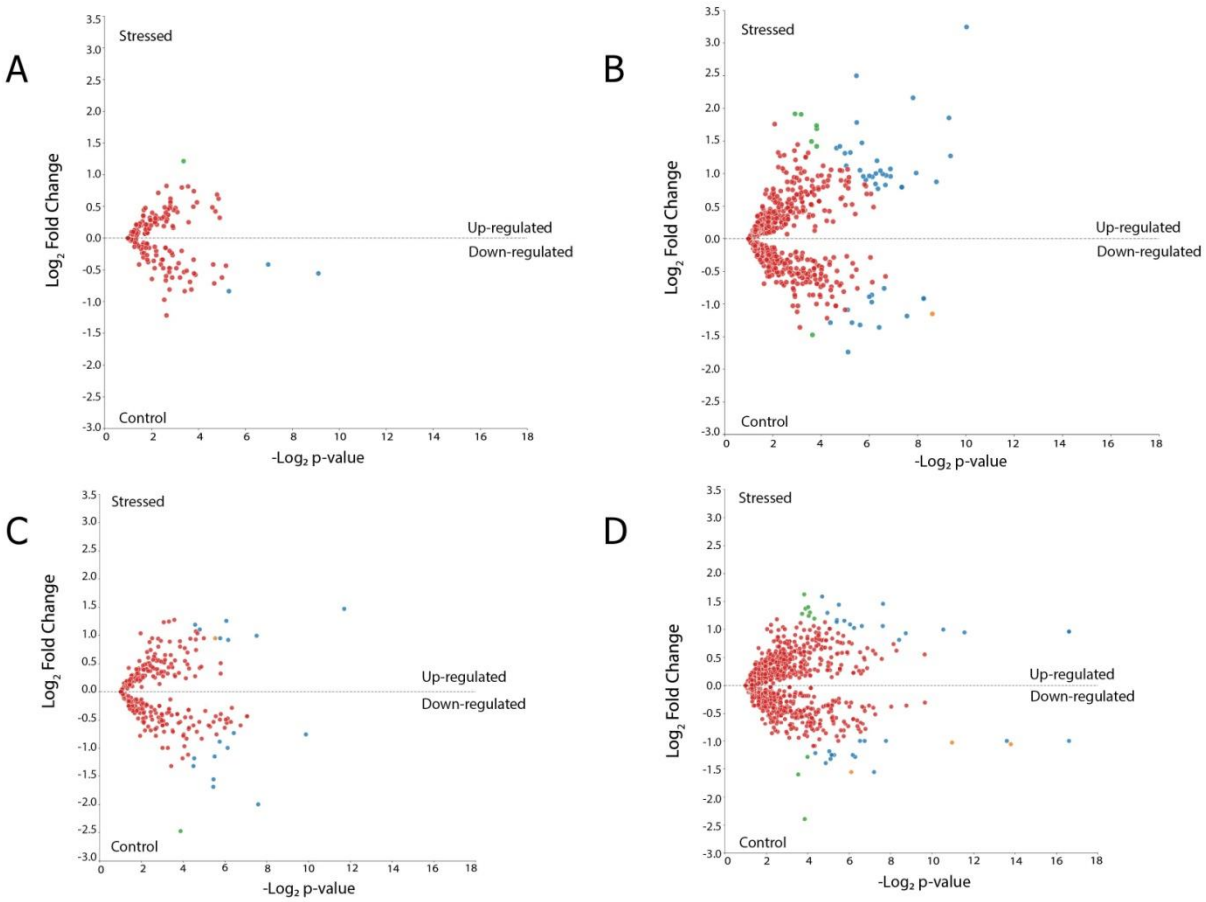

Figure 2. Volcano plots showing changes in protein expression on the leaves (A, B) and roots (C, D) of young gliricidia plants submitted to salinity stress, under two scenarios - Short-term stress (A, C) and Long-term Stress (B, D). Blue dots - proteins that satisfy the automatic fold and the statistical criteria; orange dots - proteins filtered out by the L-stringency and that deserve further experimentation to verify if they indeed are differentially expressed; green dots - proteins that did satisfy the automatic fold criteria but did not satisfy the statistical one and, for this reason, should not be considered for further analysis; and red dots - proteins that did not satisfy either one of the criteria.

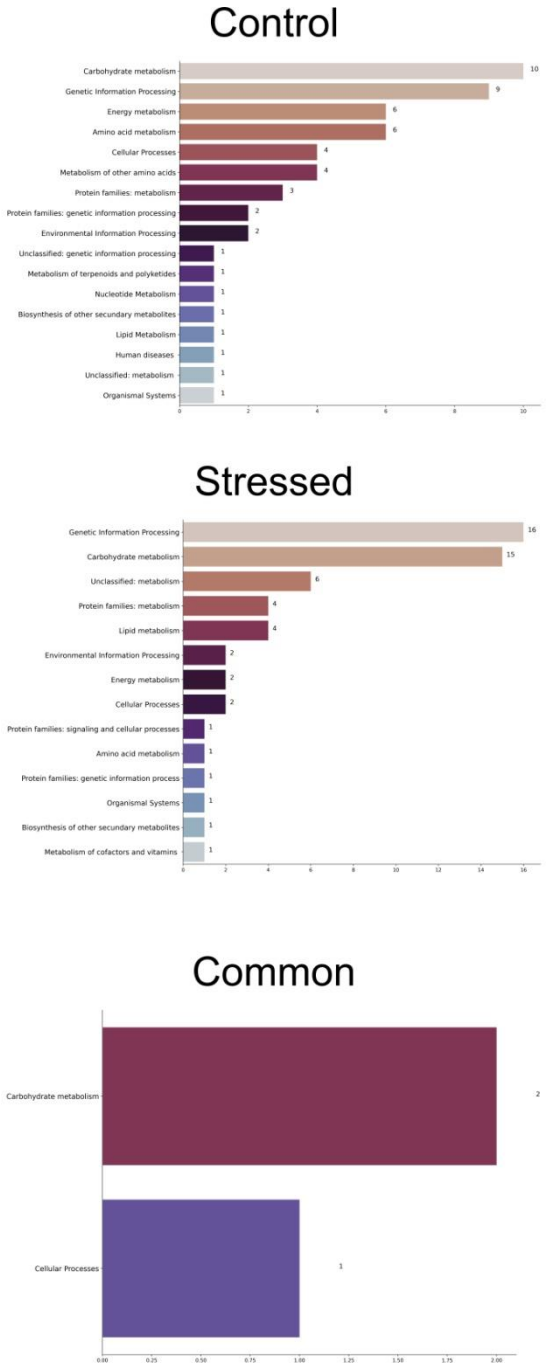

Figure 3. Annotation results from proteins in the leaves of young gliricidia plants in the short-term stress scenario, generated by the GhostKOALA annotation tool from the automatic annotation and KEGG mapping service. Results showing the number of proteins by functional category. Control - proteins present only in the control treatment; Stressed – only in the salt-stressed treatment; Common – proteins in both treatments. only the proteins present in two or more samples (replicates) per treatment are reported. As the amount of proteins

found only in one treatment, in at least two replicates, was too small, those proteins present in just one replicate were also included in the functional annotation analysis.

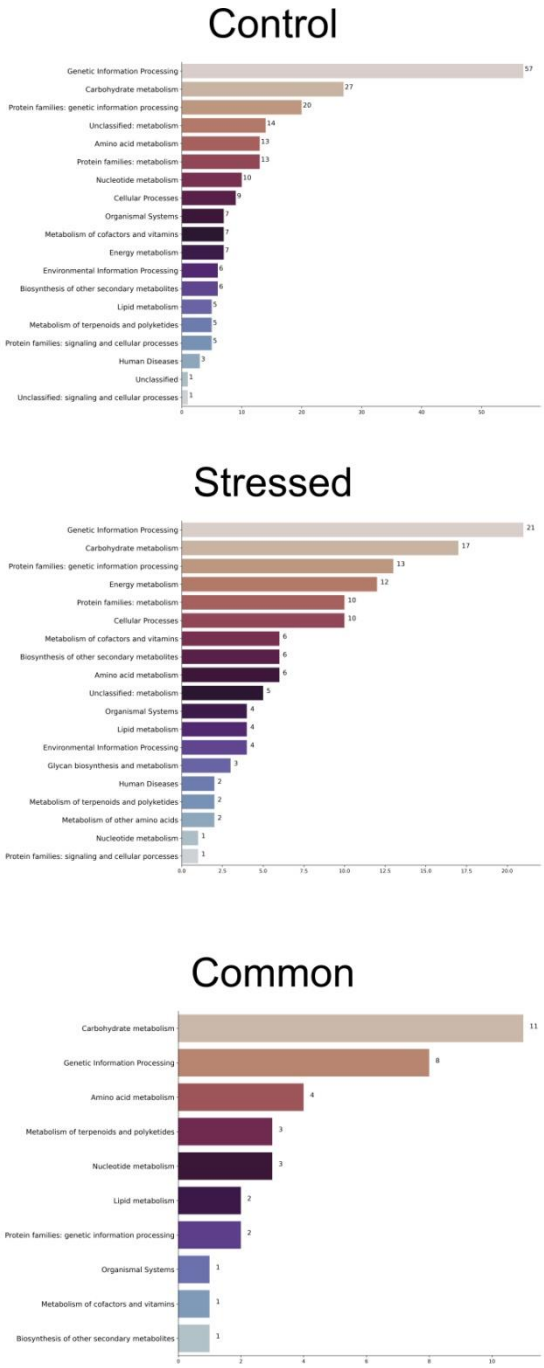

Figure 4. Annotation results from proteins in the leaves of young gliricidia plants in the long-term stress scenario, generated by the GhostKOALA annotation tool from the automatic annotation and KEGG mapping service. Results showing the number of proteins by functional category. Control - proteins present only in the control treatment; Stressed – only in the salt-stressed treatment; Common – proteins in both treatments. only the proteins present in two or more samples (replicates) per treatment are reported. As the amount of proteins

found only in one treatment, in at least two replicates, was too small, those proteins present in just one replicate were also included in the functional annotation analysis.

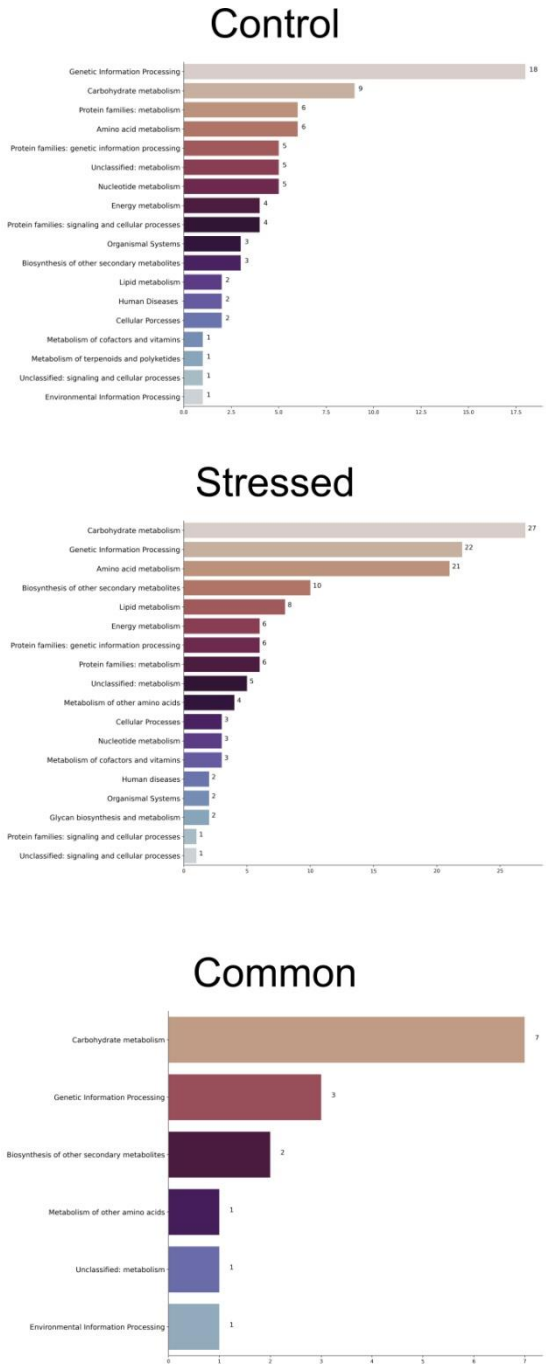

Figure 5. Annotation results from proteins in the roots of young gliricidia plants in the short-term stress scenario, generated by the GhostKOALA annotation tool from the automatic annotation and KEGG mapping service. Results showing the number of proteins by functional category. Control - proteins present only in the control treatment; Stressed – only in the salt-stressed treatment; Common – proteins in both treatments. only the proteins present in two or more samples (replicates) per treatment are reported. As the amount of proteins

found only in one treatment, in at least two replicates, was too small, those proteins present in just one replicate were also included in the functional annotation analysis.

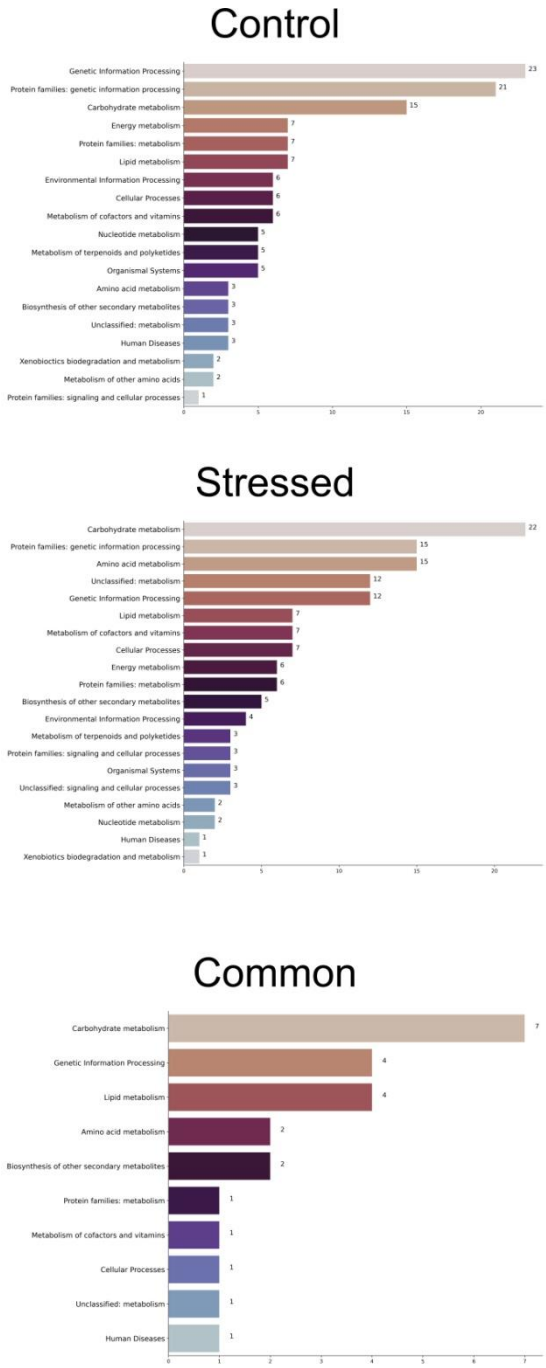

Figure 6. Annotation results from proteins in the roots of young gliricidia plants in the long-term stress scenario, generated by the GhostKOALA annotation tool from the automatic annotation and KEGG mapping service. Results showing the number of proteins by functional category. Control - proteins present only in the control treatment; Stressed – only in the salt-stressed treatment; Common – proteins in both treatments. only the proteins present in two or more samples (replicates) per treatment are reported. As the amount of proteins found only in one treatment, in at least two replicates, was too small, those proteins present in

just one replicate were also included in the functional annotation analysis.

List of Tables

Supplementary Table 5. Absolute numbers of all peptides and proteins identified in the roots of young *Gliricidia sepium* plants in the short-term scenario (Stable 05_A); list of all peptides confidently identified (Stable 05_B); list of all proteins confidently identified (Stable 05_C); and list of all proteins confidently identified with maximum parsimony criterion (Stable 05_D).

Supplementary Table 6. List of the proteins detected only in the roots of salt-stressed young *Gliricidia sepium* plants in the short-term scenario (Stable 06_A); list of the proteins detected only in the roots of control plants in the short-term scenario (Stable 06_B); and list of all differentially expressed proteins detected in the roots of gliricidia plants in both biological conditions (Stressed and Control) with statistical significance (FDR < 0.05) (Stable 06_C). Blue - proteins that satisfy the automatic fold and the statistical criteria; orange - proteins filtered out by the L-stringency and that deserve further experimentation to verify if they indeed are differentially expressed; green - proteins that did satisfy the automatic fold criteria but did not satisfy the statistical one and, for this reason, should not be considered for further analysis; and red - proteins that did not satisfy either one of the criteria.

Supplementary Table 7. Absolute numbers of all peptides and proteins identified in the roots of young *Gliricidia sepium* plants in the long-term scenario (Stable 05_A); list of all peptides confidently identified (Stable 05_B); list of all proteins confidently identified (Stable 05_C); and list of all proteins confidently identified with maximum parsimony criterion (Stable 05_D).

Supplementary Table 8. List of the proteins detected only in the roots of salt-stressed young *Gliricidia sepium* plants in the long-term scenario (Stable 08_A); list of the proteins detected only in the roots of control plants in the long-term scenario (Stable 08_B); and list of all differentially expressed proteins detected in the roots of gliricidia plants in both biological conditions (Stressed and Control) with statistical significance (FDR < 0.05) (Stable 08_C). Blue - proteins that satisfy the automatic fold and the statistical criteria; orange - proteins filtered out by the L-stringency and that deserve further experimentation to verify if they indeed are differentially expressed; green - proteins that did satisfy the automatic fold criteria but did not satisfy the statistical one and, for this reason, should not be considered for further analysis; and red - proteins that did not satisfy either one of the criteria.

CAPÍTULO 4

CONSIDERAÇÕES FINAIS

Os estudos realizados no âmbito do Programa de PD&I “Sal da Terra” permitiram, até o momento, identificar duas respostas distintas da *Gliricidia sepium* frente ao estresse salino – tolerância e adaptação (Belo Silva, 2019; Carvalho da Silva et al., 2021). A análise inicial do perfil de transcritos e metabólitos realizada por Carvalho da Silva et al. (2021) mostrou que a via metabólica da biossíntese de fenilpropanóides foi a mais afetada nas folhas de plantas jovens de gliricidia submetidas a intenso estresse salino, principalmente na fase inicial do estresse – nas primeiras 48 do início do estresse. Dando sequência a estes estudos, o trabalho atual objetivou avançar ainda mais na busca pela compreensão dos mecanismos moleculares que permitem a essa espécie vegetal a se adaptar e sobreviver em condições de salinidade superior a 20 dS m^{-1}. Para tanto, avançamos na caracterização dos perfis metabolômicos e proteômicos tanto das folhas quanto das raízes, além de avaliar estender o período do estresse de longa duração de 45 para 55 dias.

A análise dos resultados obtidos no presente estudo não só corroborou os resultados obtidos anteriormente por Belo Silva (2019) e Carvalho da Silva et al. (2021), como também ampliou o conhecimento prévio sobre as principais vias metabólicas afetadas; além de adicionar pela primeira vez a análise da proteômica neste processo. Os resultados apresentados no Capítulo 2 da presente dissertação mostraram que via metabólica da biossíntese de fenilpropanóides não só foi bastante afetada durante o estresse de curta duração, como permaneceu afetada no estresse de longa duração, quando se desenvolve todo o processo de adaptação, ou seja, começando no curto prazo e continuando no longo. No capítulo 2 também foram apresentados resultados que nos levam a postular que o acúmulo de lignina e de alguns fitoesteroides, além da biossíntese de lisina, tem um papel fundamental na promoção da adaptação ao estresse salino. Porém, estudos adicionais são necessários. Já no Capítulo 3 foram apresentados os resultados da proteômica, que não tinha sido realizada anteriormente, tanto em folhas quanto nas raízes. para caminhar em direção a compreensão da resposta de adaptação da gliricídia ao sal.

No presente estudo foi possível revelar novos metabólitos, vias metabólicas, além de proteínas responsivos ao estresse salino, e que podem estar diretamente ou indiretamente ligados ao processo de adaptação da gliricídia a condição de alta salinidade. No que diz respeito perfil metabólico das raízes, foi possível verificar diferenças e similaridades em

quatro distintos cenários (AE, STS, LTS1 e LTS2). Especial atenção foi dado ao fato de que as diferenças dos perfis metabólicos das raízes de plantas controle e estressadas aos 55 dias foi o menor possível; o que prova que após se adaptar estresse, as plantas retornam quase que totalmente à normalidade pré-estresse. E nossos dados também revelaram diferenças significativas nos padrões de expressão de proteínas tanto nas folhas quanto das raízes de plantas jovens de gliricídia submetidas ou não ao estresse por sal em comparação com as plantas controle em ambos os cenários (STS e LTS).

Por fim, durante a execução dos trabalhos, notou-se que a análise do proteoma revelou um número bem menor que o esperado, limitando assim o alcance tanto da análise singular do proteoma quanto a uma futura análise integrativa do proteoma com outras ômicas. O próximo passo neste estudo, a ser realizado no futuro, é o emprego da MOI (Multi-omic integration) a partir de dados não só de metabolômica e proteômica, como também de transcritômica, para avançar em direção a elucidação do(s) mecanismo(s) moleculares responsáveis pela resposta de adaptação ao estresse salino.