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ISABELA DOS SANTOS BEGNAMI

Desvendando os mecanismos moleculares de resistência de *Paspalum regnellii* ao ataque de cigarrinha *Mahanarva spectabilis*

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Orientadora: Profa. Dra. Anete Pereira de Souza

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RESUMO

No Brasil, as extensas áreas dedicadas à agropecuária são predominantemente compostas por pastagens. Apesar da crescente produtividade dessas forrageiras, a produção ainda sofre com uma grande limitação que é a susceptibilidade às cigarrinhas-das-pastagens, com destaque para a espécie *Mahanarva spectabilis*. A resistência é naturalmente encontrada no gênero nativo *Paspalum*, o qual pode proporcionar a descoberta de genes relacionados ao processo de defesa. O projeto visou analisar o transcriptoma das raízes de dois genótipos de *P. regnellii* em diferentes tempos de infestação de ninfas de *M. spectabilis*, com o objetivo de identificar genes de resistência. Um experimento de resistência às cigarrinhas foi realizado em campo para determinar a taxa de sobrevivência das ninfas e escolher os dois genótipos de P. regnellii mais contrastantes (BGP 248 e BGP 344). As amostras foram coletadas para ambos em triplicata e para três diferentes condições: controle (T0), após 48h (T1) e 72h de infestação (T2). Foi feita a extração de RNA total, preparação de bibliotecas de cDNA e o sequenciamento em plataforma Illumina HiSeq 2500 (*paired-end* 2x100pb). A qualidade foi analisada com o FastQC, as sequências de baixa qualidade filtradas com o Trimmomatic e o rRNA residual foi removido com o SortMeRNA. A montagem *de novo* foi realizada com o Trinity e sua qualidade garantida com o BUSCO e Bowtie2. Uma análise de genes diferencialmente expressos (DEGs) foi feita usando o pacote edgeR entre os diferentes tempos de infestação de um mesmo genótipo e entre os dois genótipos para cada condição. Os termos de processos biológicos foram enriquecidos com o Gene Ontology (GO), as vias metabólicas no Kyoto Encyclopedia of Genes and Genomes (KEGG) e também foram modeladas as redes de co-expressão para cada genótipo e uma rede metabólica das vias enriquecidas. A anatomia radicular foi analisada nos genótipos sem infestação e testes histoquímicos foram feitos para identificar os componentes de parede celular. A montagem resultou em 575.219 contigs sem redundância, com um tamanho de contig N50 de 788bp. As reads foram 80,89% alinhadas com Bowtie2 e o BUSCO encontrou 90,4% dos transcritos em plantas ortólogas. Após a filtragem, 21.508 genes permaneceram e 3.231 foram considerados genes diferencialmente expressos (DEGs) significativos, sendo a maioria deles relatados em contraste entre os genótipos. Um total de 162 termos de GOs de processos biológicos foram enriquecidos, podendo-se destacar alguns intimamente relacionados à resistência de cigarrinhas no genótipo BGP 344, como resposta a herbívoros (GO:0080027). O KEGG enriqueceu 19 vias, destacando-se uma relacionada a uma a diferenças estruturais entre os genótipos (biossíntese de cutina, suberina e cera) e duas à resistência no BGP 344 (metabolismo de purina e glutationa), com suas enzimas sendo as mais influentes na rede

metabólica. Nas redes de coexpressão pode ser notada uma maior quantidade de nós e conexões nesse mesmo genótipo, demonstrando uma maior complexidade em sua cascata de resposta. Dessa forma, foi elucidado o complexo processo de resistência de *Paspalum regnellii* à *Mahanarva spectabilis* e possíveis genes candidatos foram identificados para maiores análises.

Palavras-chave: forrageiras, defesa, pragas, RNA-Seq, redes de co-expressão

ABSTRACT

In Brazil, the extensive areas dedicated to agriculture consist predominantly of pastures. Despite the increasing productivity of these forages, production still suffers from the major limitation that is susceptibility to spittlebugs, especially *Mahanarva spectabilis* species. Resistance is naturally found in the native genus *Paspalum*, which may provide the discovery of genes related to the defense process. The project aimed to analyze the root transcriptome of two *P. regnellii* genotypes at different infestation times of *M. spectabilis* nymphs, with the objective of identifying resistance genes. A spittlebug resistance experiment was carried out in the field to determine the nymph survival rate and choose the two most contrasting *P. regnellii* genotypes (BGP 248 and BGP 344). Samples were collected for both in triplicate and for three different conditions: control (T0), after 48h (T1) and 72h of infestation (T2). Total RNA was extracted, cDNA libraries were prepared and sequenced on an Illumina HiSeq 2500 platform (paired-end 2x100bp). Quality was analyzed with FastQC, low-quality sequences filtered with Trimmomatic, and residual rRNA was removed with SortMeRNA. The assembly was carried out with Trinity and its quality guaranteed with BUSCO and Bowtie2. An analysis of differentially expressed genes (DEGs) was performed using the edgeR package between different infestation times of the same genotype and between the two genotypes for each condition. Biological process terms were enriched with the Gene Ontology (GO), the metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and co-expression networks for each genotype and a metabolic network of the enriched pathways were also modeled. Root anatomy was analyzed in genotypes without infestation and histochemical tests were performed to identify cell wall components. The assembly resulted in 575,219 redundancy-free contigs, with an N50 contig size of 788bp. The reads were 80.89% aligned with Bowtie2 and BUSCO found 90.4% of the transcripts in orthologous plants. After filtering, 21,508 genes remained and 3,231 were considered significant differentially expressed genes (DEGs), with most of them reported in contrast between genotypes. A total of 162 GO terms from biological processes were enriched, with some being able to highlight some closely related to spittlebug resistance in the BGP 344 genotype, as a response to herbivores (GO:0080027). KEGG enriched 19 pathways, highlighting one related to structural differences between genotypes (biosynthesis of cutin, suberin and wax) and two to resistance in BGP 344 (purine and glutathione metabolism), with its enzymes being the most influential in the metabolic network. In co-expression networks, a greater number of nodes and connections can be noted in the same genotype, demonstrating greater complexity in its response cascade. In this way, the complex

process of *Paspalum regnellii* resistance to *Mahanarva spectabilis* was elucidated and possible candidate genes were identified for further analysis.

Keywords: forage, defense, pest, RNA-Seq, co expression networks

Sumário

1. INTRODUÇÃO GERAL

As cigarrinhas-das-pastagens são um conjunto de insetos que despontam como as maiores pragas agrícolas de gramíneas em toda a América Latina (Paladini *et al.*, 2018), causando danos quantitativos de redução do volume de matéria seca, e qualitativos de capacidade nutricional (Congio *et al.*, 2012). Além disso, esses insetos geram perdas econômicas mundiais que podem chegar a 2,1 bilhões de dólares por ano (Bettiol *et al.*, 2017), e nacionais estimadas em 800 milhões de dólares (Souza *et al.*, 2013). Dentre as cigarrinhas podemos destacar as do gênero *Mahanarva*, composto por espécies que são encontradas em praticamente todo o país e que infectam diversos tipos de gramíneas forrageiras utilizadas como pastagens (cigarrinha *M. spectabilis*) e a cana-de-açúcar (cigarrinha *M. fimbriolata*) (Dinardo-Miranda, 2003).

As pastagens são a principal fonte nutricional da pecuária brasileira, ocupando uma área de aproximadamente 160 milhões de hectares do território nacional (IBGE, 2019). Com cerca de 194 milhões de cabeças de gado, nosso país se destaca como o maior exportador e o segundo maior produtor de carne bovina mundial, movimentando mais de 180 bilhões de reais por ano (ABIEC, 2022). Apesar desse cenário, os prejuízos de produção e qualidade das pastagens frente ao ataque de ninfas e adultos das cigarrinhas são bastante consideráveis (Aguirre *et al.*, 2012), com a produção de leite e carne prejudicada em até 54% e seu custo aumentado em 30% (Bettiol *et al.*, 2017).

Para contornar o problema de pragas agrícolas, tais como as cigarrinhas, a seleção de genótipos resistentes é a estratégia mais utilizada para diminuir as perdas de produção, visto o baixo custo do processo, a não utilização de grandes quantidades de inseticidas e a facilidade de se trabalhar com grandes quantidades de plantas (Cardona *et al.*, 1999). Desde os primórdios da agricultura conseguimos separar as plantas suscetíveis, que morrem ou não conseguem se reproduzir quando atacadas por artrópodes, das resistentes pela própria seleção natural (Smith, 2005). Atualmente, com o desenvolvimento da genética e biologia molecular, a transgenia e a edição gênica nos possibilitam explorar novas pesquisas para resistência (Fritsche-Neto & Borém, 2012).

Enquanto no melhoramento clássico grande parte do genoma é transferido por hibridação, nas técnicas de engenharia genética podemos inserir genes de interesse (Fritsche-Neto & Borém, 2012). Com a transcriptômica e tecnologias como o Sequenciamento de Nova Geração (NGS – Next Generation Sequencing), é possível alcançarmos a caracterização molecular de fenótipos de interesse a partir do estudo das expressões dos genes envolvidos em

processos biológicos, como o de resistência a estresses bióticos (Van *et al.*, 2011). Esses genes podem retardar a evolução do patógeno e serem utilizados de forma aplicada em edições genéticas, com foco no aumento da diversidade de genótipos (Dallagnol *et al.*, 2018). Como existe uma certa conservação estrutural na maioria dos genes envolvidos na resistência, espécies nativas podem ser utilizadas como fonte de genes para outras diversas espécies cultivadas de importância econômica, e essa conservação justifica o grande interesse no entendimento genético geral dos vegetais (Bobrowski *et al.*, 2003).

Diversos trabalhos realizados em campo vêm indicando a existência de certa resistência de gramíneas forrageiras, pelo mecanismo da antibiose, a diferentes espécies de cigarrinhas-das-pastagens (Cardona *et al.*, 2010; Souza Sobrinho *et al.*, 2010; Ferreira *et al.*, 2013; Mateus *et al.*, 2015; Gusmão *et al.*, 2016). Porém, por ser mais recentemente identificada como uma praga-chave agrícola, não existem muitos estudos de referência para a espécie *M. spectabilis*, resultando em lacunas sobre os mecanismos moleculares envolvidos na resistência por antibiose da interação planta-patógeno.

Paspalum L. é um gênero de gramíneas nativo, originário das regiões tropicais e subtropicais das Américas, composto por espécies com elevado potencial para serem utilizadas em pastagens para sistema de produção de gado e forragem (Acuña *et al.*, 2019; Novo *et al.*, 2016). Por ser um modelo vegetal versátil, o gênero *Paspalum* vem ganhando destaque em estudos evolutivos, genéticos, moleculares e de apomixia, além de alcançar aplicações bem sucedidas em programas de melhoramento para forragem e gramados (Ortiz *et al.*, 2020). Nesse cenário, espécies de *Paspalum* também podem apresentar grande potencial para serem utilizadas como modelo em estudos de resistência às cigarrinhas e, consequentemente, podem vir a ser fonte de genes de resistência para a espécie em estudo e para outras espécies suscetíveis ao ataque desses insetos.

Nesse contexto, este projeto visa obter e analisar os transcriptomas de raiz de genótipos de *Paspalum regnellii* com diferentes níveis de resistência às ninfas de cigarrinhas de *M. spectabilis*. Além de ser o primeiro transcriptoma para a espécie, a proposta do projeto é inédita já que até o momento não foram reportados transcriptomas de nenhuma gramínea para análise de resistência a qualquer espécie de cigarrinhas-das-pastagens.

2. REVISÃO BIBLIOGRÁFICA

2.1 Forrageiras tropicais e o gênero *Paspalum*

A alimentação dos animais na agropecuária brasileira é baseada predominantemente em pastagens, sendo o país reconhecido como um dos maiores produtores, consumidores e exportadores mundiais de sementes de gramíneas forrageiras tropicais (Fernandes *et al.*, 2017). Até a década de 1980 o cenário nacional era dominado pelas chamadas pastagens naturais, ou seja, aquelas originalmente presentes na área e que passaram a sofrer com as baixas tecnologias do setor forrageiro, as pressões ambientais e as de mercado. Consequentemente, houve um avanço das pastagens cultivadas, além de suas técnicas de melhoramento e formação de manejo (Dinardo-Miranda, 2014).

O grande potencial das pastagens para a economia brasileira resultou no surgimento de diversos programas de melhoramento genético de gramíneas forrageiras tropicais nas últimas décadas. Os principais objetivos destes programas são melhorar o desempenho e aprimorar características como produtividade, qualidade nutricional, tolerância aos estresses abióticos e resistência a pragas e doenças (Valle *et al.*, 2009). Visando alcançar esses objetivos com mais eficiência e em menos tempo, a biotecnologia vem se destacando como uma grande ferramenta tecnológica na agronomia (Carrer *et al.*, 2010).

Um dos gêneros nativos que vem sendo melhorado e utilizado em sistemas de produção de gado é o *Paspalum* L.. Ele pertence à tribo Paniceae, família Poaceae, e agrupa mais de 300 espécies distribuídas em regiões tropicais e subtropicais de continentes como Ásia, África e América (Ortiz *et al.*, 2013). No Brasil, a Embrapa Pecuária Sudeste mantém um Banco Ativo de Germoplasma de *Paspalum* (BAG *Paspalum*) composto por cerca de 550 acessos de 50 diferentes espécies (Alelo Vegetal, 2023), avaliadas para uso como forragem e cobertura vegetal.

As espécies do gênero *Paspalum* são subdivididas em grupos botânicos informais de acordo com Chase (1929). Na maioria dos casos, as espécies apresentam citótipos tetraploides apomíticos e diplóides sexuais (Ortiz *et al.*, 2013). Porém, tetraploides sexuais naturais são constatados em algumas delas, podendo ser utilizados em cruzamentos controlados como genitor sexual, viabilizando a geração de variabilidade genética. As espécies do grupo botânico Virgata se destacam por apresentar espécies com genótipos tetraplóides sexuais naturais, incluindo *Paspalum regnellii* (Acuña *et al.*, 2019).

P. regnellii é uma espécie perene, de rizomas curtos e que pode chegar a cem centímetros de altura em crescimento livre (Barro, 2011). Sua produção de biomassa é muito

boa e possui certa resistência ao sombreamento, além de apresentar um elevado potencial forrageiro e econômico (Barro *et al.*, 2012). Não existem estudos genômicos para esta espécie, e somente nove sequências de nucleotídeos estão disponíveis no banco de dados do NCBI (National Center for Biotechnology Information), sendo a maioria de cloroplastos (https://www.ncbi.nlm.nih.gov/, consultado em 27/06/2023). No entanto, *P. regnellii* vem sendo utilizada como fonte de sexualidade para cruzamentos interespecíficos em programas de melhoramento, como o da Embrapa Pecuária Sudeste (Begnami *et al.*, 2019), podendo, portanto, disponibilizar um valioso *background* genético como modelo para diferentes tipos de estudo.

Além disso, estudos recentes realizados na Embrapa Pecuária Sudeste buscaram a identificação de genótipos menos suscetíveis ao ataque de cigarrinhas-das-pastagens para seu uso em manejos integrados e no Programa de Melhoramento de *Paspalum*. Foi constatado que existe uma maior resistência em acessos da espécie *P. regnellii* conservados no Banco Ativo de Germoplasma (BAG) do que nos genótipos comumente utilizados, como os do gênero *Urochloa*.

2.2 Cigarrinha-das-pastagens

As gramíneas forrageiras são atacadas por diversas pragas que diminuem sua produção, e as cigarrinhas-das-pastagens se destacam como as principais. São herbívoros sugadores de seiva da ordem Hemiptera, subordem Auchenorrhyncha e família Cercopidae que se integram em um complexo sistema planta-hospedeiro devido à grande diversidade de espécies de forrageiras e também das próprias cigarrinhas. As espécies mais relatadas como pragas de forrageiras no Brasil são *Notozulia entreriana* (Berg, 1879), a *Deois schach* (Fabricius, 1787) e a *Deois flavopicta* (Stal, 1854) (Valério, 2009).

Embora usualmente o gênero *Mahanarva* causasse maiores danos apenas em gramíneas de grande porte, nos últimos anos seus ataques passaram a atingir também as menores, com destaque para aquelas com largas áreas de monocultura como *Urochloa brizantha* (Torres, 2022). As espécies de maior importância atualmente são a *Mahanarva fimbriolata* (Stal, 1854), pelos grandes danos causados à cultura da cana-de-açúcar, e a *M. spectabilis* (Distant, 1909) que se alimenta principalmente de forrageiras (Dinardo-Miranda, 2014), mas sendo relatada até mesmo em arroz nos últimos anos (Paladini *et al.*, 2018).

As cigarrinhas *Mahanarva*, assim como o restante dos cercopídeos, são insetos hemimetábolos, ou seja, que não passam por uma metamorfose completa. Os chamados adultos

depositam na base da planta uma quantidade variada de seus ovos alongados e de coloração amarelada (Valério, 2009). Eles irão entrar em diapausa até a chegada das épocas de chuva (janeiro a março), que é uma das condições ambientais mais favoráveis à sua eclosão em ninfas (Balsalobre *et al.*, 2009). O desenvolvimento ninfal passa por cinco ínstares de crescimento de tamanho durante cerca de 40 dias, nos quais as ninfas sempre se instalam acima do solo e se alimentam da seiva das raízes, até que a forma juvenil se transforma em adulto (Schöbel & Carvalho, 2021).

Ao colonizar os vasos lenhosos das raízes, a alimentação das ninfas já inicia o processo predatório desses herbívoros, visto que pode ocasionar desordens fisiológicas que irão dificultar ou até mesmo impedir o fluxo de água e nutrientes na planta (Ravaneli *et al*., 2011). Além disso, elas ficam protegidas de seus predadores e também do ressecamento durante todo o seu ciclo de vida por uma espécie de espuma, que é produzida em suas cavidades abdominais e secretada pelos túbulos de Malpighi (Schöbel & Carvalho, 2021).

Posteriormente, a transformação em adulto ocorre e os animais vivem de 10 a 15 dias se alimentando das partes aéreas das plantas, como folhas e brotos (Fonseca *et al.*, 2016). Essa forma alada continua a predação, agora sugando seiva das folhas e injetando toxinas, o que gera distúrbios fisiológicos que reduzem tanto a produção de matéria seca como a qualidade das pastagens, podendo deixá-las impalatáveis para o gado (Resende *et al.*, 2013).

Para controlar essa praga, atualmente temos como possibilidade o chamado controle químico a partir de inseticidas, que é econômica e ecologicamente inviável no caso das cigarrinhas-das-pastagens devido à extensão das áreas de forrageiras no território nacional (Auad *et al.* 2012). Uma alternativa agronômica é o manejo integrado de pragas (MIP), em que se procura preservar e intensificar os fatores que causam a mortalidade natural das cigarrinhas-das-pastagens com o uso de métodos de contenção (Picanço, 2010). Porém, a falta de informações comportamentais e biológicas básicas das espécies do gênero *Mahanarva* restringe o desenvolvimento de estratégias de manejo. Mudanças em características metabólicas, por exemplo, podem provocar rupturas no ciclo de vida dos insetos e são essenciais para o entendimento das suas preferências ambientais (Auad & Carvalho, 2009), mas são desconhecidas neste gênero.

Dessa forma, a melhor alternativa para solucionar os problemas causados pelas cigarrinhas-das-pastagens é o investimento em gramíneas alternativas e genótipos resistentes. A resistência das plantas contra organismos infectantes pode ser capaz de originar uma diminuição dos insetos sem desequilibrar drasticamente o ecossistema, além de não causar custos adicionais às produções (Souza *et al.*, 2012). Além disso, estudos com *M. spectabilis*

vêm mostrando uma tendência da espécie de ser afetada por certos mecanismos de resistência gerados pelas plantas forrageiras (Auad *et al.*, 2007), os quais também são observados considerando outros gêneros de cigarrinhas, tais como o *Notozulia* (Lapointe *et al.*, 1992; Gusmão *et al.*, 2016).

2.3 Mecanismo geral de resistência à herbívoros

Sob as mesmas condições de ataque, uma planta pode responder de diferentes formas para impedir que a infestação pela praga resulte em problemas de produtividade da cultura. Estas estratégias são tradicionalmente divididas entre a antixenose ou não preferência, a tolerância e a antibiose (Aoyama & Labinas, 2012). A antixenose, ou não-preferência, é identificada quando uma planta, embora em mesma condição de outras, não é alvo de alimentação e oviposição dos herbívoros devido a características estruturais e morfológicas, ou ainda estímulos elaborados pelas plantas (Lara, 1991). Já a tolerância consiste na capacidade de uma planta de sofrer menor perdas de produção ainda que sob as mesmas condições de ataque, ou seja, o genótipo possui características que diminuem o prejuízo do ataque (Fritsche-Neto & Borém, 2012).

Outro tipo de resistência é a antibiose, a qual se baseia, principalmente, em efeitos negativos à biologia do inseto, como ações para mortalidade das larvas e ninfas, redução de crescimento, fertilidade, longevidade e até aberrações fisiológicas da praga (Morais & Pinheiro, 2012). Essas complicações são causadas, em sua maioria, por composto orgânicos denominados metabólitos secundários, que são produzidos pelos vegetais para tentar reduzir o estresse causado pelos agentes infecciosos (Boiça Júnior *et al.*, 2017).

Essa variedade de estratégias de defesa é resultante de diferentes características físicas e químicas que retardam o ataque dos insetos de maneira constitutiva ou induzida (Scalschi *et al.*, 2013). Os componentes já existentes na planta atuam como uma primeira linha de defesa, funcionando como uma barreira morfológica natural que dificulta a herbivoria (Walters, 2011). Alguns exemplos deste mecanismo são a presença de estruturas vegetais como tricomas, pêlos radiculares, espessura de parede celular e até mesmo deposição de substâncias como cera, cutina e lignina (Aoyama & Labinas, 2012). Já a parte induzida da defesa se inicia apenas com o ataque do inseto e consiste na liberação de metabólitos secundários de diferentes classes químicas, como terpenóides, compostos fenólicos e nitrogenados (Kabera *et al.*, 2014). Estes compostos podem gerar toxicidade ao patógeno ou ainda liberar os chamados Voláteis de Plantas Induzidos por Herbívoros (VPIHs), que irão atrair os inimigos naturais do inseto e gerar uma resposta generalizada no hospedeiro, podendo inclusive alertar as plantas ao redor (War *et al.*, 2011).

Caso um inseto ultrapasse as dificuldades morfológicas de uma planta e inicie a herbivoria, um mecanismo geral de resistência (Figura 1) é ativado. Ele se inicia com a ligação das secreções liberadas pelo inseto aos padrões moleculares associados a herbívoros (HAMPs) e a danos (DAMPs) (Acevedo *et al.*, 2015). Ambos são posteriormente reconhecidos pelos receptores de reconhecimento padrão (PRRs), que catalisam a ação das proteínas quinases ativadas por mitógenos (MAPKs) e alteram a expressão dos fatores de transcrição WRKYs (Li *et al.*, 2019). Além disso, esse reconhecimento gera uma despolarização da membrana plasmática das células, abrindo os canais voltaicos de cálcio e aumentando a concentração dos íons Ca2+ no citoplasma. Estes íons serão reconhecidos por sensores, como por exemplo proteínas quinases dependentes de cálcio (CDPKs), para desencadear o processo de resposta (Erb & Reymond, 2019).

Concomitantemente, uma outra via de sinalização contra a herbivoria é ativada com o rompimento das primeiras linhas de defesa e a identificação dos elicitores – a das espécies reativas de oxigênio (ROS) (Block *et al.*, 2018). Esses compostos são produzidos por uma NADPH oxidase localizada na membrana plasmática, chamada RBOH, que tem seu funcionamento estimulado também pelo influxo dos íons de cálcio e suas quinases relacionadas (Erb & Reymond, 2019).

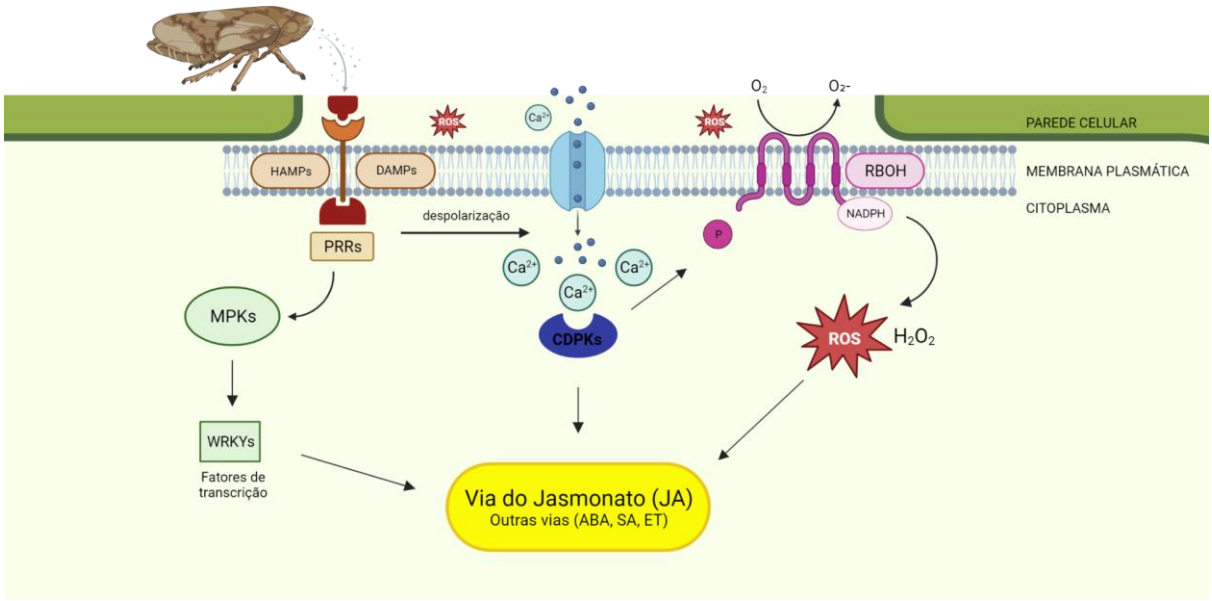

Figura 1. Esquema geral do processo de sinalização inicial da resistência contra herbívoros construído no BioRender.

As sinalizações iniciais descritas, então, vão possibilitar o processo de resistência pelas vias hormonais, sendo a do ácido jasmônico (JA) a mais associada à defesa contra herbivoria (Wang *et al.*, 2021). Outros fitormônios que são bem estabelecidos e relacionados ao estresse biótico, como o ácido abscísico (ABA), salicílico (SA) e etileno (ET), tem sua importância variando dependendo do herbívoro e seu modo de ataque (Erb & Reymond, 2019).

Visto que existem lacunas no conhecimento do processo de resistência à herbívoros e ele apresenta muitas variações e relativizações, uma comparação entre genótipos que apresentam diferentes estratégias, sob uma mesma condição, permite a compreensão de suas características de funcionamento para uma possível aplicação em programas de melhoramento genético (Celin *et al.*, 2017). Nesse contexto, a integração do conhecimento já existente, obtido em experimentos de análise de resistência em ambiente protegido e a campo, com informações inéditas em nível genômico, pode gerar um melhor entendimento dos processos genéticos e moleculares envolvidos na resistência, e tornar mais eficiente a seleção de genótipos resistentes.

2.4 Transcriptômica

A associação do conhecimento de diferentes áreas como bioquímica, genética e informática, visando a identificação de genes, proteínas e suas interações, possibilitou o estudo das chamadas ciências ômicas. A transcriptômica, por exemplo, consiste no entendimento das relações entre o genoma funcional e todas as informações codificadas (Espindola *et al.*, 2010).

O desenvolvimento e avanço das tecnologias de sequenciamento possibilitou o surgimento do Sequenciamento de Nova Geração (NGS), com maior capacidade operacional e custo reduzido por cada base, quando comparado com os de primeira geração (Davies, 2010). Com as inovações de técnicas, o sequenciamento total de RNA (RNA-seq) tem se destacado por permitir a obtenção de perfis completos de todos transcritos, inclusive os raros, de qualquer espécie, sem a necessidade de um genoma ou transcriptoma prévio de referência (Egan *et al.*, 2012).

A técnica do RNA-seq baseia-se na ideia de que a quantidade de *reads* derivados do sequenciamento de cDNA é proporcional a quantidade de transcritos desse mesmo gene na amostra original (Ekblom & Galindo, 2011). Como as espécies de gramíneas forrageiras tropicais, incluindo *Paspalum* spp., geralmente não têm genoma de referência, a montagem *de novo* pode possibilitar a montagem dos transcritos sem sequências prévias (Egan *et al.*, 2012). Além disso, esse tipo de análise vem sendo aplicado com sucesso em trabalhos visando à prospecção de genes de resistência a pragas em diversos tipos de plantas, incluindo espécies da

família Poaceae (Zhao *et al.*, 2017), e tem gerado resultados de expressão diferencial relacionados às respostas das plantas quando infestadas (Becker *et al.*, 2017; Cui *et al.*, 2017; Wang e*t al.*, 2018).

2.5 Redes Complexas

O conceito de rede, ou seja, a investigação da interação entre componentes de um processo, é muito presente no mundo atual (Boguñá *et al.*, 2009). Seja para tentar explicar o funcionamento de dinâmicas sociais ou uma rede de internet, o seu estudo sempre esteve presente na esfera matemática com a teoria dos grafos (Boccaletti *et al.*, 2006). Porém, nos últimos anos, esse conhecimento passou a ser usado também para análises de biologia molecular, a princípio de forma exclusivamente estatística, mas depois integrando dados funcionais como perfis de regulação transcricional, dados de expressão gênica e reconstrução de vias regulatórias (Luscombe *et al.*, 2004).

A grande quantidade de dados gerada pelas análises de transcriptoma pode ser, com as redes de co-expressão, integrada de forma a revelar os agrupamentos gênicos e padrões de expressão entre eles em determinadas condições, além de possibilitar a identificação de genes ainda não caracterizados (Serin *et al.*, 2016). Em plantas, essas análises comparativas estão permitindo o entendimento de diversas características nas mais variadas espécies (Jones & Vandepoele, 2020).

Em uma exploração de rede, os nós representam os genes, as arestas a conexão entre esses componentes e cálculos são realizados a fim de evidenciar o grau de similaridade no padrão entre genes e, assim, mostrar a correlação de suas expressões gênicas (Rao & Dixon, 2019). Por fim, a análise de redes de co-expressão também é aplicada para descobrir os genes mais importantes de um processo biológico específico a partir da identificação dos chamados *hubs*, que têm uma grande quantidade de conexões e ocupam uma função principal na estabilidade contra perturbações e evidenciam sua relevância (Amrine *et al.*, 2015).

Desta forma, a análise de co-expressão gênica pode favorecer o preenchimento de lacunas de conhecimentos na literatura de alguns processos, como é o caso da resistência às cigarrinhas. A partir da elaboração das redes, inferências poderão ser feitas relacionando os genes mais relevantes na defesa e suas conexões e cascatas de sinalização.

3. OBJETIVOS

3.1 Objetivo Geral

Analisar e comparar o perfil transcricional de raiz em três diferentes tempos de infestação de dois genótipos de *Paspalum regnellii* com diferentes níveis de resistência ao ataque de ninfas de cigarrinhas (*Mahanarva spectabilis*), visando caracterizar o processo molecular de defesa.

3.2 Objetivos específicos

- Obtenção e anotação do primeiro transcriptoma de Paspalum regnellii;
- Determinação das diferenças constitutivas entre os genótipos;
- Exploração dos genes diferencialmente expressos em cada genótipo com e sem infestação;
- Identificação dos genes co-expressos e das proteínas que contribuem ao processo de defesa;
- Caracterização das estratégias moleculares de resistência.

4. ARTIGO

Lignin, glutathione and kinases play important roles in *Paspalum regnellii* defense against spittlebug (*Mahanarva spectabilis*) attack

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Summary

- Spittlebugs cause large production losses that affect agribusiness worldwide. Understanding plant-herbivore interactions at molecular level may be the key to developing resistant cultivars.
- After a nymph survival experiment, root transcriptomes were assembled from two *Paspalum regnellii* genotypes (BGP 248 and 344) with different levels of resistance, with no infestation and in two times of spittlebug (*Mahanarva spectabilis)* nymph infestation, integrating differential expression analysis and complex network modeling, and supplemented by root anatomical analysis.
- GO terms related to different stress responses were enriched in BGP 248, such as salicylic acid catabolic process, while some specific to spittlebugs like response to herbivores were enriched in BGP 344. KEGG enriched pathways were related to structural differences between genotypes, like cutin, suberin and wax biosynthesis. BGP 344 also presented pathways related to induced defense, such as glutathione metabolism. Metabolic networks highlighted kinases, and coexpression networks demonstrated a complex cascade response which includes lncRNAs.
- This study provides the first molecular insights into the defense mechanisms of *P. regnellii* against *M. spectabilis.* It was identified that the genotype with the highest nymph mortality (BGP 344) has constitutive barriers, such as lignin, which delay the attack. In addition to presenting an enriched glutathione pathway and kinases presence.

Keywords: complex network, forage, herbivory, lignin, plant-sap-feeding insects, resistance, RNA-seq, root.

INTRODUCTION

Worldwide, livestock is responsible for ensuring the livelihood of approximately 1.3 billion people. In addition, pasture areas total more than half of the planet's land surface for cattle raising or animal feed cultivation (FAO). Brazil stands out as one of the largest producers, exporters and consumers of beef in the world. In 2022, approximately 194 million cattle were distributed in more than 160 million hectares of planted pastures (Abiec Beef Report, 2022).

The main agricultural pest of tropical pastures are spittlebugs (Hemiptera: Cercopidae), causing global economic losses of more than US$ 2 billion a year (Thompson, 2004) because of quantitative and qualitative damage that reduces the dry matter volume and decreases nutritional capacity (Congio *et al.*, 2012). In Brazil, the genus *Mahanarva* became highly reported as an agricultural pest of forage grasses, mainly Marandu palisade grass (*Urochloa brizantha* cv. Marandu), and sugarcane in the 1990s (Congio *et al.*, 2020), with *Mahanarva spectabilis* (Distant, 1909) being responsible for limiting livestock production of beef and milk (Auad *et al.*, 2007).

In the spittlebug infestation, the nymph form settles in the roots and feeds on the xylem, damaging the vessels and preventing full nutrient transport, while the adults attack the leaves, breaking the cellular wall and reducing the amount of chlorophyll until it leads to tissue death (Alvarenga *et al.*, 2019). Plant defense strategies to deal with herbivores generally consist of constitutive factors that are always present, such as morphological characteristics and chemically induced defenses (Walters, 2011). These secondary metabolites are important for recognizing and responding to environmental stressors, such as herbivory. They are not synthesized all the time due to high energy expenditure, but they start to be produced when the plant detects attack, allowing plant survival and preventing insects from developing (Singh *et al.*, 2021).

The chemical control of insects, common in agriculture worldwide, is ecologically and economically unfeasible in the case of spittlebugs due to the large area of cultivated tropical pasture. As a result, the best management involves the development of resistant forage cultivars (Silva *et al.*, 2019). This process traditionally involves crossing individuals to generate hybrids and fixing and establishing them in the field for testing, but it is time-consuming and does not guarantee the achievement of the desirable characteristic without another trait loss (Lenaerts *et al.*, 2019). In addition, herbivore resistance is a quantitative feature (Dinardo-Miranda *et al.*, 2014), a kind of trait that is difficult to completely fix in natural ways because it is controlled by many genes and affected by environmental pressures (Zhang *et al.*, 2020).

An alternative is to use a native genus that proves to be more resistant than other forages to spittlebugs, such as *Paspalum* (Gusmão *et al.*, 2016), both as forage cultivars and for resistance genes prospection. This genus originates from the tropical and subtropical regions of the Americas and is composed of species with high potential for forage (Novo *et al.*, 2016; Acuña *et al.*, 2019). Embrapa Pecuária Sudeste maintains a *Paspalum* germplasm bank that allowed breeding program development using *Paspalum regnellii* as a source of sexuality in crosses for the apomictic breeding program (Matta *et al.*, 2022). Moreover, it is a perennial species with good biomass production, some resistance to shading and high forage potential (Bortolin *et al.*, 2019).

Currently, genomic information is essential in breeding programs to increase the gains and reduce the required time to release a new cultivar, but the application of these tools has been very limited until now (Pereira *et al.*, 2018). *P. regnellii*, for example, has only a few nucleotide sequences publicly available in the National Center for Biotechnology Information (NCBI) and no published genetic studies. Transcriptome analysis is an approach that can provide the complete transcript expression profile in a specific response condition (Egan *et al.*, 2012), including new and rare transcripts even for species without prior genomic information (Wang *et al.*, 2009). This analysis has been successfully applied in works that aimed to identify pest resistance genes in different plants, including species from the Poaceae family (Zhao *et al.*, 2017), and has generated interesting differential expression results related to responses in infested plants (Becker *et al.*, 2017; Cui *et al.*, 2017; Wang *et al.*, 2018).

In addition to the lack of genomic studies in the species that are the subject of this study, no expression analysis of resistance to spittlebugs has been reported until now, representing an important information gap for such a complex process. In this context, this research aimed to characterize the roots of two *P. regnellii* genotypes, with different resistance level to *M. spectabilis* spittlebug nymphs, in the scopes of nymph survival, root anatomy, transcriptomic analysis and coexpression and metabolic networks to elucidate the genetic and molecular mechanisms involved in plant defense.

MATERIALS AND METHODS

Resistance experiment

Bioassays were conducted to determine the resistance level of *P. regnellii* accessions to *M. spectabilis* in a greenhouse at Embrapa Pecuária Sudeste, São Carlos, SP, Brazil, at 21°96'17" S and 47°84'21" W, at a mean altitude of 856 m. The effects on genotypes were evaluated using the nymphal survival parameter to test the antibiosis resistance hypothesis with

methodology adapted from Lapointe *et al.* (1989), Valério *et al.* (1997) and Gusmão *et al.* (2016). Genotypes BGP-215, BGP-248, BGP-258, BGP-341, BGP-344, BGP-345 and BGP-397 were established by seeds from plants retained in the *Paspalum* Active Germplasm Bank at Embrapa Pecuária Sudeste and compared with the susceptible controls *Urochloa brizantha* (Hochst. ex A. Rich.) R. D. Webster cv. Marandu and *U. decumbens* (Stapf) R. D. Webster cv. Basilisk.

The seeds were sown in a 162-cell JKS® model tray with 50 cm^3, filled with substrate based on sphagnum peat, expanded vermiculite, dolomitic limestone, agricultural plaster and NPK fertilizer (Carolina®). After 30 days, seedlings were transplanted to 480 ml Styrofoam cups (DART® 480J32) containing the substrate for Vivatto® Plus plants. Each one was covered with J32 DART® Styrofoam cups containing a central hole for the plant aerial part to maintain the absence of light on the lap and allow superficial root growth to ensure local feed for nymphs hatched.

The experimental design was completely randomized, with ten replicates. Plants were infested with insect eggs 30 days after transplanting using five eggs obtained according to Auad *et al.* (2007). At the time of plant infestation, the eggs showed complete embryonic development, that is, the appearance of the operculum and reddish ocular and glandular spots (Valério, 2009). The nymphal survival evaluations occurred weekly, from plant infestation with eggs until the adult insects emerged.

Finally, the nymph survival percentage was calculated for each plant genotype and transformed into SQUARE ROOT (X + 0.5) and submitted to variance analysis, and the variable averages were compared by Tukey's test ($p<0.05$) based on the procedures of SAS, PROC-GLM (SAS Institute, 2010).

RNA-seq experimental design and plant material

The experiment was carried out in a greenhouse at Embrapa Pecuária Sudeste in the season 2019/2020. Two sexual tetraploid genotypes (BGP 248 and BGP 344) of *P. regnellii* (2n=4x=40) were selected based on the contrast identified in the previous results of spittlebug resistance evaluation in different genotypes of the species.

Seedlings of the two genotypes were obtained from tillers of the same adult plant already established to ensure genetic identity, and the plants were cultivated in Styrofoam cups for six weeks, so the root system was well developed. Adult insects of *M. spectabilis* were collected from pasture areas of *U. brizantha* cv. Marandu, and the eggs were obtained in the laboratory and incubated in the B.O.D chamber (T 25°C, RH 60% and photophase of 12 hours) until the

complete development of the embryonic phase. Ten completely developed eggs were confined to the root system of the plant through an aperture made on the side of the styrofoam cup. After infestation, the system was closed with tape to guarantee nymph fixation. The control samples went through the same procedure but without infestation with eggs to simulate the same stress on the plants.

Root samples were collected in triplicate, for both genotypes, under three treatments: without egg infestation (TC), after egg infestation and nymph eclosion (T1: 48 h after TC) and with nymph colonization on the roots (T2: 72 h after T0), totaling nine samples per genotype.

RNA sequencing, data quality and filtering

Total RNA (totRNA) from the roots was extracted with the RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA) according to the manufacturer's protocol. A NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to quantify the RNA. To assess the extraction efficiency and integrity, totRNA samples were evaluated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, EUA). The mRNA sequencing libraries were made for the 18 samples and sequenced on Illumina HiSeq 2500 v4 2x100 bp.

FastQC version 0.11.9 software (Babraham Bioinformatics) was used to assess the quality of the libraries sequenced. The reads were filtered with Trimmomatic version 0.39 (Bolger *et al.*, 2014) to remove the adapters with the Illumina Adapters parameter and SlidingWindow 4:20. In addition, the software SortMeRNA version 2.1b (Kopylova *et al.*, 2012) was used to eliminate the ribosomal RNA (rRNA) with default parameters.

Transcriptome assembly

Since *P. regnellii* does not have a publicly available reference genome, a *de novo* assembly was performed using Trinity version 2.5.1 (Grabherr *et al.*, 2012) with the target K-mer coverage set to 30 (normalize_max_read_cov 30) and other parameters in default. The longest isoform (unigenes) for each transcript was selected with its own software.

An assembly using the genome sequence of diploid *Paspalum notatum* (available at NCBI Genome under accession ASM2253091v1) as a reference was also tested. STAR version 2.7.3a (Dobin *et al.*, 2013) was used to align the reads to the genome, and then StringTie version 2.1.6 (Kovaka *et al.*, 2019) was used to assemble the transcriptome.

Assembly quality was analyzed using the *Viridiplantae* dataset of BUSCO version 5.2.2 (Simão *et al.*, 2015), comparing the sequences of the transcripts with a set of orthologous genes

conserved in plants, and Bowtie2 version 2.3.3.1 (Langmead & Salzberg, 2012), mapping the reads in the transcriptome to determine the transcript abundance with default parameters.

Differential gene expression analysis

Salmon software version 0.14.1 (Patro *et al.*, 2017) was used with default parameters to quantify the longest isoform expression. The differential gene expression (DGE) analysis was performed using the edgeR package version 3.38.4 (Robinson *et al.*, 2010) implemented in R (R Development Core Team, 2011). Raw count data were first normalized with at least 10 counts per million (CPM) and then in a minimum of three samples. For each differential gene expression test we used a false discovery rate (FDR) cutoff ≤0.05 and a minimum log2-*fold change* (logFC) of 2.

For the analysis, samples were coded as 248 (BGP 248) or 344 (BGP 344) followed by C (control without infestation), 1 (48 h after infestation) and 2 (72 h after infestation). Tests for identifying differentially expressed genes (DEGs) were made comparing time points for each genotype, for example, BGP 248 in control treatment against after 48 h of infestation (coded as 248_C vs. 248_1), and between different genotypes at the same time points, such as BGP 248 in control treatment against BGP 344 in control (coded as 248_C vs. 344_C). A total of nine comparisons were performed, following the same codification cited before: 248_C vs. 248_1, 248_C vs. 248_2, 248_1 vs. 248_2, 344_C vs. 344_1, 344_C vs. 344_2, 344_1 vs. 344_2, 248_C vs. 344_C, 248_1 vs. 344_1 and 248_2 vs. 344_2. As there is a biotic stressor in the plants, a Principal Component Analysis (PCA) was performed and a hierarchical cluster based on the Euclidean distance and grouped with the weighted pair group method with arithmetic mean (WPGMA) was organized to confirm the similarity between the replicates.

Functional annotation and enrichment

Trinotate software version 3.2.1 (Bryant *et al.*, 2017) was used to predict the open reading frames (ORFs) and translate the coding sequences to peptides. Transcript functional annotation was performed using diamond software version 2.0.14.152 (Buchfink *et al.*, 2021) against the SwissProt database (UniProt - https://www.uniprot.org/) with at least 60% similarity. Moreover, protein domains were found using HMMER software version 3.3.2 (Mistry *et al.*, 2013), and finally, all results were integrated into the Trinotate extract reports.

Gene Ontology (GO) (Ashburner *et al.*, 2000) terms were retrieved from the sequence that was functionally annotated in the database. We restricted the results for plants (*Arabidopsis thaliana*) and enriched the DEGs in each contrast with the topGO R package (Alexa &

Rahnenfuhrer, 2022) for biological processes using Fisher's exact test (p value < 0.01). The enriched terms were submitted to REVIGO (Supek *et al.*, 2011) with medium similarity allowed (0.7) for data summarization.

Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) database was used to map all DEGs with the *Panicum hallii* reference. Fisher's test (p value < 0.01) was performed to obtain enriched metabolic pathways.

Gene coexpression and metabolic network analysis

Two distinct network models to infer biological relationships were performed: the construction of two gene coexpression networks based on RNA-seq data obtained from each genotype and the establishment of a metabolic network incorporating pathways associated with the DEGs.

For the creation of gene coexpression networks, we employed the highest reciprocal rank (HRR) methodology proposed by Mutwill *et al.* (2010). Initially, we computed pairwise correlations between genes using the R Pearson correlation coefficient. To ensure robust associations, we set a minimum absolute correlation coefficient threshold of 0.8 and considered a maximum of 30 strongest edges for the network connections. The network construction process was performed using R statistical software (R Development Core Team, 2011) and the igraph library version 1.3.5 (Csardi & Nepusz, 2006). Subsequently, we calculated the hub score for each gene using Kleinberg's hub centrality algorithm (Kleinberg, 1999).

For the metabolic network, we identified enzyme commission (EC) numbers associated with the annotations of all DEGs. Leveraging this information, we used the KEGG database (Kanehisa & Goto, 2000) to select the metabolic pathways in which these enzymes are involved. Subsequently, a unified metabolic network was constructed using BioPython version 1.78 (Cock *et al.*, 2009), and centrality measures were evaluated using Cytoscape version 3.91.1 (Shannon *et al.*, 2003).

Quantitative reverse transcription PCR (RT–qPCR)

The DEGs were validated by RT–qPCR. Ten target genes were selected from the DEG list by the fold-change value. In addition, five housekeeping genes were chosen based on the literature (Gimeno *et al.*, 2014; Andrade *et al.*, 2017; Liu *et al.*, 2017; Takamori *et al.*, 2017). Primer pairs (Supplementary Table 1) were designed using Primer3Input (Untergasser *et al.*, 2012) with the following parameters: size between 18 and 22 bases, amplification product size between 75 and 175 bp, melting temperature (Tm) between 57 and 59°C and GC content of

approximately 50%. Dimers, heterodimers and hairpin were checked in NetPrimer (Premier Biosoft).

The cDNA synthesis was performed with the GoScript™ Reverse Transcription System A5000 (Promega, Madison, WI, USA) according to the manufacturer's protocol. RT–qPCR was performed on QuantStudio 6 Pro (Applied Biosystems, Waltham, MA, EUA) using GoTaq® qPCR Master MixA6001 (Promega, Madison, WI, USA) with the following protocol: 95°C for 120 s, followed by 40 cycles of 95°C for 15 s and 60 s at 60°C. Melting curves were obtained by heating from 60 to 95°C to verify the products after amplification. All qPCR experiments were performed using two technical and three biological replicates and analyzed with Design and Analysis Software 2.6.0 (Thermo Fisher Scientific Inc., Waltham, MA, EUA).

Anatomical analysis of the roots

As in the RNA-seq experiment, the seedlings were obtained from tillers of the same plant and cultivated for six weeks for the root system to develop. Roots of both genotypes, without spittlebug infestation, were fixed in BNF (phosphate buffer solution, formalin; 9:1 v/v) for 48 hours (Lillie, 1965) and stored in 70% ethanol. The material was dehydrated in an ethanol series including hydroxyethyl-methacrylate (Historesin® Leica) following the manufacturer's recommendations. Cross-sections 5 μm thick were made with the aid of a Microm HM 340E rotary microtome (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

The slides were stained with 0.05% toluidine blue in citrate buffer (pH = 4.5) (O'Brien *et al*., 1964) and finally mounted on Entellan® synthetic resin (Merck KGaA, Darmstadt, Germany). Images were captured with a digital camera (Olympus DP71) coupled to an Olympus BX51 optical microscope (Olympus Optical Co., LTD, Japan). To detect the main chemical compounds present in the cell walls of root cells, we used the following histochemical tests: acidified phloroglucinol for lignin detection (Johansen, 1940) and Nile red fluorochrome for lipid substances under a DAPI-LP filter (ex377/50 nm; em447/60 nm) (Greenspan *et al.*, 1985).

RESULTS

1. **Spittlebug resistance experiments**

The nymph survival average, percentage and standard error were calculated from the seventh day of plant infestation with *M. spectabilis* eggs until the emergence of the last adult insects at 56 days after infestation (Fig. 1a). The percentages in the controls, *U. brizantha* and *U. decumbens*, were higher than those verified in *P. regnellii* genotypes. Between *P. regnellii*

accessions, there were significant differences in nymph survival until 21 days after infestation, with mortality being more intensive in BGP 344 (Fig. 1a).

Significant differences were observed from the average nymph survival percentage for each *P. regnellii* genotypes after days, highlighting nymph mortality between BGP 344 and BGP 248, mainly young nymphs of the 1st and 2nd instars until the 21st day after infestation (Fig. 1b).

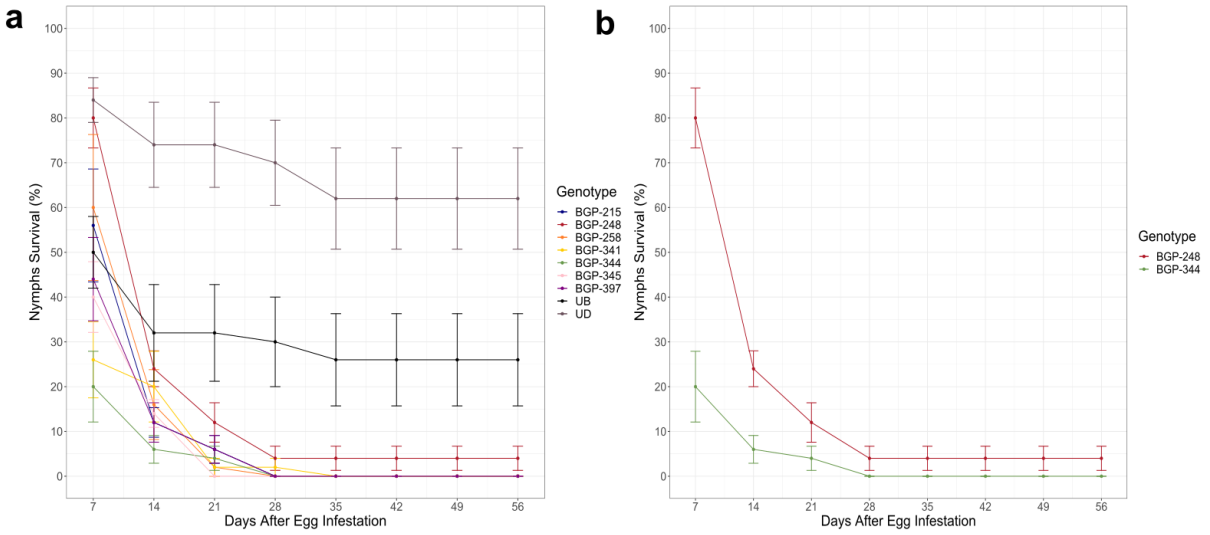


Figure 1. Survival percentage (average ± standard error) of *M. spectabilis* nymphs **(a)** in seven *Paspalum regnellii* genotypes in comparison with susceptible controls *Urochloa brizantha* and *U. decumbens*. **(b)** The two genotypes (BGP-248 and BGP-344) selected for transcriptome analysis based on their contrasting behavior to *M. spectabilis* nymph survival.

2. Transcriptome assembly and annotation

The sequencing from 18 samples, from two genotypes in three different time points, generated a total of 806,942,117 paired-end reads from the *P. regnellii* root transcriptome. Sequence quality analysis performed with FastQC software revealed that all 18 samples presented a similar quality profile. With the filtering procedures, ~19.97% of the reads were discarded as low-quality sequences (remaining 645,827,820 reads) and ~4.95% as ribosomal RNA residues (remaining 613,888,166 reads).

Both assemblies, *de novo* with only the longest isoform (unigenes) and the reference based assembly using the *P. notatum* genome, were performed and their statistics were compared (Table 1). The second method generated good transcript results, but in the initial stage of aligning the reads with the reference, we obtained only 55.96% success, losing a large

amount of data. In addition, it was possible to observe with BUSCO that the assembly generated many duplicate transcripts (44.9%), probably because of the different ploidy levels between *P. notatum* (diploid) and *P. regnellii* (tetraploid).

Table 1. Comparison between the *de novo* assembly (Trinity - Unigenes) and the genome reference (StringTie) followed by their quality statistics.

Software	Alignment (STAR)	Transcripts	N50	Mean size (pb)	BUSCO	Bowtie2
Trinity (Unigenes)	-	575,219	788	568.39	Complete: 90.4% Duplicate: 8%	80.89%
StringTie	55.96%	73,887	2003	1335.87	Complete: 95.2% Duplicate: 44.9%	65.51%

Despite the large transcript number and lower values of N50 and mean size, transcriptome *de novo* assembly in polyploid plants is naturally complex because of the homeologous presence that creates many transcripts with structural abnormalities (Payá-Milans *et al*., 2018). Trinity software lost less data and generated successful results in other *Paspalum* species (de Oliveira *et al*., 2020), so this assembly was selected for the following analyses. Almost 16% (92,035) of the transcripts were annotated against the SwissProt database, and 7.3% (42,040) of the proteins were predicted with Transdecoder.

3. Differential Expression Analysis and Enrichment

After CPM normalization and expression filtering of the raw count data for all samples, we obtained a total of 21,508 genes that will be the data universe for the next analysis. In both PCA and WPGMA analysis (Fig. 2), it was possible to observe a clear genotype separation in addition to a proximity between the samples of the control treatment and 48 h after plant infestation, while the last treatment was more distant.

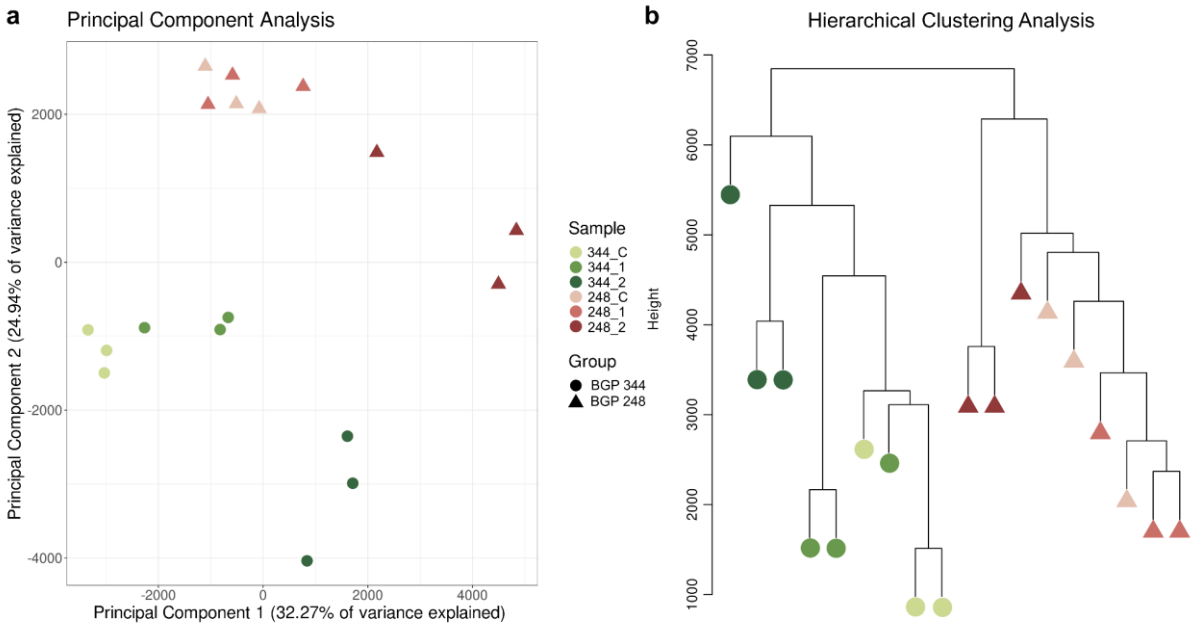


Figure 2. Sample distribution by **(a)** Principal component analysis. **(b)** Hierarchical clustering analysis performed with Euclidean distance and weighted pair group method with arithmetic mean (WPGMA).

The genes were submitted to differential expression analysis (Supplementary Table 2), and contrasts between genotypes at the same treatment time obtained many DEGs even in the control, showing a considerable basal difference (Table 2). In the treatment comparison, neither genotype showed DEGs between the TC and T1, but many genes changed their expression after 72 h of infestation (T2), mostly in the BGP 344 genotype.

Table 2. Number of total, up- and downregulated DEGs obtained in each treatment comparison.

DEGs	R0vsR1	R0vsR2	R1vsR2	S0vsS1	S0vsS2	S1vsS2	R0vsS0	R1vsS1	R2vsS2
Total	0	408	30	0	65	41	865	783	1039
Up-regulated	0	1	0	0	14	13	533	469	500
Down-regulated	0	407	30	0	51	28	332	314	539

Venn diagrams were created to investigate the intersection of DEGs between the comparisons (Fig. 3). The contrasts in the same genotype showed that only some genes participated in more than one treatment, and most were only after 72 hours (Fig. 3a-b). On the other hand, in genotype contrasts, 616 genes were shared between all three treatments, demonstrating the existence of an important natural difference in expression among plants even with no infestation (Fig. 3c).

Aiming to exclusively select genes related to resistance, an analysis of the intersection between the genotypes in time points was performed. Some transcripts had increased expression over time in both genotypes, but even in the control, they were still more highly expressed in BGP 344 (Table 3), and their annotations are possibly related to resistance: suppressor cell death (LSD1), resistance gene analogs (RGA5R) and one domain-containing protein (DUF4220). Other transcripts expand their expression in BGP 344 and are naturally more abundant in BGP 248: transcription factors that regulate secondary cell wall (NST1) and response inducers to ozone and pathogens (AtOZI1). Last, those that increased the expression in the BGP 248 genotype were mostly related to abiotic stress (PLA7).

Table 3. DEGs intersection and their relation to resistance in both genotypes.

Gene name	Annotation	Genotype with more expression	After BGP 344 treatments	After BGP 248 treatments
TRINITY_DN200773_c0_g1	LSD1	BGP 344	-	Upregulates
TRINITY_DN221780_c5_g1	DUF4220		Upregulated	
TRINITY_DN219172_c0_g1	RGA5R		-	
TRINITY_DN212267_c2_g2	PLA7	BGP 248	-	Upregulates
TRINITY_DN189406_c0_g1	PLA7			
TRINITY_DN190702_c3_g1	NST1	BGP 248	Upregulates	-
TRINITY_DN205618_c0_g1	AtOZI1			

a) GOs

The functional classes of all DEGs were enriched in 89 different GO terms of biological processes in topGO (Supplementary Table 3). The BGP 344 genotype after spittlebug infestation showed terms closely related to the resistance process (Fig. 3a), such as the chitin catabolic process (GO:0006032), response to oxidative stress (GO:0006979), response to other organisms (GO:0051707) and response to herbivores (GO:0080027).

In contrast, in BGP 248, there were some terms related to resistance (Fig. 3b), including salicylic acid catabolic process (GO:0046244) and regulation of anthocyanin biosynthetic process (GO:0031540), but they were not very specific. Furthermore, some terms revealed that the plant ignores infestation and continues its normal growth, such as seed maturation (GO:0010431) and positive regulation of lateral root development (GO:1901333).

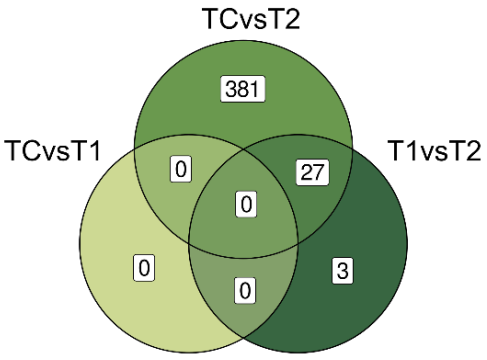
When we compared the genotypes in the last infestation treatment (Fig. 3c), we obtained some interesting terms that had different expression levels between them, such as defense response (GO:0006952), plant-type hypersensitive response (GO:0009626), positive regulation of circadian rhythm (GO:0042753), retrotransposition (GO:0032197) and protein phosphorylation (GO:0006468).

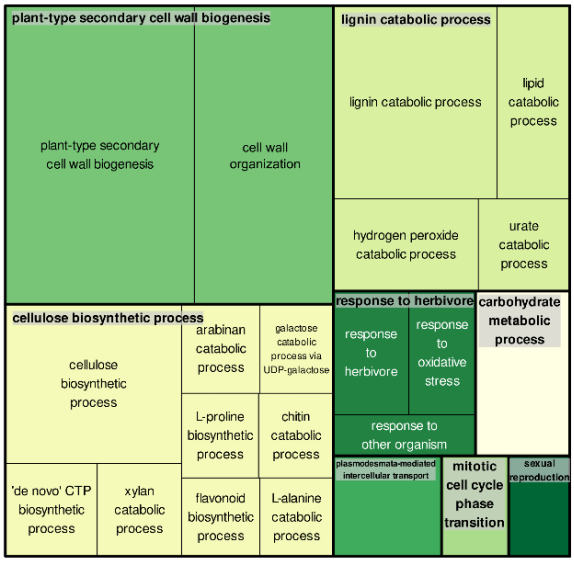
a) KEGG

Furthermore, the DEGs were enriched in 13 metabolic pathways by the KEGG database (Table 4). Of these, some were related to basal cellular mechanisms and enriched in the comparisons of both genotypes: glycolysis/gluconeogenesis, fatty acid degradation, tyrosine metabolism, alpha-linolenic acid metabolism and pyruvate metabolism (Table 4). Enrichment of the DNA replication pathway, as it is a more structural part, was not selected for further study.

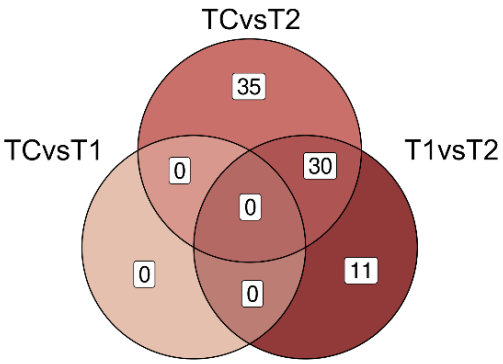
The other seven pathways that occurred in just one treatment comparison and are possibly related to resistance were chosen to make a unique metabolism network: purine metabolism, caffeine metabolism, glutathione metabolism, galactose metabolism, cutin, suberin and wax biosynthesis, and circadian rhythm - plant, starch and sucrose metabolism.

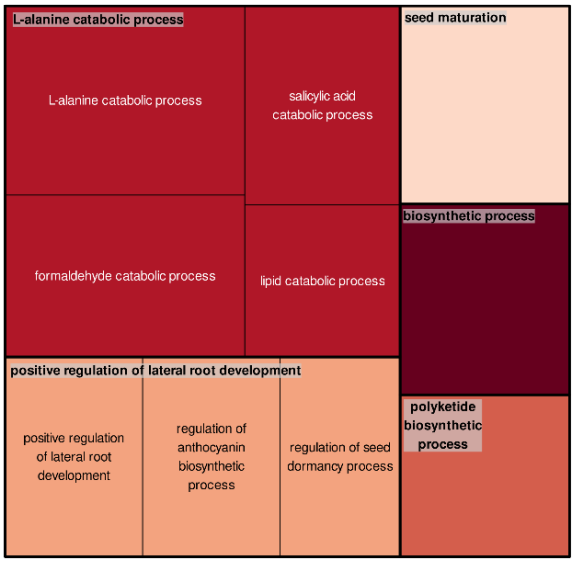
a BGP 344



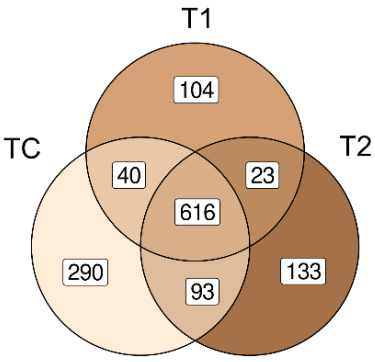


b BGP 248





c BGP 248 vs BGP 344



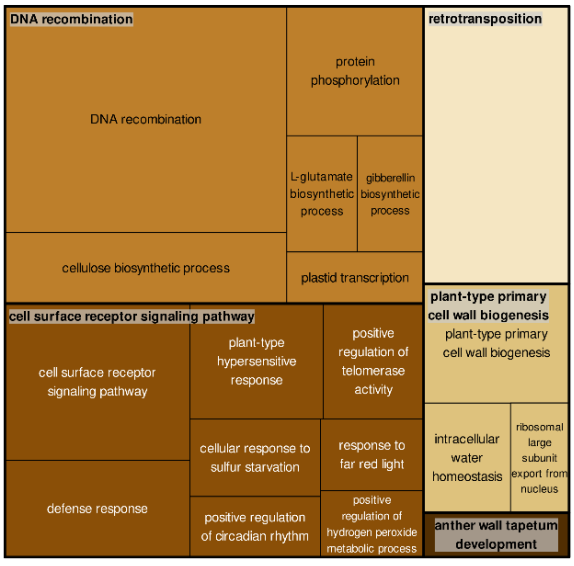


Figure 3. Venn Diagram of DEGs and summarized terms of Gene Ontology in TreeMap. **(a)** Venn Diagram for all BGP 344 comparisons and GO terms enriched after 72h (upregulated in 344_2). **(b)** Venn Diagram for all BGP 248 comparisons and GO terms enriched after 72h (upregulated in 248_2). **(c)** Venn Diagram between genotypes and GOs TreeMap for 344_2 vs. 248_2.

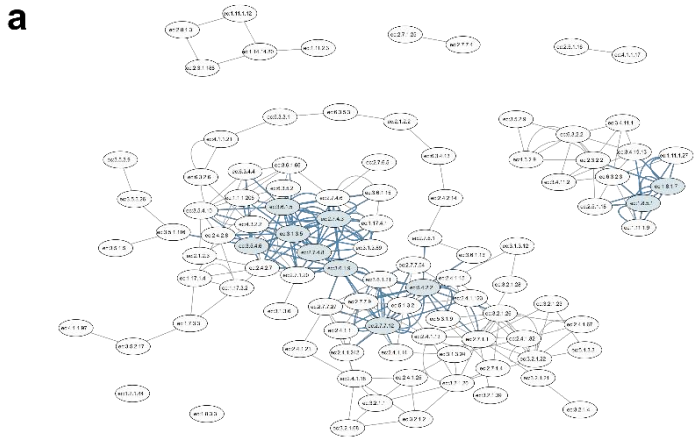
Table 4. KEGG enriched pathways arranged in decreasing *p*-value order and their respective related genotype contrast comparison.

KEGG ID	Pathway	Contrast	*p* value
map00500	Starch and sucrose metabolism	344_C vs. 344_2	0.044433
map00480	Glutathione metabolism	344_1 vs. 248_1	0.041352
map00071	Fatty acid degradation	248_C vs. 248_2	0.038649
		344_1 vs. 344_2	0.025122
map00620	Pyruvate metabolism	248_C vs. 248_2	0.038649
		344_1 vs. 344_2	0.025122
map04712	Circadian rhythm - plant	344_C vs. 248_C	0.027134
map00592	alpha-Linolenic acid metabolism	248_C vs. 248_2	0.026678
		344_1 vs. 344_2	0.017209
map00073	Cutin, suberin and wax biosynthesis	344_C vs. 248_C	0.026091
map00010	Glycolysis / Gluconeogenesis	248_1 vs. 248_2	0.023988
		248_C vs. 248_2	0.018660
		344_1 vs. 344_2	0.009861
map00230	Purine metabolism	344_1 vs. 344_2	0.017209
map00350	Tyrosine metabolism	248_C vs. 248_2	0.016575
		344_1 vs. 344_2	0.010610
map00232	Caffeine metabolism	344_1 vs. 344_2	0.010610
map03030	DNA replication	344_C vs. 248_C	0.010392
map00052	Galactose metabolism	344_C vs. 344_2	0.000021

4. Network Analysis

A metabolic pathway was prepared from the KEGG enriched pathways and resulted in 109 enzymes related to the resistance process (Supplementary Table 4). The enzymes with greater influence on the network ("EdgeCount") and in relationship with other enzymes (In and Outdegree) are highlighted in blue in Figure 4a and listed here: adenylate kinase (ec:2.7.4.3), glutathione reductase (ec1.8.1.7), guanylate kinase (ec:2.7.4.8), AMP deaminase (ec:3.5.4.6), apyrase (ec:3.6.1.5), nucleotide diphosphatase (ec:3.6.1.9), AMP phosphatase (ec:3.1.3.5) phosphoglucomutase (ec:5.4.2.2), glutathione dehydrogenase (ec:1.8.5.1) and uridyl transferase (ec:2.7.7.12).

Gene coexpression networks were constructed for each genotype separately. The BGP 344 network (Fig. 4b) had 7,410 nodes (Supplementary Table 5), so we observed many more hubs and connections in its network compared to that of BGP 248 (Fig. 4c), which had 5,275 nodes (Supplementary Table 6). This suggests a large difference in transcript number expression between genotypes as well as in DEG analyses, showing the complexity of the genetic response. Furthermore, some of the transcripts with the greatest impact on the networks because of the higher "Degree" number were not characterized or not annotated.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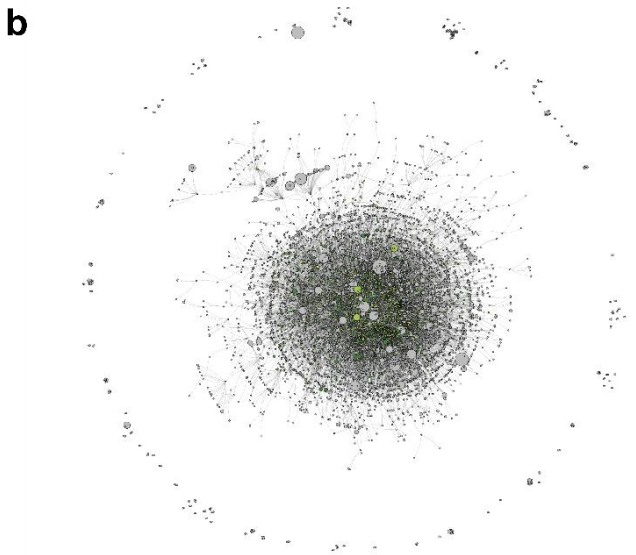
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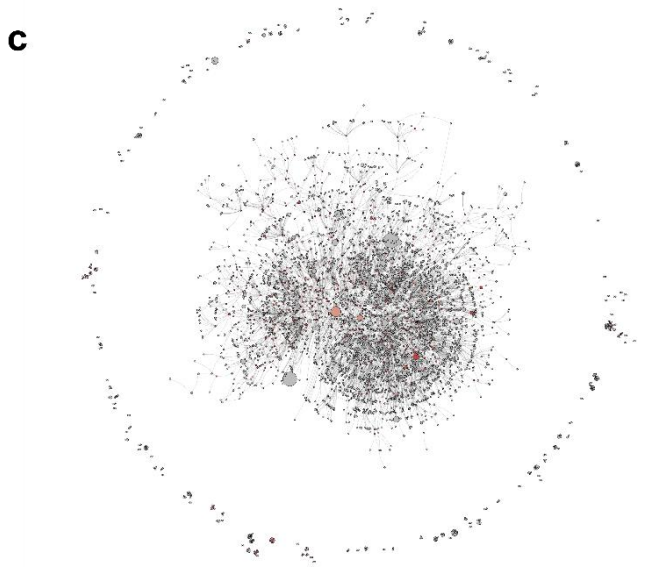
c

Figure 4. Network representations. **(a)** Metabolic network, with the most significant enzymes of enriched pathways and their connections in blue. **(b)** BGP-344 coexpression network. The green nodes represent hub genes. **(c)** BGP-248 coexpression network. The pink nodes represent hub genes.

5. RT–qPCR validation

A total of ten transcripts were selected to validate the differential gene expression, and in two of them, flavonol 3-sulfotransferase (F3) and IQ domain-containing protein (IQ), primers did not score properly, so they were discarded. Validation was performed with two unknown transcripts (D1 and D2) and six annotated genes - phospholipase D alpha 2 (PL), enhancer of mRNA-decapping protein 4 (VC), acyclic sesquiterpene synthase (AC), lipoxygenase 2 (LO), protein reveille 2 (RE) and protein iron-related transcription (IR) - chosen because the considerable difference in expression.

In addition, two of five normalizers were selected as endogenous controls in the experiment based on lowest quantification cycle (Cq) variation values using five methods collected by RefFinder (Xie *et al.*, 2012): NADH dehydrogenase ubiquinone 1 alpha (NDAU1) and splicing factor U2af small subunit B (U2AFB) (data not shown). The expression patterns generated by RT–qPCR analysis agree with the transcriptome data, proving the accuracy of the bioinformatic results (Figure 5a), in addition to the data showing a good correlation (Figure 5b).

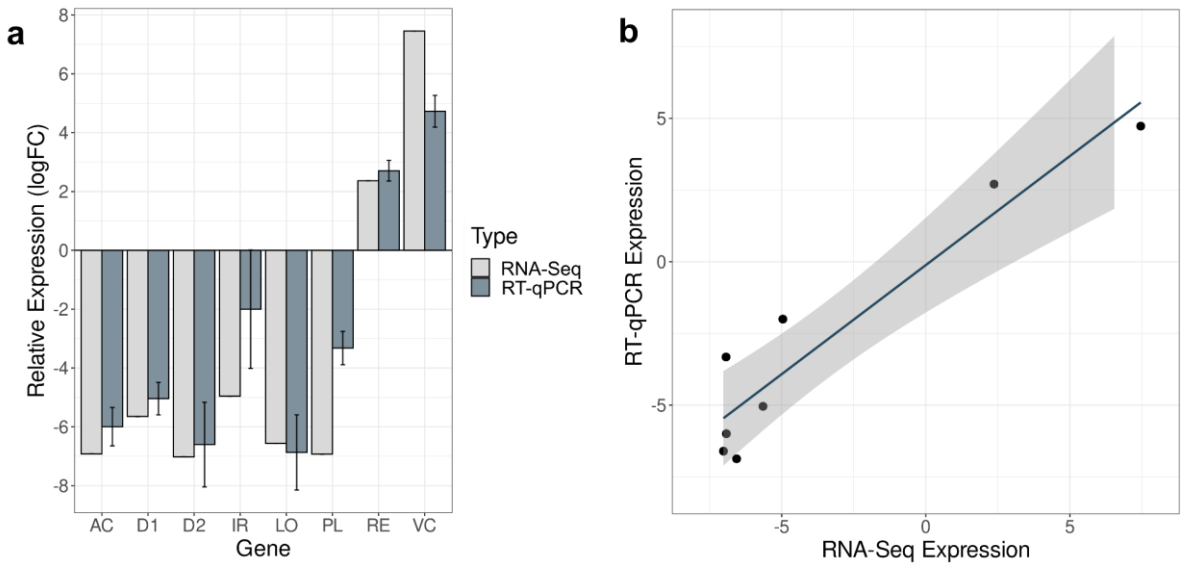

Figure 5. RT–qPCR transcriptome validation results. **(a)** Relative expression (log2-fold change) of RT–qPCR and transcriptome data represented by the mean expression value ± SEM (n = 3) relative to BGP 344 without infestation (344_C). **(b)** Correlation between RNA-seq and

RT–qPCR analysis. Abbreviations - AC: acyclic sesquiterpene synthase; D1: unknown transcript 1; D2: unknown transcript 2; IR: protein iron-related transcription; LO: lipoxygenase 2; PL: phospholipase D alpha 2; RE: protein reveille 2; VC: enhancer of mRNA-decapping protein 4.

6. Anatomical analysis of the roots

We performed a comparative anatomical analysis between roots of the two *P. regnellii* genotypes without biotic stress to investigate whether transcriptomic variations are evidenced in the fine root structure, where spittlebugs prefer to feed.

Both genotypes present a uniseriate epidermis, with a slightly thickened external periclinal cell wall and the presence of secretion (Fig. 6a-b). Regarding the cortex, we observed the presence of an exodermis in both genotypes, characterized by a layer of cells with differences in the cell wall composition (Fig. 6a-b). In BGP 248, the exodermis has a thickened cell wall consisting of lipid material (Fig. 6c-d), and in BGP 344, the cells show lignified secondary cell wall deposition, as confirmed by the acidified phloroglucinol test (Fig. 6e). Additionally, we observed that the exodermis cells of this genotype collapse and acquire irregular shapes, a process that begins close to the root apex (Fig. 6b).

The cortical parenchyma in both genotypes has wide intercellular spaces and forms an aerenchyma, which is more developed in BGP 344, contributing to the formation of irregular cells of the exodermis (Fig. 6a-b). In the BGP 344 genotype, the internal cellular layers of the cortical parenchyma present globular cells arranged in a spiral pattern around the endodermis (Fig. 6b). The endodermis has Casparian strips, and the cells form “U”-shaped lignified wall thickenings (Fig. 6a-b). The pericycle is uniseriate, and the vascular system is composed of few protoxylem poles alternating with phloem strands and few central metaxylem elements (Fig. 6a-b).

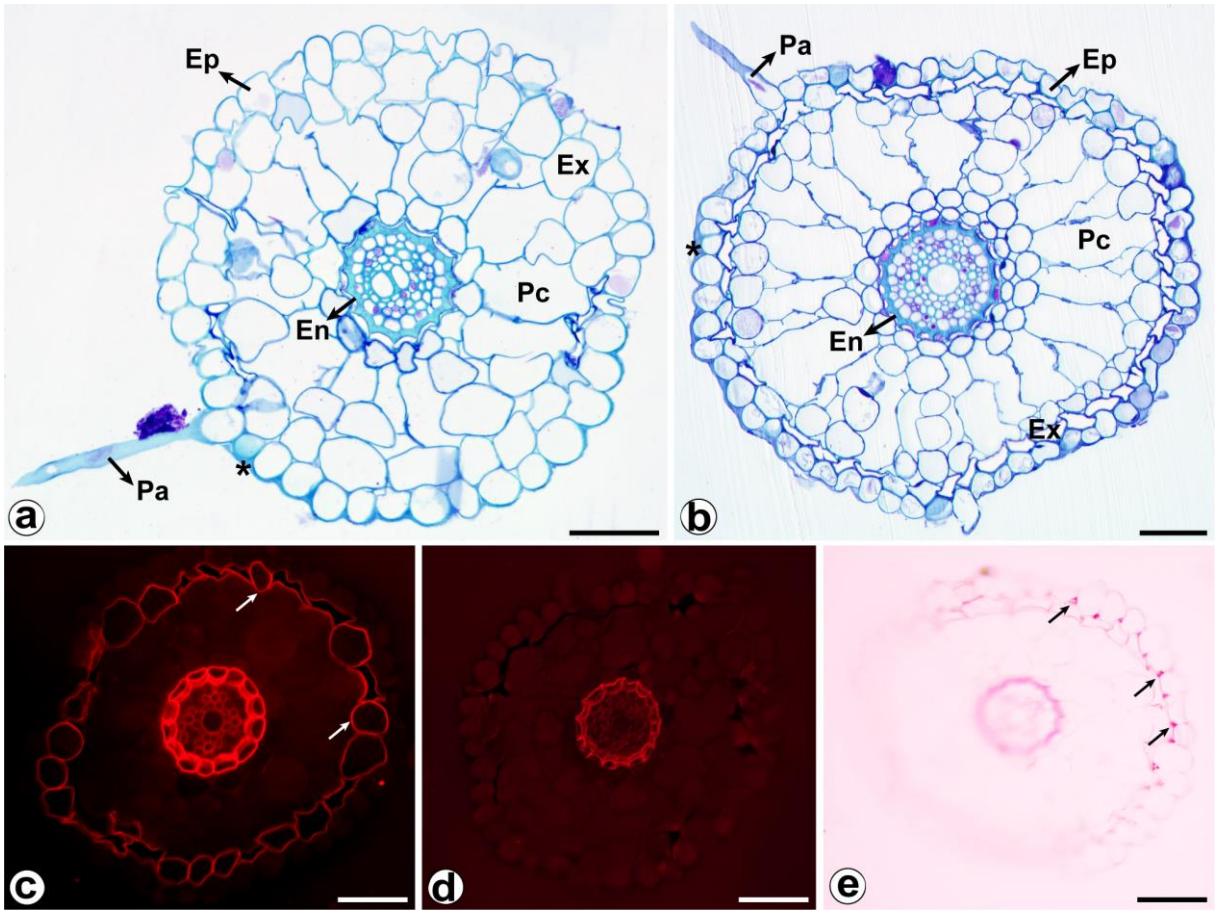

Figure 6. Anatomical structure and histochemical tests in root cross-sections of different *Paspalum regnellii* genotypes. **(a)** BGP 248 and **(b)** BGP 344, with asterisks indicating secretion covering the epidermis. **(c)** Positive reaction to Nile red fluorochrome, with arrows showing wall thickenings with lipid composition in BGP 248. **(d)** Negative reaction to Nile red fluorochrome on the cell walls of BGP 344 cells. **(e)** Positive reaction to acidified phloroglucinol showing lignin deposition in the cell walls of BGP 344. Abbreviations - En: endoderm; Ep: epiderm; Ex: exoderm; Rh: root hairs; Cp: cortical parenchyma. Scale bars: 50 µm (a-e).

DISCUSSION

Desirable characteristics for a superior forage plant consist of, in addition to resistance to biotic and abiotic stresses, high nutritional value and biomass production, and good palatability, regrowth ability and adaptation to infertile soils (Matias *et al.*, 2018). However, complex genetic architectures are obtained by combining these traits because of the correlation from close linkages between genes or when they are responsible for multiple characteristics. Therefore, the main focus of forage breeding is still biomass production, which is the trait that is present in practically all cultivar studies (Casler & Santen, 2010), and for that reason, the

largest pasture area in South America is composed of African *Urochloa* species (Matias *et al.*, 2021). Despite their productivity qualities, these forage grasses are more susceptible to many spittlebug species than other grass species, such as those from the genus *Paspalum* (Gusmão *et al.*, 2016).

Gaining resistance through interspecific crossings may not be so simple and may lead to the loss of other good traits. In addition, current biotechnologies such as gene editing enable specific gene expression into plant varieties and accelerate the breeding cycle for improving genetic transformation in grass species (Ramstein *et al.*, 2019). An interesting approach is the transference of resistance genes (Jacob *et al.*, 2018), such as identifying targets related to herbivore resistance present in *Paspalum* and inserting them in cultivars of interest that do not have natural resistance to spittlebugs, such as *Urochloa* grasses.

Despite the importance of some species of the genus *Paspalum* as forage, turf and grain (Acuña *et al.*, 2019), knowledge of their molecular genetics is still lacking, with low genomic resources available. In this study, the *de novo* transcriptome assembly represents the first large-scale molecular resource for *P. regnellii* and generates reliable novel information on gene expression. The low alignment (Table 1) with another species of the same genus (*P. notatum*) suggests genomic variation even within the genus itself, showing a large difference in genome composition, which was also confirmed by the low number of annotated transcripts. This was expected because of the great genomic complexity and diversity present in *Paspalum* that resulted in a separation of the two species into botanical informal groups primarily by Chase (1929), a phylogenetic segregation in distinct clades (Delfini *et al.*, 2023) and the different genomic composition by comparing a diploid genome from the Notata group (NN) and an allotetraploid from the Virgata group (IIJJ) (Cidade *et al*., 2013).

Tropical grasses tend to have well-established defense mechanisms against insect pests and pathogens, highlighting secondary metabolites, but first, the physical barriers delay infestation with, for example, cell wall adaptations (Zhang *et al*., 2020). Among the cell wall components that can cause mechanical difficulties in infestation is lignin, which reduces the palatability of plants for herbivores (Xiao *et al*., 2023). It increases cellular rigidity, and its higher concentration has been described as a resistance factor (Lynch *et al.*, 2021; Wu *et al*., 2022), avoiding colonization of the plant by insects, which characterizes the antixenosis resistance mechanism (Kogan and Ortman, 1978). In our root histochemical analyses, it was possible to observe that lignification occurs only in the walls of BGP 344 (Fig. 6e), confirming that there is already a natural anatomical predisposition that makes it less likely to be a host. Moreover, in this same genotype, we can observe a differentiation of the sclerenchymatous

tissue into aerenchyma (Fig. 6a-b) that can contribute to the resistance process, since it has already been reported as responsible for improving the gas displacement in the cellular environment when there is an oxygen shortage, helping to avoid hypoxia (Yamauchi & Nakazono, 2022; Teixeira *et al.*, 2022).

Secondary metabolites reduce insect survival and development, preventing the insect from feeding and characterizing resistance by antibiosis (Valério *et al.*, 2001). However, it is necessary that the plant has a certain tolerance to damage since spittlebug resistance is a quantitative trait, with more than one gene controlling it (Dinardo-Miranda *et al.*, 2014). For this reason, through DEGs analysis (Table 2), we observed that after 48 h of infestation, the anatomical tolerance and the time for nymphal tissue feeding were influential. After 72h, the genes are mostly related to defense signaling and analyses with longer infestation times could show the metabolites and phytohormones production.

With the beginning of insect feeding on plant tissues, changes first occur in the cell membrane conformation and the production of reactive oxygen species (ROS) is triggered (Walters, 2011). The second defense line then begins, with secondary metabolites and herbivore-induced plant volatiles (HIPVs), which stimulate defense in neighboring plants and bring predators, both controlled by phytohormones with emphasis on jasmonic acid (Lin *et al.*, 2022). This is consistent with the GO enrichment results obtained in BGP 344 (Fig. 3a) since we found cell configuration terms, such as cell wall organization and plant-type secondary cell wall biogenesis, together with some terms with a regenerative character, such as cellulose biosynthetic process and lignin catabolic process, both related to the root anatomic findings. ROS-related terms were also identified as hydrogen peroxide catabolic process, response to oxidative stress and response to herbivores.

An interesting GO found in BGP 248 (Fig. 3b) is the salicylic acid (SA) catabolic process. This phytohormone is usually linked to the stress response and its degradation is possibly justified because SA alone does not seem to play a large role in spittlebug defense. Compounds present in *M. spectabilis* saliva may impede its signaling, although in some susceptible species (such as *U. decumbens*) the SA has been identified after infection (Barros *et al.*, 2021). In addition, it is common for plants to focus their energy expenditure on resisting herbivores, increasing the production of resistance mechanisms to the detriment of their development (Divekar *et al.*, 2022); this does not occur in the BGP 248 genotype, which continues to promote root growth and seed development, for example. With this, it is possible to understand that this genotype has a late recognition of the pest because of the different defense mechanism presence.

This difference in response level becomes more evident with the pathway analysis (Table 4). The metabolism of starch and sucrose, purine and caffeine were more active over time only in BGP 344 and are possibly related to the plant response to herbivores. Starch is an energy reserve that releases it in the form of sucrose (Du *et al.*, 2020). Under environmental stress, starch provides plant resources to recover and alleviates the problems generated by carbon consumption (Qiu *et al.*, 2021; Zhang *et al.*, 2021), consequently being mainly related to the response and tolerance to abiotic factors (Thalmann & Santelia, 2017; Chen *et al.*, 2023). In addition, purine metabolism generates secondary metabolites called purine alkaloids, and caffeine is one of them (Ashihara *et al.*, 2008). This compost plays a role in biotic stress plant defense, inducing the production of hormones such as salicylic and abscisic acid (Wang *et al.*, 2016). Thus, the increased activity of these three metabolisms in the BGP 344 genotype when infected by spittlebugs suggests involvement in the plant's defense response, generating energy and releasing secondary metabolites.

The pathways enriched in the contrasts between the genotypes in the control sample (circadian rhythm - plant and cutin, suberin and wax biosynthesis) are related to morphological characteristics intrinsic to each genotype that confer plant tolerance. The circadian rhythm regulates plant physiological processes according to environmental differences, providing survival advantages (Xu *et al.*, 2022). By regulating carbon metabolism and hormone signaling pathways, it is closely linked to stress responses for stabilizing plant development (Bhattacharya *et al.*, 2017), including metabolite liberation against herbivore attack (Hua, 2013); that is, the plant seems to be naturally more prepared to deal with spittlebugs. In the second, cutin, suberin and wax constitute the cuticle structure, which is responsible for stiffening the cell wall and forming the first layer of plant immunity (Mahatma *et al.*, 2021). Studies suggest that cutin is involved in plant defense (Fich *et al.*, 2016) and that suberin plays an important role as a plant root cell barrier against adverse environmental situations (Xiao *et al.*, 2020) and pathogens (Guo *et al.*, 2022). In treatments with infestation, an elicited response commanded by the glutathione pathway is enriched. It is one of the major contributors to antioxidant defense, controlling ROS under biotic and abiotic stress conditions (Gong *et al.*, 2018; Huang *et al.*, 2019). That is, in the BGP 344 genotype, in addition to structural differences such as wall constitution and a greater release of compounds from the purine and caffeine pathways, there is an induction of the glutathione pathway after infestation, unlike in BGP 248, in which it is not present.

Herbivore resistance, in addition to secondary metabolites and phytohormones, generally includes important proteins such as kinases and transcription factors (Wang *et al.*,

2020). To find this specific information, network analyses can bring pontual inferences. Metabolic networks (Fig. 4a) highlight two enzymes from the ascorbate-glutathione (AsA-GSH) cycle, dehydroascorbate reductase (DHAR) and glutathione reductase (GR), that handle oxidative stress conferring plant tolerance but also regenerate AsA and GSH to maintain the redox state (Hasanuzzaman *et al.*, 2019; Dorion *et al.*, 2021). Furthermore, some important enzymes from the kinase family responsible for protein phosphorylation are constantly associated with stress tolerance (Patil & Senthil-Kumar, 2020). Some of these enzymes were identified in our data and can be good resistance targets: adenylate kinase and guanylate kinase. Both enzymes participate in the cellular homeostasis process, converting adenine and guanine nucleotides (Dzeja & Terzic, 2009; Sekulic *et al.*, 2002). The first is reported in maize with a regulatory role against salt stress, maintaining normal growth (Chen *et al.*, 2022), and in *U. decumbens* roots, high enzyme expression is related to a constant supply of necessary substrates to deal with aluminum resistance (Arroyave *et al.*, 2018). Second, guanylate kinase genes change their expression when sugarcane is under armyworm herbivory attack, assisting the resistance process (Wang *et al.*, 2021). With this, the importance of glutathione pathways and the relation between the kinase family and herbivore defense are demonstrated, and the metabolic network provides new interesting targets of the resistance process.

Coexpression network construction highlighted hub genes that were not identified in the other analyses but are essential to the complex process of spittlebug resistance, influencing several others by connections. The first hub in the BGP 344 network (Fig. 4b) is a *Setaria viridis* hypothetical gene of ncRNA; that is, it does not encode proteins but acts post-transcriptionally to modify gene expression in plants according to abiotic and biotic stress (Hou *et al.*, 2019; Song *et al.*, 2021). Furthermore, we obtained a retrotransposon gag protein and a retrovirus-related Pol polyprotein from transposon TNT 1-94 (RPPT) that regulate the transcription levels of adjacent genes when exposed to environmental changes (Yang *et al.*, 2020; Hao & Zhang, 2022). Finalizing the cytosol changes, putative ubiquitin-like-specific protease 1 (ULP1B) is a post-translational gene modification responsible for stress defense in eukaryotes (Pedley *et al.*, 2018; Morrell & Sadanandom, 2019). To complete the main hubs, we annotated the trehalose 6-phosphate synthase gene, which is related to abiotic response (Sarkar & Sadhukhan, 2022), and two others are described as uncharacterized because we had no correspondence in the literature. This indicates how networks can bring new discoveries and indicates that not only do the transcribed genes influence the defense process against herbivores but RNAs also affect several expression genes linked to resistance, since they are central in the networks and have

many connections. Therefore, specific studies of these ncRNAs can provide important information for unveiling the resistance mechanism for spittlebug resistance.

With this work, it was possible to conclude that the process of *P. regnellii* resistance to *M. spectabilis* spittlebugs is extremely complex, being governed by morphological characteristics that make infection difficult and secondary metabolites, enzymes and noncoding RNAs that are produced for plant defense. These findings will be useful to direct future genomic studies and for breeders to select new spittlebug-resistant tropical forage cultivars.

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Competing interests

None declared.

Author contributions

MRG, WMJ and BBZV conceived the work. ISB, DSG, MRG, WMJ and BBZV prepared the data. ISB, AHA and RCUF analyzed the transcriptome and network data, ISB and WMJ analyzed the RT–qPCR results, and DSG and SMC-G analyzed the anatomy. MRG, BBZV and APS acquired funding and oversighted the work. ISB led the writing of the manuscript, and all authors contributed to the writing. All authors revised the work critically and approved the submitted version.

Data availability

The data that support the findings of this study are openly available in Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/sra, reference number PRJNA999588.

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5. CONCLUSÕES GERAIS

A metodologia de RNA-Seq foi realizada com êxito, gerando de forma *de novo* o primeiro transcriptoma para a espécie *Paspalum regnellii* reportado até então. Assim como o primeiro estudo de investigação genético de resposta ao ataque de cigarrinhas *Mahanarva spectabilis*.

A partir da comparação dos diferentes genótipos em condição controle, pudemos obter o perfil transcricional de cada um deles e comparar, evidenciando as diferenças estruturais já existentes entre eles. Além disso, com as comparações entre os tempos de infestação pela praga, conseguimos elucidar como ocorre o processo de sinalização celular que responde ao estresse provocado pela cigarrinha-das-pastagens, corroborando com dados gerais de herbivoria encontrados na literatura. Foi possível estabelecer os mecanismos gerais de defesa e sua complexidade, desde as diferenças morfológicas e sistêmicas já presentes constitutivamente nos genótipos, como por exemplo a deposição de lignina nas paredes celulares de raiz, como também as defesas iniciais induzidas e seu processo de sinalização, como o metabolismo da glutationa envolvido na reação ao estresse oxidativo.

6. PERSPECTIVAS

Foram identificados pela primeira vez os genes, seus respectivos co-expressos e as enzimas que podem estar intimamente relacionados com o processo de resistência de *Paspalum regnellii* ao ser atacado por cigarrinha-das-pastagens da espécie *Mahanarva spectabilis*. Desta forma, os resultados obtidos indicaram potenciais genes para serem utilizados nos processos de edição gênica não apenas para *Paspalum*, como também para outras forrageiras tropicais importantes para as quais não se conhece fonte de resistência, como *Megathyrsus maximus*, *Urochloa* spp. ou até mesmo cana-de-açúcar, que também é afetada por cigarrinhas do gênero *Mahanarva*.

As análises também elucidaram que o processo de resistência é intimamente afetado pela presença dos outros tipos de RNA, como os microRNAs e RNAs não codificantes, os quais estão sendo identificados em estudos recentes como mecanismos de interferência de expressão e poderiam trazer novos vislumbres importantes. Além disso, os resultados gerados são principalmente das primeiras etapas do processo de defesa, portanto investigações de infestações mais prolongadas seriam capazes de produzir mais metabólitos secundários e fitohormônios envolvidos com a resistência.

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ANEXO I – Depósito no bioRxiv

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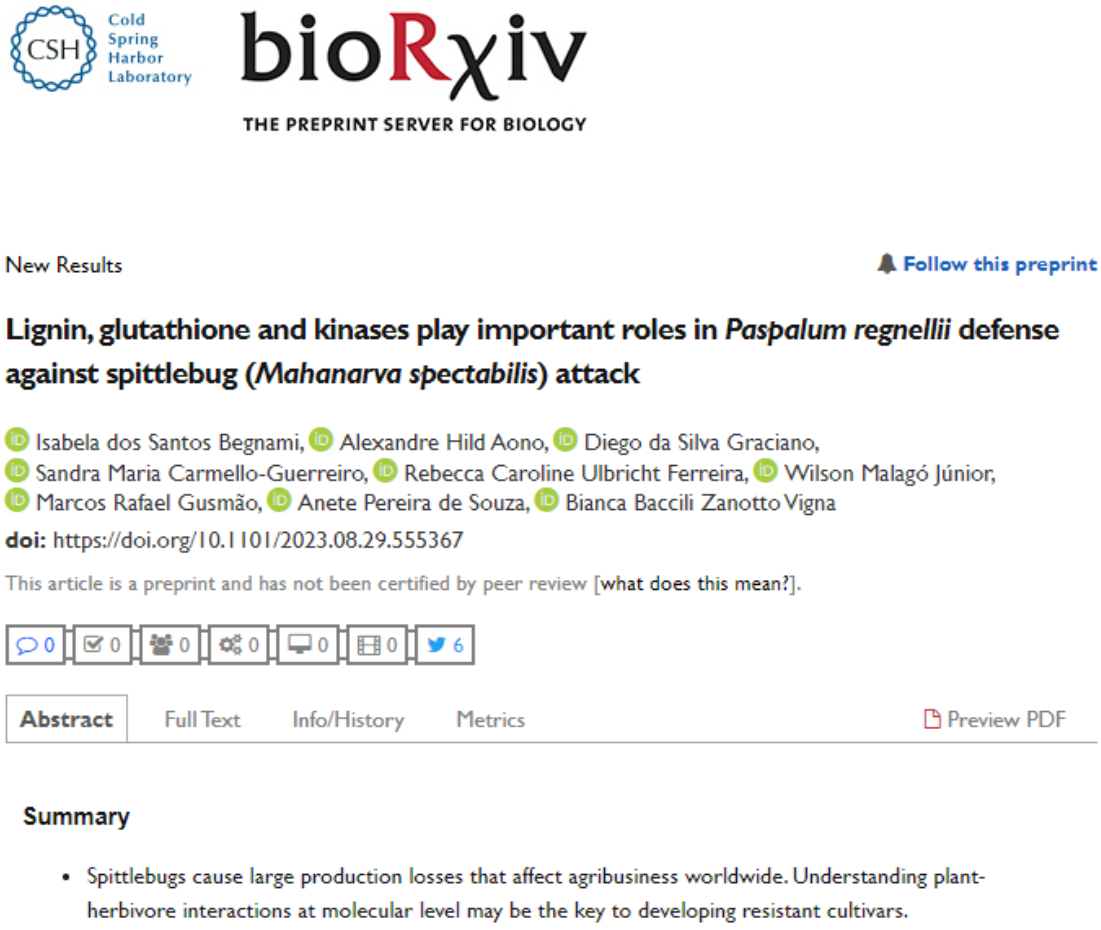

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Lignin, glutathione and kinases play important roles in *Paspalum regnellii* defense against spittlebug (*Mahanarva spectabilis*) attack

Isabela dos Santos Begnami, Alexandre Hild Aono, Diego da Silva Graciano, Sandra Maria Carmello-Guerreiro, Rebecca Caroline Ulbricht Ferreira, Wilson Malagó Júnior, Marcos Rafael Gusmão, Anete Pereira de Souza, Bianca Baccili Zanotto Vigna

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Summary

- Spittlebugs cause large production losses that affect agribusiness worldwide. Understanding plant-herbivore interactions at molecular level may be the key to developing resistant cultivars.

ANEXO II - Declaração Bioética e Biossegurança

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DECLARAÇÃO

Em observância ao **§5° do Artigo 1° da Informação CCPG-Unicamp/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada: **"Desvendando os mecanismos moleculares de resistência de *Paspalum regnellii* ao ataque de cigarrinha *Mahanarva spectabilis*".**
Desenvolvida no Programa de Pós-Graduação de Genética e Biologia Molecular do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

Campinas, 20 de setembro de 2023.

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ANEXO III - Declaração Autoria

DECLARAÇÃO DE AUTORIA

As cópias de artigos de minha autoria ou de minha coautoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação de Mestrado, intitulada "**Desvendando os mecanismos moleculares de resistência de *Paspalum regnelli* ao ataque de cigarrinha *Mahanarva spectabilis***", não infringem os dispositivos da Lei nº9. 610/98, nem o direito autoral de qualquer editora.

Campinas, 20 de setembro de 2023.

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