LUCAS LEAL LIMA

SINAIS SONOROS DE MASTIGAÇÃO DESENCADEIAM AJUSTES METABÓLICOS E DE EXPRESSÃO GÊNICA EM FOLHAS DE SOJA SEMELHANTES AOS PRODUZIDOS PELA HERBIVORIA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Doctor Scientiae.*

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RESUMO

LIMA, Lucas Leal, D.Sc., Universidade Federal de Viçosa, outubro de 2021. **Sinais sonoros de mastigação desencadeiam ajustes metabólicos e de expressão gênica em folhas de soja semelhantes aos produzidos pela herbivoria.** Orientador: Humberto Josué de Oliveira Ramos. Coorientadoras: Elizabeth Pacheco Batista Fontes, Maria Goreti de Almeida Oliveira, Juliana Lopes Soares Ramos e Flávia Maria da Silva Carmo (*in memorian*).

Ondas sonoras são sinais mecânicos que podem promover mudanças fisiológicas e moleculares em plantas, afetando o crescimento, conteúdos fitoquímicos e respostas ao estresse. No entanto, a especificidade dessas respostas ao som produzido por um emissor de origem biológica ainda foi pouco explorada em relação a maior quantidade de estudos já realizados com frequências sonoras singulares em plantas. A interação da planta com insetos, outros organismos e o ambiente produzem distintos sinais sonoros que podem ser relevantes e refletir na fisiologia e comportamento do organismo vegetal, contribuindo principalmente com sua adaptação e evolução. Visto essa necessidade de maior esclarecimento no reflexo da interação de plantas com sinais sonoros oriundos de seu habitat (sons ecológicos), foram avaliadas se alterações metabólicas e moleculares podem ser diferencialmente reguladas e reajustadas por diferentes tipos de sons ecológicos; e se sons de mastigação de lagarta desencadeiam respostas bioquímicas em acordo com o observado quando plantas são atacadas por herbívoros. Para responder essas perguntas, ajustes bioquímicos foram avaliados em plantas de soja tratadas com dois tipos de sons ecológicos: mastigação de herbívoro (lagarta) e ambiente da floresta. Em geral, as respostas foram bem distintas, embora algumas similaridades tenham sido observadas, indicando que os sinais sonoros oriundos de sistemas ecológicos podem desencadear cascatas específicas dependentes do espectro de frequências acústicas da fonte emissora. Enzimas envolvidas no metabolismo oxidativo responderam a ambos os sons, e o ácido salicílico (SA) foi induzido apenas pelo som de mastigação. Após 12 e 18 horas de exposição aos sons ecológicos não houve significância na alteração da atividade da lipoxigenase (LOX) e nem na abundância relativa do ácido jasmônico (JA). No entanto, os genes *SKTI* e *BBI*, que codificam inibidores de protease, foram induzidos pelos sinais sonoros de mastigação. Por outro lado, os tratamentos sonoros promoveram regulações em diferentes ramificações das vias de biossíntese de fenilpropanóides e flavonoides, evidenciando uma tendência de aumento de alguns conjugados de flavonóis em plantas tratadas com o som de mastigação. Em concordância, os genes *PR10 / Bet v-1* e *gmFLS1* envolvidos na biossíntese de flavonoides e flavonóis também foram induzidos pelo som de mastigação. Finalmente, os resultados possibilitam propor que o sinal acústico de mastigação pode atuar como uma primeira linha de defesa à herbivoria; e sons ecológicos podem desencadear cascatas de sinalização distintas dependentes do espectro de frequências acústicas da fonte emissora. No entanto, os ajustes metabólicos ocasionados pelos sons ecológicos devem ser expandidos para outros sistemas de interação inseto-planta para revelar a aplicabilidade, magnitude e relevância ecológica de sinais sonoros presentes em ecossistemas.

Palavras-chave: Efeito do som. Relação inseto-planta. Flavonoides. Fenóis. Hormônios vegetais. Enzimas. Genes. Ondas sonoras. Vibração – Efeito fisiológico.

ABSTRACT

LIMA, Lucas Leal, D.Sc., Universidade Federal de Viçosa, October, 2021. **Chewing sound signals trigger specific metabolic and gene expression changes in soybean leaves resembling the plant response to the caterpillar attack** Adviser: Humberto Josué de Oliveira Ramos. Co-advisers: Elizabeth Pacheco Batista Fontes, Maria Goreti de Almeida Oliveira, Juliana Lopes Soares Ramos and Flavia Maria da Silva Carmo (*in memorian*).

Sound waves are mechanical signals that can promote physiological and molecular changes in plants, affecting growth, phytochemical contents and stress responses. However, the specificity of these responses to the sound produced by a biological emitter has been little explored in relation to the greater number of studies already carried out with singular sound frequencies in plants. The plant's interaction with insects and other organisms produces different sound signals in the environment that may be relevant and reflect on plant physiology and behavior, contributing mainly to its adaptation and evolution. Given this need for further clarification of the interaction of plants with sound signals from their habitat (ecological sounds), we evaluated whether the signaling cascades are differentially regulated and readjusted by ecological sounds containing different frequencies; and whether caterpillar chewing sounds trigger molecular responses in agreement those produced by herbivore attack. To answer these questions, biochemical and molecular adjustments were evaluated in soybean plants treated with two types of ecological sounds: herbivore chewing and forest environment. In general, the responses were quite distinct, although some similarities were observed, indicating that sound signals from ecological systems can trigger specific cascades depending on the acoustic frequency spectrum of the emitting source. Enzymes involved in the oxidative metabolism were responsive to both sounds, and salicylic acid (SA) was responsive only for chewing sound. After 12 and 18 hours of sound exposure lipoxygenase (LOX) activity and jasmonic acid (JA) did not change. However, *SKTI* and *BBI* genes, encoding for protease inhibitors, were induced by chewing sound signals. Otherwise, the sound treatments promoted modifications in different branches of the phenylpropanoid and flavonoid pathways, highlighting a tendency for increased of some flavonol conjugates for plants under chewing sounds. In accordance, a *PR10/Bet V1-like* and *gmFLS1* genes involved in biosynthesis of flavonoids and flavonols were also induced by chewing sounds. Finally, our results make possible propose that acoustic chewing signal may be acting as a first line of defense to herbivore attack, and different ecological sounds can trigger distinct signaling cascades.

However, it need be evaluated for other plant-insect systems to reveal their broadly applicable, magnitude and ecological relevance.

Keywords: Vibrational signaling. Sound vibration. Plant–insect interactions. Phytohormonal response. Phenolic compounds. Flavonoids. Sound-induced enzymes. Sound-induced genes.

SUMÁRIO

INTRODUÇÃO GERAL

Para superar um estilo de vida livre séssil, plantas apresentam mecanismos biológicos que examinam e processam as informações presentes no ambiente, promovendo um ajuste fisiológico responsável em proporcionar uma aclimatação em direção a condições indesejáveis iminentes (BRAAM; DAVIS, 1990; MISHRA; GHOSH; BAE, 2016). As plantas dispõem de adaptações moleculares, fisiológicas, citológicas e morfológicas especializadas na captação e processamento de sinais do meio circundante, sendo ferramentas responsáveis por sensibilizar e fornecer a elas uma capacidade de perceber fatores abióticos (vento, chuva, toque e vibrações) e bióticos (reconhecimento de patógenos) que são de extrema importância para que se comuniquem com o meio e interpretem os sinais presentes na natureza essenciais no estabelecimento de sua sobrevivência (BRAAM; DAVIS, 1990).

A comunicação, um fenômeno natural onipresente e característico de todos organismos vivos, desperta a atenção para a investigação científica por longos tempos em vista de sua importância no estabelecimento da complexa rede de interações e padrões de comportamento dos seres vivos. Diversas formas de comunicação são observadas nos ecossistemas, e todas elas, transmitem informações e sinais ambientais determinantes para o processo adaptativo e evolutivo das espécies. As vibrações sonoras, um fenômeno ondulatório de origem mecânica, são uma das fontes de comunicação encontradas na natureza e utilizada por diversos organismos como forma de alertar um eventual perigo, permitir uma relação entre seres da mesma espécie e de espécies distintas e perceber diversas situações ocasionadas pelo meio(GAGLIANO et al., 2012; GAGLIANO; MANCUSO; ROBERT, 2012).

Na natureza, os nichos ocupados por plantas não são livres de sons e outros sinais vibratórios de origem mecânica. Pelo contrário, são repletos de vibrações sonoras que englobam um espectro de frequências e amplitudes oriundas de diversas fontes, tanto físicas como biológicas. Sons são energias acústicas sob a forma de ondas de pressão (ondas mecânicas) transmitida através de gases, líquidos e sólidos. Devido a essa propriedade, as ondas acústicas podem potencialmente provocar vibrações na interface de objetos e seres vivos (HASSANIEN et al., 2014; MISHRA; GHOSH; BAE, 2016). Assim sendo, é muito provável que as plantas apresentem mecanismos biológicos adaptados na percepção de vibrações sonoras dentro de um espectro de frequências e amplitudes para facilitar a compreensão dos inúmeros sinais de relevância ecológica (GHOSH et al., 2016).

Diversos estudos com a aplicação de sons em plantas têm demonstrado efeitos metabólicos, fisiológicos, morfológicos e comportamentais que podem afetar o desenvolvimento e crescimento vegetal (APPEL; COCROFT, 2014; DE LUCA; VALLEJO-MARÍN, 2013; GHOSH et al., 2016, 2017; JEONG et al., 2008; KIM et al., 2016, 2021; KWON et al., 2014; QIN et al., 2003; YI et al., 2003; ZHAO et al., 2002). Logo, é provável que as vibrações sonoras possam servir como um estímulo que disparam mecanismos de sinalização molecular responsáveis em alterar o fluxo metabólico celular e, a longo prazo, afetar a interação planta/ambiente (MISHRA; GHOSH; BAE, 2016). Porém, o mecanismo biológico envolvido na percepção e processamento do som e a importância ecológica da interação som/plantas permanecem inconclusivos pelas dificuldades em mapear as vias de percepção e transdução de sinal. Desta forma, a falta de marcadores de resposta dificulta a expansão do estudo em diversas plantas, bem como a compreensão de como a percepção sonora pode refletir na adaptação e evolução dos organismos vegetais (APPEL; COCROFT, 2014; MISHRA; GHOSH; BAE, 2016).

No início das pesquisas com percepção e processamento de sinais sonoros acreditava-se que plantas não possuíam a capacidade de perceber ondas acústicas, tema inclusive bastante polêmico e controverso na comunidade científica da época. Com o passar das décadas, muitas evidências foram levantadas indicando a sensibilidade dos organismos vegetais em responder estímulos sonoros, e principalmente, utilizá-los como forma de comunicação para reconhecimento do ambiente e interação tanto intra como interespécie (GAGLIANO et al., 2012). Em 1950, Dr. TC Singh deu início aos estudos em bioacústica avaliando o efeito da música em plantas e, em 1973, Dra. Retallack publicou um livro com o título "*The Sound of Music and Plant'*, onde descrevia experimentos envolvendo música e plantas. Em seus trabalhos, ela chegou à conclusão que os melhores resultados foram alcançados quando as plantas eram submetidas ao som de música clássica (CHOWDHURY; LIM; BAE, 2014).

Muitos trabalhos conduzidos com plantas submetidas a diferentes estilos musicais já foram feitos e muitos resultados positivos evidenciam o efeito da música em culturas agrícolas (CHOWDHURY; LIM; BAE, 2014; HASSANIEN et al., 2014). Porém, pesquisas no campo da bioacústica não se restringem apenas a utilização de música como fonte sonora, mas compreende também sons de frequências singulares e *green music* (sons de ambientes naturais) (GHOSH et al., 2016; QIN et al., 2003). Sons ambientais são vibrações acústicas que reproduzem cantos de vertebrados e invertebrados, mastigação de herbívoros, zumbidos de insetos, ambiente de uma floresta e demais sons de relevância ecológica. O fenômeno da polinização por zumbido tem sido observado em diferentes espécies vegetais e indica uma grande importância ecológica da influência dos sons naturais na biologia comportamental de plantas. Pesquisas têm mostrado que zumbidos de abelha induzem a abertura de anteras para liberação do pólen somente em uma frequência acústica particular produzida pela abelha polinizadora da espécie (DE LUCA; VALLEJO-MARÍN, 2013).

Com o avanço das técnicas bioquímicas e biotecnológicas muitos trabalhos no campo da percepção e processamento de sinais sonoros em plantas empregaram essas tecnologias para caracterizar eventos moleculares e fisiológicos envolvidos na resposta às vibrações sonoras. Hoje em dia, há um número considerável de pesquisas que apontam alterações no metabolismo de plantas submetidas ao som. Trabalhos evidenciam ativação em cascatas de transdução de sinal dependentes de cálcio e de espécies reativas de oxigênio (GHOSH et al., 2016), alteração na estrutura secundária de proteínas de membrana (ZHAO et al., 2002), aumento na fluidez do estado físico das membranas (YI et al., 2003), controle da expressão e regulação gênica a nível de promotor (GHOSH et al., 2016; JEONG et al., 2008; KIM et al., 2016; KWON et al., 2014; SAFARI et al., 2013), ajustamento hormonal (GHOSH et al., 2016; KIM et al., 2016) e elevação nos níveis de aminoácidos, açúcares solúveis e poliaminas (QIN et al., 2003; YI et al., 2003).

Jeong e colaboradores (2008) avaliando a influência de vibrações sonoras na expressão gênica de plantas revelaram um conjunto de genes responsivos regulados por ondas sonoras de frequências singulares. Foi observado que as plantas tratadas com vibrações sonoras sob condições de luz e escuro apresentaram aumento na expressão de dois genes (*rbcS e ald*) que sofrem regulação pela luz. Desta forma, os autores sugerem que o som pode representar uma forma alternativa de energia à luz no processo de regulação gênica. Em outro trabalho, onde também foi investigada a expressão diferencial de genes em plantas tratadas com ondas sonoras em cinco frequências distintas, foi observado variação na expressão de genes pertencentes a seis grupos distintos: genes responsivos a estímulo mecânicos, genes de vias de sinalização celular, fatores de transcrição, regulação da homeostase, vias biossintéticas e vias relacionadas na defesa contra patógenos (GHOSH et al., 2016).

Um dos principais motivos pelo qual a percepção sonora em plantas é algo pouco investigado pela a ciência, é a falta de um órgão sensitivo em plantas especializado na captação de vibrações oriundas do ar, como o ouvido humano. No entanto, um crescente número de evidências surgindo em pesquisas com aplicação de sons em plantas indicam que organismos vegetais são altamente sensíveis a vibrações acústicas, possuindo adaptações que os permitem emitirem e captarem ondas sonoras (JUNG et al., 2018).

Mesmo as plantas não apresentando um órgão especializado na percepção de vibrações sonoras, mecanismos moleculares sofisticados podem ser responsáveis por esse fenômeno. Ghosh e colaboradores (2016, 2017) investigando alterações transcricionais em plantas *de Arabdopsis thaliana* tratadas com som e toque, observaram que ambos os estímulos mecânicos testados influenciaram a expressão de genes que codificam canais iônicos e/ou proteínas mecanosensitivas. Foi notado que tais estímulos são responsáveis em alterar a expressão de genes correlacionados, pertencentes à mesma família, apresentando em ambos os casos semelhanças em níveis transcricionais. Porém, diferenças no perfil de expressão gênica entre os estímulos mecânicos foi suficiente para mostrar que o processamento desses sinais ocorre por mecanismos biológicos distintos, apesar da percepção do som e do toque compartilharem algumas proteínas mecanosensitivas e vias de transdução de sinal de Ca^2.

Com tais fatos, pode-se levantar a hipótese de que o mecanismo de percepção de vibrações sonoras em plantas pode envolver proteínas membranosas mecanosensitivas e formadoras de canais iônicos; e a emissão do sinal sonoro pelo tecido vegetal pode ocorrer por transmissão elétrica formada pela diferença de potencial na membrana devido ao fluxo de cálcio nos canais iônicos ao longo das células. A membrana plasmática de células vegetais é equipada com um enorme número de canais proteicos mecanossensíveis responsivos a sinais mecânicos. Assim sendo, devido ao comportamento mecânico das ondas sonoras e sua capacidade de vibrar superfícies, a alteração na tensão da membrana biológica pelas ondas acústicas podem evocar uma cascata de sinalização através da ativação desses canais, permitindo que o sinal sonoro seja processado e uma resposta bioquímica e fisiológica seja elaborada como consequência da exposição ao som (GHOSH et al., 2016, 2017; MISHRA; GHOSH; BAE, 2016).

As pesquisas com bioacústica de plantas não necessariamente procuram responder se as plantas são sensíveis às vibrações acústicas ou não. Os esforços são principalmente direcionados para elucidar os mecanismos moleculares envolvidos na percepção e para verificar a capacidade das mesmas em diferenciar os distintos sinais sonoros encontrados na natureza (sons ecológicos) (MISHRA; GHOSH; BAE, 2016). Em um trabalho onde foi investigado se as plantas podem reconhecer som de alimentação de herbívoros, Appel e Cocroft (2014) relataram que as vibrações sonoras causadas pela alimentação de insetos podem desencadear defesas químicas. Foi observado que o som de mastigação de lagarta promoveu o aumento na produção de metabólitos (Glicosinolatos e Antocianinas) relacionados a defesa contra herbivoria. Esses resultados demostram a importância da percepção sonora como uma ferramenta adaptativa e evolutiva (MISHRA; GHOSH; BAE, 2016), bem como o potencial que os organismos vegetais têm em diferenciar e elaborar respostas metabólicas específicas em direção a cada tipo de som ecológico presente em seu ambiente. Porém, mais pesquisas devem ser realizadas visando a compreensão dos mecanismos envolvidos na percepção e processamento de sinais sonoros para estabelecer a importância da comunicação acústica em plantas.

Apesar de atualmente termos um acúmulo considerável de informações e descrições de processos biológicos em plantas tratadas com ondas sonoras, pouco sabe sobre o mecanismo molecular envolvido na sinalização e o reflexo desse ajuste bioquímico e fisiológico no desenvolvimento e crescimento vegetal. Identificada essa lacuna no conhecimento de como as plantas percebem e processam os sinais sonoros ambientais, e se são capazes de diferenciar esses sinais sonoros, esta tese tem como objetivo investigar os reajustes metabólicos provocados em plantas de soja após a exposição de sons ecológicos; e verificar se a resposta molecular e bioquímica é direcionada de acordo com a informação contida nas ondas acústicas. Para verificar essa hipótese, plantas de soja da cultivar EMBRAPA 48 foram tratadas com dois tipos diferentes de sons de relevância ecológica: som de mastigação de herbívoro e som ambiente de mata; e moléculas estimulantes como hormônios, aminoácidos, poliaminas, flavonóis, flavonoides foram identificadas e quantificadas, bem como atividades enzimáticas e expressões de genes alvos foram avaliados para encontrar os principais reajustes metabólicos ocasionados pela exposição aos sons ecológicos.

Com as principais moléculas responsivas determinadas pelas análises estatísticas, vias que sofreram reajustes devido a exposição sonora foram caracterizadas para verificar as principais diferenças e similaridades na resposta elaborada para cada tipo de som. Em seguida uma investigação foi realizada e evidências mostraram uma possível capacidade de plantas em elaborar uma resposta bioquímica direcionada a informação contida no som. Os resultados indicaram uma maior produção de moléculas estimulantes da interação planta-herbívoro no som de mastigação e, no som ambiente de mata, foi encontrado um perfil metabólico com algumas similaridades, no entanto, bem diferente do perfil encontrado para o som de mastigação, revelando que para cada tipo de som ecológico uma resposta metabólica específica foi elaborada pela planta. Desse modo, o trabalho evidencia uma possível capacidade das plantas em responder a ondas sonoras de conteúdo ecológico e evidencia uma possível capacidade do som de mastigação em atuar como primeira linha de defesa contra o ataque de herbívoros.

Em síntese, o presente trabalho possibilitou esclarecer sobre a potencial de plantas em perceber e processar sons ecológicos e evidenciou uma provável atuação de ondas sonoras como um sinal necessário para as plantas compreenderem o ambiente circundante.

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CAPÍTULO ÚNICO

Chewing sound signals trigger specific metabolic and gene expression changes in soybean leaves resembling the plant response to the caterpillar attack

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CHEWING SOUND SIGNALS TRIGGER SPECIFIC METABOLIC AND GENE EXPRESSION CHANGES IN SOYBEAN LEAVES RESEMBLING THE PLANT RESPONSE TO THE CATERPILLAR ATTACK

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ABSTRACT

Sound waves are mechanical signals that can promote physiological and molecular changes in plants, affecting the growth, phytochemical contents and stress responses. However, the specificity of these responses to sound produced by a specific emitter has been partially investigated. Insects interacting with the plant produce different sounds in the environment which may be relevant for the plant behavior. Thus, was evaluated whether signaling cascades are regulated differently by ecological sounds containing different frequencies, and whether trigger molecular responses in accordance to the sounds produced by herbivorous insects. To verify this, soybean plants were treated with two types of ecological sounds: chewing herbivore and forest ambient, then their molecular responses were evaluated. In general, the responses were markedly distinct, despite of some similarly have been observed, indicating that sound signals may also triggered specific cascades. Enzymes involved in the oxidative metabolism were responsive to both sounds, and salicylic acid (SA) was responsive only for chewing sound. After 12 and 18 hours of sound exposure lipoxygenase (LOX) activity and jasmonic acid (JA) did not change. However, *SKTI* and *BBI* genes, encoding for protease inhibitors, were induced by chewing sound signals. Otherwise, the sound treatments promoted modifications in different branches of the phenylpropanoid and flavonoid pathways, highlighting a tendency for increased of some flavonol conjugates for plants under chewing sounds. In accordance, a *PR10/Bet v-1-like* and *gmFLS1* genes involved in biosynthesis of flavonoids and flavonols were also induced by chewing sounds. Finally, our results allow us to propose that acoustic chewing signal may be acting as a first line of defense to herbivory, and different ecological sounds can trigger distinct signaling cascades. However, it need be evaluated for other plant-insect systems to reveal their broadly applicable, magnitude and ecological relevance.

Keywords: Vibrational signaling Sound vibration Plant–insect interactions Phytohormonal response Phenolic compounds Flavonoids Sound-induced enzymes Sound-induced genes

INTRODUCTION

Communication is an ubiquitous natural phenomenon characteristic of all living organisms. Thus, it calls attention to the scientific research due to its importance in establishing a complex network of interactions and determining patterns of behavior. Several forms of communication are observed in ecosystems, and all of them convey important information for the adaptation and evolution of species (GAGLIANO et al., 2012). Sound vibrations (SVs) are wave phenomena of mechanical origin and are one of the sources of communication found in nature. They are used by various organisms as a way to alert a danger, to allow a relationship between organisms from the same or diferent species, and to help to establish the complex network of interaction between the individual and the environment (GAGLIANO; MANCUSO; ROBERT, 2012).

In nature, the niches occupied by plants are not free from sounds and other vibrational signals of mechanical origin. They are full of sound vibrations that encompass a spectrum of frequencies and amplitudes from different physical and biological sources. Sounds are acoustic energies in the form of pressure waves that propagate through gas, liquids and solids. Due to this physical characteristic of sound vibration they can potentially cause vibrations at the interface of objects and living beings (GHOSH et al., 2017; HASSANIEN et al., 2014). Thus, it is very likely that plants present biological mechanisms adapted to the perception of sound vibrations within a spectrum of frequencies and amplitudes to facilitate the understanding of the numerous signs of ecological relevance (GHOSH et al., 2016).

Several studies with the application of sounds in plants have shown metabolic, physiological, morphological and behavioral effects that can affect plant development and growth. Different sounds have been shown to activate calcium- and reactive oxygen species-dependent signal transduction cascades (GHOSH et al., 2016), alter the secondary structure of membrane proteins (ZHAO et al., 2002), increase the fluidity of the physical state of membranes (YI et al., 2003), adjust the expression and gene regulation at the promoter level (GAGLIANO; MANCUSO; ROBERT, 2012; GHOSH et al., 2016; JEONG et al., 2008; KIM et al., 2016; KWON et al., 2014; SAFARI et al., 2013) and increase the levels of amino acids, soluble sugars and polyamines (QIN et al., 2003; YI et al., 2003). Therefore, it is likely that sound vibration can serve as a long-range stimulus that triggers molecular signaling mechanisms, responsible for altering cellular metabolic flow and affecting the plant-environment interactions (MISHRA; GHOSH; BAE, 2016). However, the biological mechanism involved in sound perception and processing and the ecological importance of the sound-plant interactions remain inconclusive due to the difficulties in mapping the perception and signal transduction pathways. The lack of response markers hinders the expansion of the study in several plants, as well as the understanding of how sound perception can reflect on the adaptation and evolution of plants (APPEL; COCROFT, 2014; MISHRA; GHOSH; BAE, 2016).

Although plants do not have an organ specialized in the perception of sound vibrations, sophisticated molecular mechanisms can be responsible for this phenomenon. Ghosh and collaborators (2016, 2017), investigating transcriptional alterations in *Arabidopsis thaliana* treated with sound and touch, observed that both mechanical stimuli induce the expression of ion channels genes. However, differences in gene expression profile between mechanical stimuli was enough to show that the processing of these signals occurs by different biological mechanisms, despite the perception of sound and touch share some mechanosensitive proteins and Ca^{2+} signal transduction pathways. These observations raised the hypothesis that the mechanism of sound vibration perception in plants may involve mechanosensitive proteins and ion channel-forming proteins (GHOSH et al., 2017; MISHRA; GHOSH; BAE, 2016) . The emission of the sound signal by the plant tissue can occur due to the difference in membrane potential generated by the sound stimulus. The plasma membrane of plant cells is equipped with an enormous number of mechanosensitive protein channels responsive to mechanical signals. Due to the mechanical behavior of sound waves and their ability to vibrate surfaces, the change in biological membrane tension by acoustic waves can evoke a signaling cascade through the activation of ion channels, allowing the sound signal to be processed and a biochemical and physiological response is elaborated as a consequence of exposure to the sound (GHOSH *et al.*, 2016, 2017; MISHRA et al., 2016).

Research on plant bioacoustics does not necessarily seek to answer whether plants are sensitive to acoustic vibrations or not. Efforts are mainly directed to elucidate the molecular mechanisms involved in perception and to verify the ability of plants to differentiate the sounds present in nature (sounds of ecological relevance) (MISHRA et al., 2016). In a study investigating whether plants can recognize herbivore feeding sounds, Appel and Cocroft (2014) reported that vibrations caused by insect feeding can trigger chemical defenses. The sound of caterpillar chewing promoted an increase in the production of metabolites (glycosinolates and anthocyanins) responsive to attack by herbivores. In another study, showed that Bee hums has also been shown to induce the opening of anthers for pollen release only at a particular acoustic frequency produced by the pollinator bee (De Luca and Vallejo-Marín, 2013). These results demonstrate the importance of sound perception as an adaptive and evolutionary tool, as well as the potential that plants have to differentiate and elaborate specific metabolic responses towards each type of sound of ecological relevance.

Although we currently have a considerable accumulation of information and descriptions of biological processes in plants treated with sound waves, little is known about the molecular mechanism involved in signaling and the reflection of this biochemical and physiological adjustment in plant metabolism. Due to this gap in knowledge of how plants process environmental sound signals, this investigation aimed to investigate the metabolic adjustments caused in soybean treated with ecological sounds and to verify whether the molecular and biochemical response is targeted by the information from the acoustic waves. First, to verify the main similarities and differences in the metabolic and molecular response in plants treated with caterpillar chewing sound and forest sound, and then, to investigate whether the responses found in the chewing sound resembling to the response observed for the caterpillar attack. To investigate this hypothesis, soybean plants of the EMBRAPA 48 variety were treated with two different types of ecological sounds: herbivore chewing sound and forest ambient sound. Elicitor molecules, including hormones, amino acids, polyamines, flavonols, and flavonoids, were evaluated and quantified and so were enzymatic activities and expressions of target genes responsive to herbivory in soybean. Finally, the metabolic and molecular profiles generated from the chewing sound and forest sound treatments were contrasted with those observed and characterized during the caterpillar attack.

MATERIAL AND METHODS

Plant growth conditions

*Glycine ma*x (Embrapa 48) seeds were supplied by the seed collection of the Plant Molecular Biology Laboratory belonging to the Department of Biochemistry and Molecular Biology of the Federal University of Viçosa/UFV, Brazil. Seeds were germinated in 40 polyethylene pots (3.5L) containing a mixture of substrates for growing seedlings (Tropstrato) and latosol soil in a 2:1 ratio. After germination, three seedlings were maintained by pot and corresponded to a biological repetition of sound treatments. Plants were maintained in greenhouse conditions for 45 days (V7) before starting the sound treatments in the acoustic treatment boxes.

Ecological Sounds

The sound of the bioacoustic landscape was recorded in Mata da Biologia on a spring morning. Mata da Biologia is located within the campus of the Universidade Federal de Viçosa, in the city of Viçosa, Minas Gerais, Brazil. The chewing sound it was downloaded from the ZAPSPLAT page. The audio file contains the sound frequencies of a non-specific soybean caterpillar chewing a plant leaf. The downloaded audio file was edited, mixed and mastered using the package Reaper. The sounds were standardized to be reproduced in the acoustic treatment boxes at an intensity (volume) of 70 decibels (dB). The intensity of 70 dB was chosen because it represents the average value of the ambient sound of the forest where the audio was recorded. The chewing sound was also played at 70 dB to prevent the measured response from being a consequence of the difference between the intensities of the two ecological sounds.

Sound treatments

The sound treatments were carried out in sound treatment boxes equipped with an internal sound and illumination system for the reproduction of ecological sounds (**Fig. 1A**). Plants were treated with sound waves for an exposure time of 1 hour followed by a rest period of 15 minutes. Six one-hour sessions of sound were applied per day over a two and three-day period totaling 12 and 18 hours of treatment, respectively. The rest time was used to avoid the accumulation of carbon dioxide in the box, as it remained closed during the treatment. The boxes were kept hermetically closed to avoid interference from external sounds and leakage of internal sound generated by the speaker. The sound treatment boxes were opened during the rest time to eliminate excess carbon dioxide and to change the biological repetitions of boxes, preventing them from always remaining in the same box. (**Fig. 1B**).

The experiment was carried out in the morning and afternoon period. At the end of the sound treatments, the plants were placed back on the greenhouse benches until the experiment was restarted the following day. The sound treatment boxes were installed in the greenhouse next to the plant bench (**Fig. S1**). Due to the fact that only 10 sound treatment boxes were available, the experiment was time-divided into two independent blocks: one experiment for the chewing sound and another for the forest sound.

The first experiment was carried out with forest sound, which was divided into four treatments: two sound exposure times (12 and 18 hours), in the presence or absence of sound (soundless control). Five boxes were used for the sound treatment and another five were for the soundless control. In each box, there was a biological replicate of the 12-hour treatment and another of the 18-hour treatment (two biological replicates per box). On the last day of experimentation, only the biological repetition of the 18-hour treatment remained, because the 12-hour treatment biological repetitions were discarded after the collection on the second day (**Fig. 1**).

The plants from the control group were also placed in sound treatment boxes so that the environment was the same as the sound treatments. The absence of sounds was the only difference between the controls and the sound treatments.

The second experiment was carried out in the same way as the first experiment. However, the chewing sound was used instead of the forest sound. Thus, two independent experiments were created. One experiment for the forest sound and the other for the chewing sound, each containing two sound treatments classified according to the exposure time of the plants to the sound – 12 hours and 18 hours.

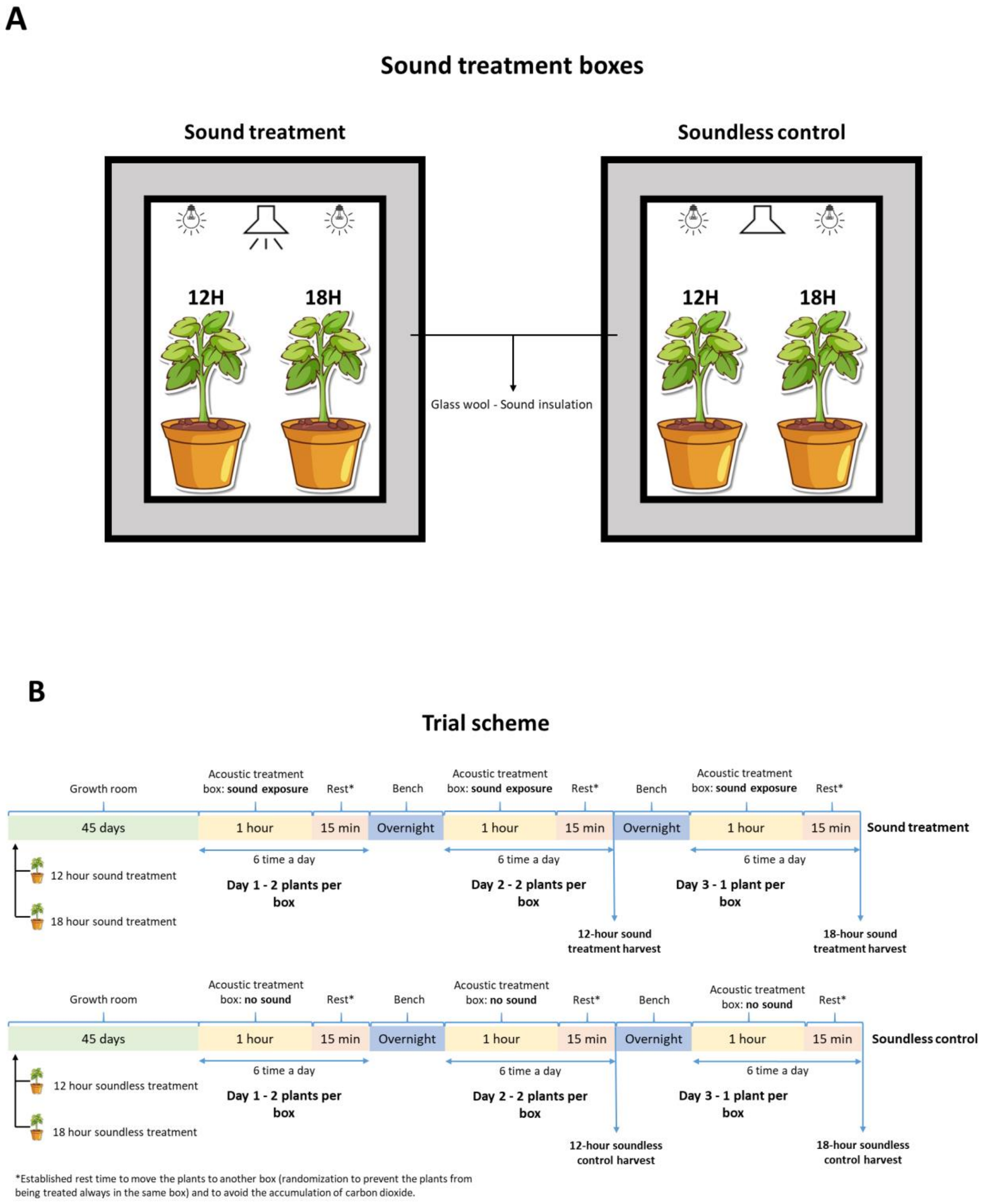


Figure 1. A. Acoustic treatment box. **B.** Schematic drawing of how sound treatments were done in the chewing and forest sound experiments.

RNA extraction, cDNA synthesis and expression analysis by qRT-PCR

Pulverized leaves (50 mg) were processed to extract total RNA from the control and sound treatments with CTAB (cetyltrimethylammonium bromide) reagent, following the protocol established by Guertler et al. (2013), with some modifications. RNA quality was examined in 1.0 % (m v^{-1}) denatured agarose gels stained with 0.1 µg/mL ethidium bromide (EtBr) and quantified using a Thermo Scientific NanoDrop 2000c. A total of 2 µg of RNA were used for cDNA synthesis with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions. Gene expression was evaluated using an ABI 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) and Fast Master SYBR Green Master Mix (Thermo Fisher Scientific). The amplification reactions were performed with the cycling conditions: 15 s at 95 °C, 40 cycles at 95 °C for 3 s; 30 s at 60 °C and final denaturation at 95 °C for 20 s, followed by a melting curve. RT-qPCR-specific primers were designed using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with a melt temperature (T) of 59 to 61 °C, a length of 18 to 23 bp, an amplifier product size of 120 to 150 bp, and a 40 to 60 % GC content. Three technical replicates were performed for each one of the three biological replicates. Gene expression was quantified using the ΔCT method and the expression levels were calculated as $2^{-\Delta CT}$ (PFAFFL, 2001).

Determination of lipoxygenases, PPO and CAT activity and protease inhibitors

For the lipoxygenase (LOX), polyphenol oxidase (PPO), catalase (CAT) activity determination, 100 mg soybean leaf powder was homogenized with 100 mM sodium phosphate buffer, pH 7.0, containing 1 % polyvinylpolypyrrolidone (PVPP) and 1 mM phenylmethylsulfonyl fluoride (PMSF) in the ratio of 100 mg of leaf per 1 mL of buffer. The extract was kept on ice at rest for 30 min and sonicated at 4 ºC for 10 min. The samples were centrifuged at 12,000 rpm and the supernatant was collected.

The lipoxygenase activity on linoleic acid was measured by the formation of the conjugated double-bond system of hydroperoxide, detected at 234 nm. The reaction mixture consisted of 10 µL of the supernatant (plant extract), 10 µL of 10 mM sodium linoleate, and 100 µL of 50 mM sodium phosphate buffer, pH 6.5. The relative activity was measured after 2 min of reaction at 234 nm and was calculated by the ratio between the absorbance value and the concentration of total protein in µg/µl (AXELROD; CHEESBROUGH; LAAKSO, 1981). PPO activity was assayed by measuring the increase rate in absorbance at 420 nm. Absorbance measurements were collected every 20 seconds within 3 minutes of reaction, and the relative activity was determined by calculating the ratio of the change in absorbance (ΔAbs = Abs*final*-Abs*initial*) by the concentration of total proteins in µg/µl. The reaction medium contained 20ul of sample (leaf extract) and 60ul of substrate buffer (100mM Potassium Phosphate Buffer and catechol, pH 7). CAT activity was assayed by measuring the rate of decrease in absorbance at 240 nm. Absorbance measurements were collected every 15 seconds within 1 minute and 20 seconds of reaction, and the relative activity was determined by calculating the ratio of the change in absorbance (ΔAbs = Abs*initial*-Abs*final*) by the concentration of total proteins in µg/µl. The reaction medium contained 10 µl of sample (leaf extract) and 60µl of substrate buffer (100mM Potassium Phosphate Buffer and 12.5 mM hydrogen peroxide, pH 7) (DE ARAÚJO et al., 2021). The protein concentration from leaves was determined using bovine serum albumin (BSA) as standard and the analyses were performed at 595 nm (BRADFORD, 1976).

For the protease inhibitors experiments, 100 mg soybean leaf powder was homogenized with extraction buffer 0.1 M Tris-HCl buffer pH 8.2 containing 20 mM $CaCl_2$ in the ratio of 100 mg of leaf per 1 mL of bufferand centrifuged at 17,200 *g* for 30 min at 4 °C. The protease inhibitors were determined by measuring the inhibition of purified trypsin when mixed with the supernatant (plant extract) and using 1.2 mM L-BApNA as substrate. Two controls were used to determinate the total inhibitor activity: the trypsin control consisted of 20 µL of trypsin 4.7 x 10^{-5} M, 90 µL of substrate and 100 µL of extraction buffer, and the substrate control consisted of 90 µL of substrate and 120 µL of extraction buffer. The test sample with 10 µL of trypsin, 10 µL of plant extract and 100 µL of extraction buffer were incubated at ambient temperature for 5 min, then was added 90 µL of substrate and the change in absorbance (Abs*f*-Abs*i*) at 410 nm was measured after 3 min (one absorbance collection point every 20 seconds). Relative activity was calculated by the difference in absorbance between the trypsin control and the test sample (ΔAbscontrol –ΔAbs*sample*) and divided by the concentration of total protein in mg/ml. The protein concentration from leaves was determined using bovine serum albumin (BSA) as standard and the analyses were performed at 595 nm (BRADFORD, 1976).

Phytohormone and metabolite profiling analysis by LC/MS

Phytohormones and metabolites extractions were performed from soybean leaves according to the procedure described by Muller and Munné-Bosch (2011) with some modifications (COUTINHO et al., 2019; LIMA et al., 2019). Leaves extracts were prepared by powdering approximately 180 mg fresh weight in liquid nitrogen and 500 µl of extraction solution [methanol, isopropanol and acetic acid 20:79:1 (v/v/v)]. Then, the samples were shaken in a vortex four times for 20 s (on ice), subjected to ultrasound treatment for 5 min, placed on ice for 30 min and then centrifuged at 14000 × g for 30 min at 4 ºC. The supernatant was removed and transferred to a new tube. A new extraction cycle was carried out with the resulting pellet from the previous extraction to increase the extraction efficiency, and then the supernatants were pooled. Finally, the total supernatant was filtered through 0.45 mm filters. About 400 µL of the extracts were placed in vials, and 5.0 µL were injected into the LC/MS system (Agilent Eclipse Plus, RRHD, 1.8 µm, 2.1 × 50 mm) from NuBioMol (Center for Biomolecules Analysis-UFV, Brazil), with the continuous flow (Φ = 0.3 mL/min), coupled online to a mass spectrometer QQQ triple quadrupole (Agilent).

The leaf extracts were separated by ultra-high performance chromatography (UHPLC, Agilent) using chromatography column C18 (50 mm x 1.0 mm ID, 1.7 µm particle and 300 *A*) coupled to the mass spectrometer (QqQ Agilent) alternating negative/positive modes according each metabolite. The samples were scanned by MRM (multiple reaction monitoring) for detecting some target metabolites: phytohormones (jasmonic acid, abscisic acid, salicylic acid, indolacetic acid, ACC, trans-zeatin, cis-zeatin, methyl jasmonate, GA3, GA4, 20 E, brassinolide-1, brassinolide-2), polyamines (putrescine, spermine, spermidine) , flavonoids (hesperidin, rutin, naringin, isoorientin (homoorietin), isovitexin (saponaretin), myricetin, morin, hesperetin, quercitin, kaempferol, luteolin, phloretin, epicatechin, catechin, narigenin, genistein, apigenin, and daidzein), phenolic compounds (4-hydroxybenzoic acid, 4-hydroxyflavone, 4-hydroxy-3-methoxy-cinnamaldehyde, 7-hydroxyflavone, benzoic acid, caffeic acid, catechol, chalcone, chlorogenic acid, coumarin, ferulic acid, isoferulic acid, neochlorogenic acid, n-propyl gallate, p-coumaric acid, quercetin, sinapic acid, sinapyl alcohol, syringic acid, trans-cinnamic acid, vanillin, 3,5dihydroxy benzoic acid) and proline.

The tests were carried out with four biological replicates for each treatment. The mass spectra were processed using the Skyline software according to the methods described by Coutinho et al., (2019). The XIC area values by MRM were used to determine the relative abundance of the phytohormones in the Metaboanalyst platform (**Table S1** and **S2**).

Bioinformatics Analysis: Characterization of Sound Response

As two experiments were carried out independently, one for the chewing sounds and the other for the forest sounds, the results of each experiment were analyzed independently. Thus, a statistical contrast was not performed between the forest and chewing sound data, the comparison occurred only within each type of sound, between soundless control and sound treatment. However, the statistical significance for the metabolite profiles were used to perform a comparison between the response to chewing sound and the forest sound.

The platform MetaboAnalyst was used to perform the following statistical analyses of the metabolomics data: heatmap, random forest, test t ($\alpha < 0,05$) and pathway analysis. Furthermore, the data were normalized according to their type of distribution (normalization by the sum of the peaks or normalization by the median), by the logarithmic function and by the pareto scaling method. In the analysis of pathways for mapping the phenolic compounds and flavonoids, the main pathways affected by the sound treatment were classified. The Pathway Analysis module combines results from powerful pathway enrichment analysis with pathway topology analysis to help identify the most relevant pathways involved in the conditions under study. Pathway enrichment analysis usually refers to quantitative enrichment analysis directly using the compound concentration values, as compared to compound lists used by over-representation analysis. As a result, it is more sensitive and has the potential to identify subtle but consistent changes amongst compounds involved in the same biological pathway (GOEMAN; BÜHLMANN, 2007; HUMMEL; MEISTER; MANSMANN, 2008). The pathway topology analysis uses two well-established node centrality measures to estimate node importance - degree centrality and betweenness centrality. The node importance measure selected for topological analysis was the betweenness centrality. The betweenness centrality measures the number of shortest paths going through the node. Since the metabolic network is directed, was used the relative betweenness centrality for a metabolite as the importance measure. The degree centrality measure focuses more on local connectivity, while the betweenness centrality measure focuses more on global network topology (AITTOKALLIO; SCHWIKOWSKI, 2006).

For the statistical analysis of the data from expression gene and enzyme activities, the JMP Statistical Discovery software was used. Student's t-test (normal distribution and homogeneous variance) and Welch's t-test (normal distribution and heterogeneous variance) were performed to identify the significant variables ($\alpha < 0,05$) of each sound treatment.

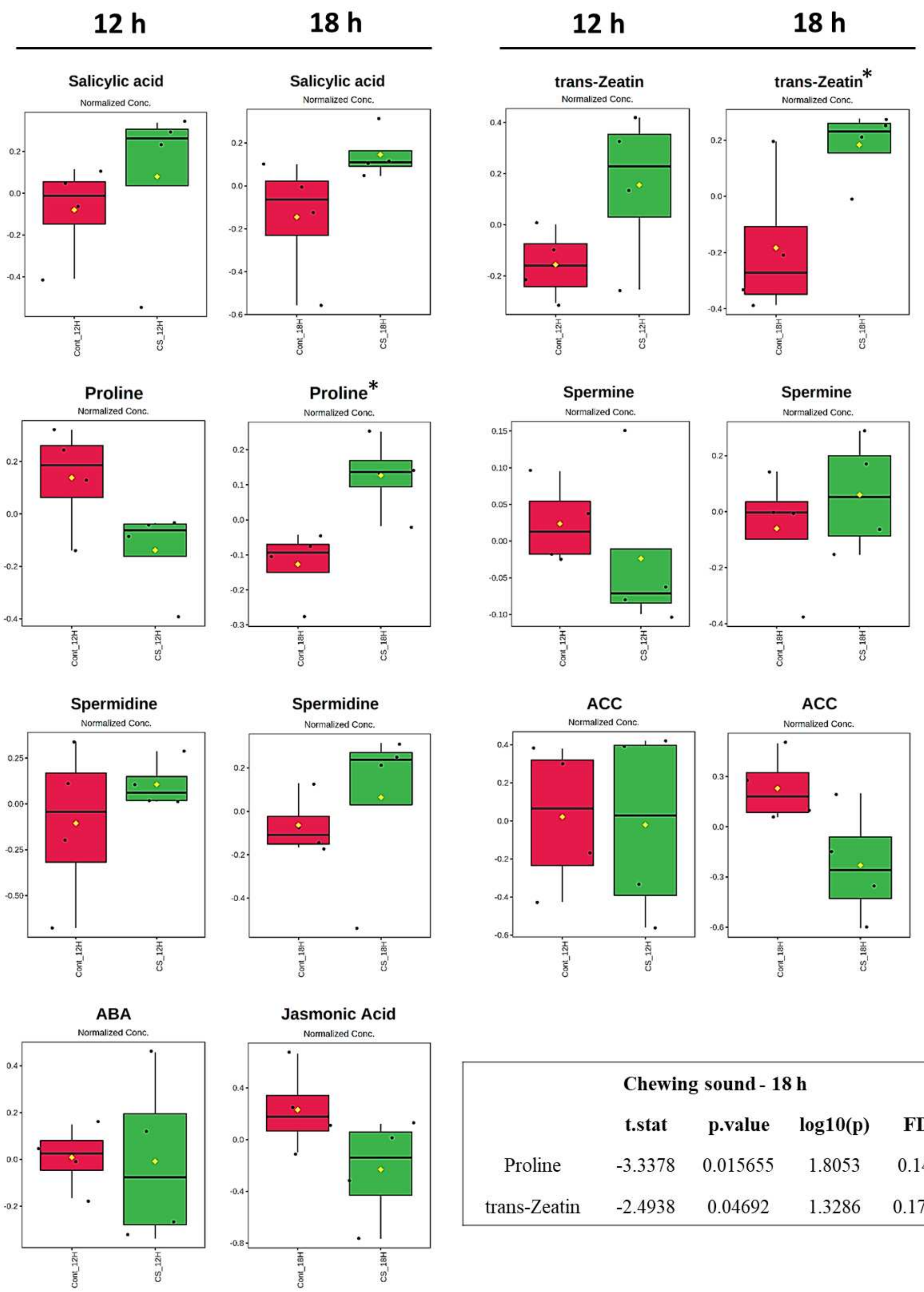
RESULTS

Soybean plants under different ecological sounds signals showed distinct phytohormonal profiles

Plant responses against herbivorous insects involve the biosynthesis of chemical defenses and protease inhibitors (PIs), which depend on the signal perception and induction of phytohormone biosynthesis. As acting synergistically to activate signaling cascades that regulate downstream transcriptional responses (NGUYEN et al., 2016), was evaluated the absolute concentration of some phytohormones and stress-responsive metabolites by LC/MS QqQ from soybean leaves (**Fig. 2** and **3**).

Jasmonic acid (JA) has been reported as the most responsive phytohormone in plant-insect interactions (YANG et al., 2019) and its level depend on the activation of the lipoxygenase (LOX) pathway. When the plants were elicited by sounds producing during caterpillar chewing, the JA levels did not change after 12 and 18 hours (**Fig. 2**). However, others phytohormones responsive to insect attach salicylic acid (SA) and ACC showed changes in the levels under chewing sound treatment. SA levels increased after 12 and 18 hours, while the ACC levels decreased, although they are not statistically significant. SA have been observed to acting positively during plant-insect interactions, while ACC negatively. Zeatin (ZA) levels were also responsive to chewing sound signals and it increased after 18h ($p < 0,05$). For the stress responsive metabolites, proline (chewing sound treatment) and spermidine (forest sound treatment) showed significative alterations in the abundances related to soundless control (**Fig. 2** and **3**), and their levels also increased after 18-hours treatment. Thus, phytohormones profiles indicate that chewing sound signals appeared not to trigger JA cascades, however those involving ZA and SA may be activated.

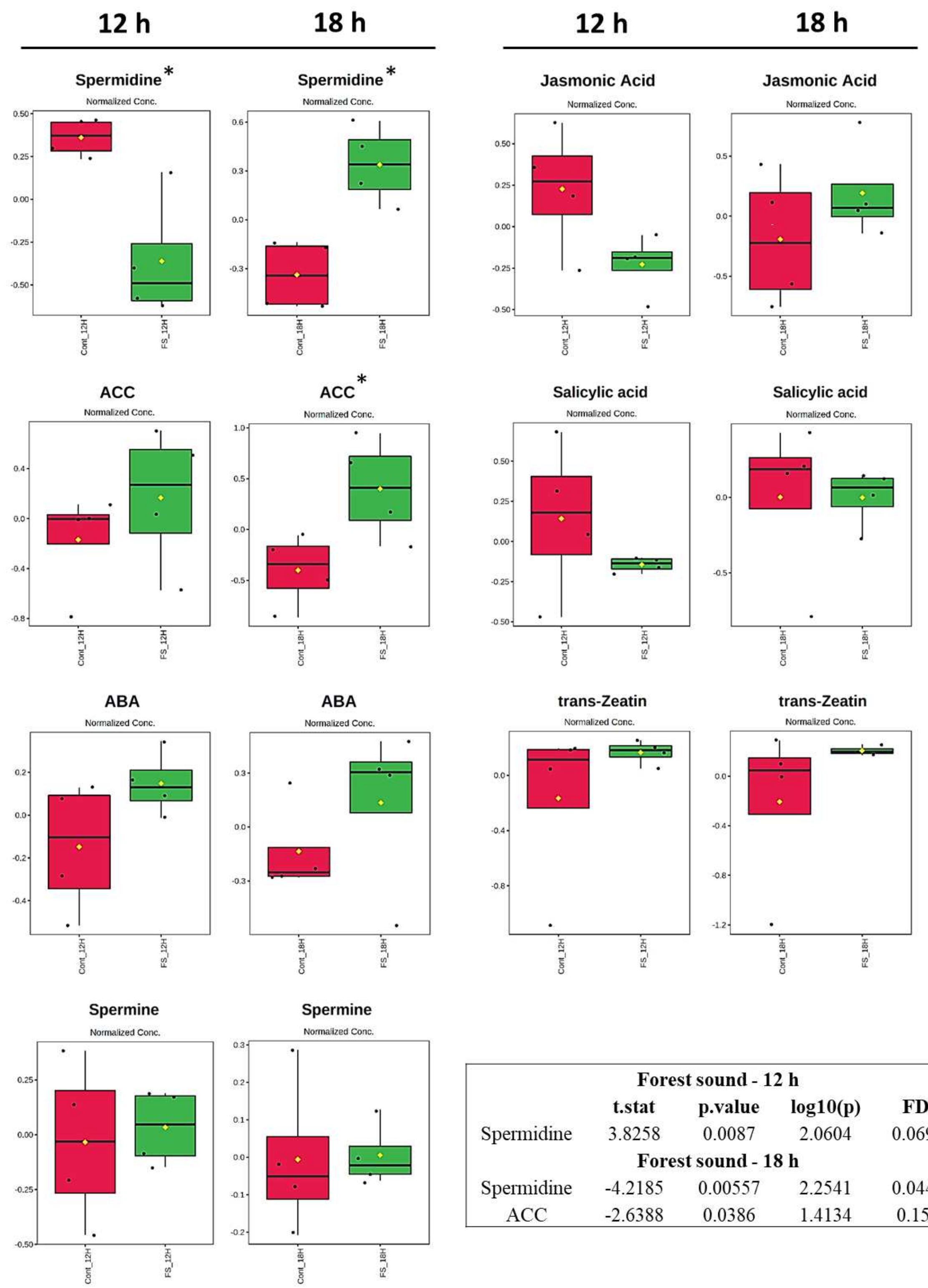
To verify whether the phytohormonal responses were specific for the chewing sound frequencies, we evaluated the phytohormone profiles under treatment of forest sound, containing different frequencies (**Fig. 3**). The changes observed for forest sound treatments were markedly different for all phytohormones. abscisic acid (ABA) and ACC levels were increased after 18hs while SA were not changed under forest sound signals (**Fig. 3**). In contrast, JA levels showed a trend of reduction after 12 hours. Like for proline under chewing sound signals, the polyamine spermidine levels were increased after 18-hour of forest sound.



Chewing sound - 18 h

	t.stat	p.value	log10(p)	FDR
Proline	-3.3378	0.015655	1.8053	0.1409
trans-Zeatin	-2.4938	0.04692	1.3286	0.17971

Figure 2: Box plots showing normalized relative concentrations of the phytohormones, polyamines and proline from soybean leaves by LC/MS after 12 and 18 hours under chewing sound treatments. Cont_12H and Cont_18H indicate plant in absence of sound. CS_12H and CS_18H indicate plants in the presence of chewing sound. **t. stat**: Negative values indicate greater relative abundance for the Sound Treatment and positive values greater abundance for the Soundless Control. The higher the $|t.stat|$, the greater the variable significance. **p-value**: Important features selected by t-tests with threshold 0.05. Note the *p values* are transformed by **-log10** so that the more significant features (with smaller p values) will be plotted higher on the graph. **FDR**: False Discovery Rate. *Significant features (α < 0,05).



	Forest sound - 12 h			
	t.stat	**p.value**	**log10(p)**	**FDR**
Spermidine	3.8258	0.0087	2.0604	0.06961
	Forest sound - 18 h			
Spermidine	-4.2185	0.00557	2.2541	0.04456
ACC	-2.6388	0.0386	1.4134	0.1544

Figure 3 Box plots showing normalized relative concentrations of the phytohormones, polyamines and proline from soybean leaves by LC/MS after 12 and 18 hours under forest sound treatments. Cont_12H and Cont_18H indicate plant in absence of sound. FS_12H and FS_18H indicate plants in the presence of forest sound. **t. stat**: Negative values indicate greater relative abundance for the Sound Treatment and positive values greater abundance for the Soundless Control. The higher the |*t.stat*|, the greater the variable significance. **p-value**: Important features selected by t-tests with threshold 0.05. Note the *p values* are transformed by **-log10** so that the more significant features (with smaller p values) will be plotted higher on the graph. **FDR**: False Discovery Rate. *Significant features (α < 0,05).

The data of concentration from all phytohormone and stress responsive metabolites (**Fig. 2** and **3**) were also used for clustering analysis by Heatmap, aiming to visualize the overall tendencies of the metabolites related to response to sound treatments (**Fig. 4A** and **4B**). These results indicated that sounds with different frequencies promoted different response. After 18 hours, it was possible to observe a tendency to increase the levels for the proline and spermidine under both sound signals (**Fig. 4B**). For phytohormones, the profiles indicated antagonistic tendencies, except for ZA that showed an increase after 12- and 18-hour treatment for both sounds (**Fig. 4B**). A random forest test (mean decrease accuracy as rank parameter) was applied to classify the most responsive variables to sound treatments (**Fig. 5**). For chewing sounds, SA showed higher abundance alterations for both 12- and 18-hour treatment (**Fig. 5A** and **5B**), while for the forest sounds spermidine were the most responsive. Thus, we conclude that the different ecological sounds may be triggering distinct signaling cascades.

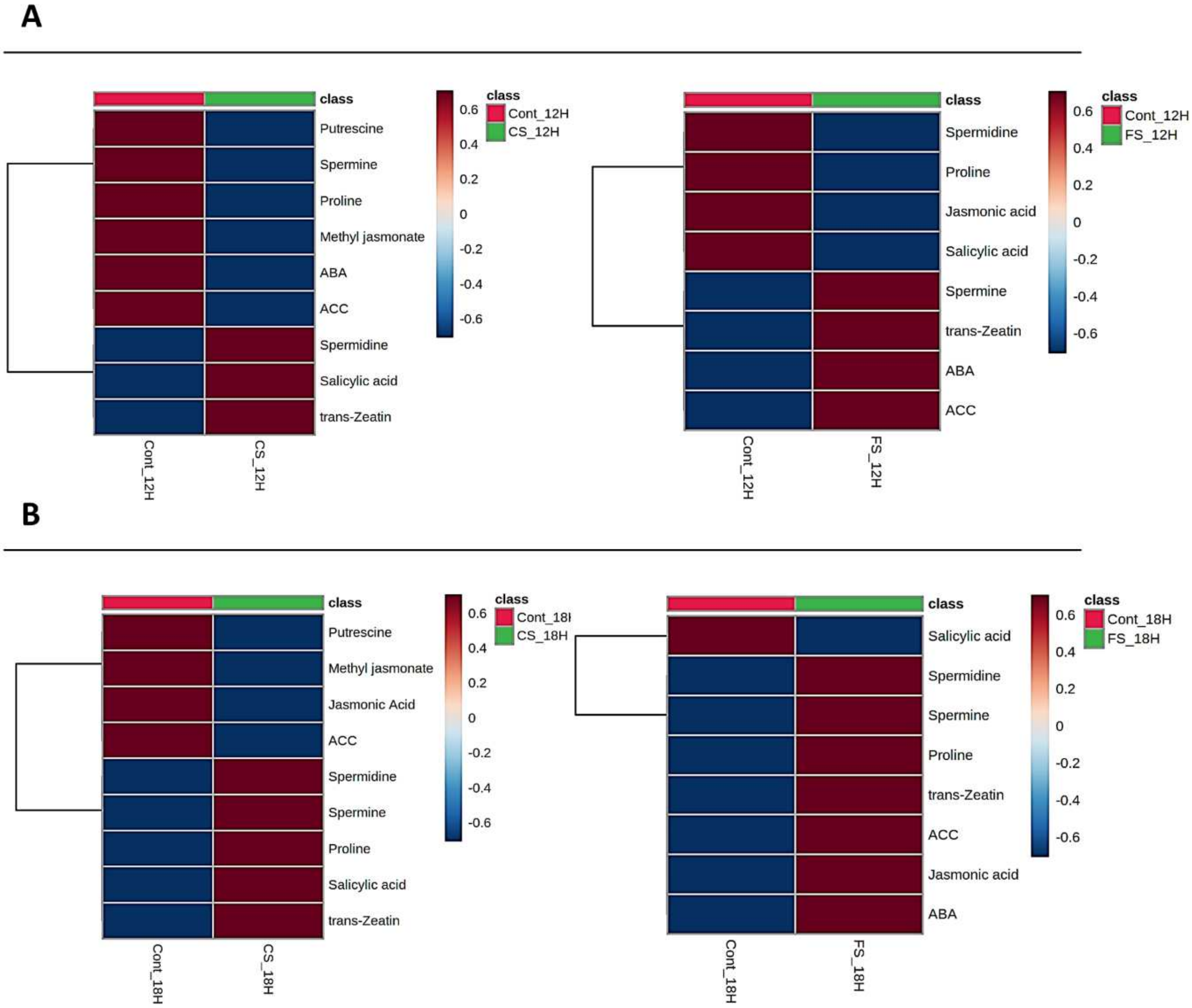


Figure 4. Heatmap of the characterized metabolites and their abundance fluctuations due the ecological sound exposures. CS_12H: 12-hour chewing sound treatment; CS_18H: 18-hour chewing sound treatment; FS_12H: 12-hour forest sound treatment; FS_18H: 18-hour forest sound treatment; Cont_12H: 12-hour soundless control treatment; Cont_18H: 18-hour soundless control treatment.

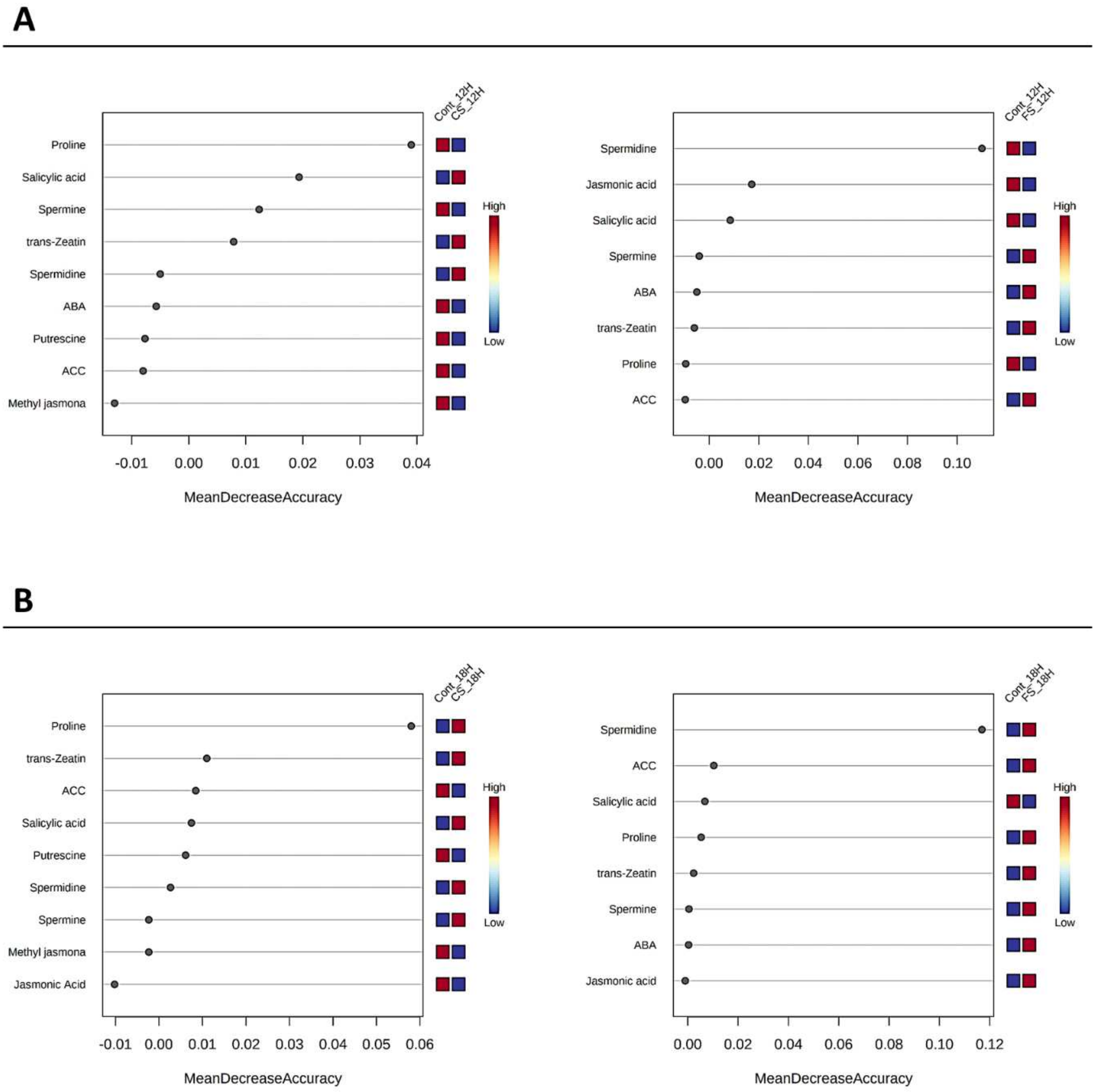


Figure 5. Random Forest of characterized metabolites showing the most responsive metabolites and their abundance fluctuations due to ecological sound exposures. Mean Decrease Accuracy: Level of importance of the variable. More responsive variables have higher values for this parameter. CS_12H: 12-hour chewing sound treatment; CS_18H: 18-hour chewing sound treatment; FS_12H: 12-hour forest sound treatment; FS_18H: 18-hour forest sound treatment; Cont_12H: 12-hour soundless control treatment; Cont_18H: 18-hour soundless control treatment.

PPO and CAT enzymatic activities from soybean leaves showed alterations under sound treatments

Alterations on the activity of some enzymes have been related with responses of the plants to environmental stresses. Thus, we evaluated the activities for lipoxygenases (LOX), polyphenol oxidases (PPO), catalase (CAT) and protease inhibition (PI) of the soybean leaves under sound treatments (**Fig. 6**). LOX activities were not affected by sound signals, despite an increase after 12 h under forest sound treatment (**Fig. 6**). These results for LOX activities correlated with JA levels (**Fig. 2** and **3**). Induction of the PIs synthesis have been triggered by JA for soybean plants under insect attack (GÓMEZ et al., 2020). However, PI activities did not modify also under chewing and forest sounds (**Fig. 6**). Thus, the plant responses to sound treatment did not involve LOX and JA cascades, at least for the evaluated exposures time.

After 12hours, PPO activities levels were decreased under chewing sound (24 %, p = 0,0715) and increased under forest sound (22 %, p = 0,154). In both cases the variations were not significant, but a trend in enzymatic activity was observed in the two ecological sounds. However, after 18 hours, PPO activities were more contrasting, increasing 35% in response to chewing sound (p = 0,051) and decreasing 56% (p = 0,035) in forest sound, being more responsive to the forest sound (**Fig. 6**). Although the chewing sound did not stimulate the JA- and LOX-dependent herbivory signaling pathway, the significant increase in PPO activity under the 18-hour chewing sound may indicate a possible activation of other herbivory signaling cascades. Oxidation of phenols catalyzed by PPO is a potential defense mechanism in plants against herbivorous insects. Quinones formed by oxidation of phenols bind covalently to leaf proteins, and inhibit the protein digestion in herbivores (KAUR, et al., 2017; WAR *et al.*, 2012).

CAT activities also showed alterations in response to sound treatments, showing a decrease in response to both sound signals, especially after 12 h of chewing sounds (p = 0,02) and after 18 h of forest sound treatments (p = 0,021). Although both treatments caused a decrease in the CAT activity, responses were faster for chewing sound (**Fig. 6**).

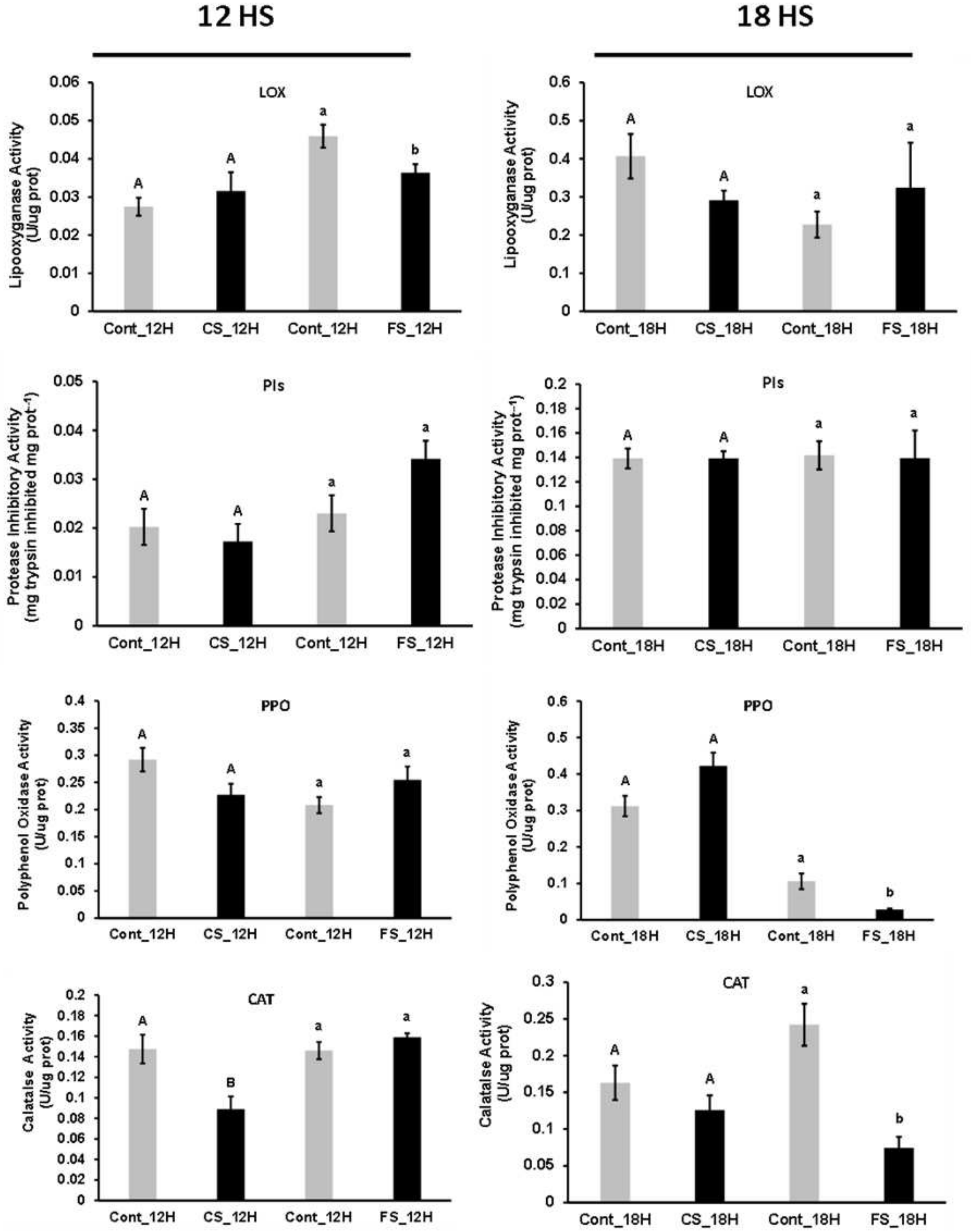


Figure.6: Bar graph for the mean values of relative enzymatic activity in the 12- and 18-hour sound treatments. Significant variables ($p < 0{,}05$) are represented by different letters and non-significant variables by the same letters. As the experiments are independent, the comparisons took place within each sound treatment. Uppercase letters are for comparisons within the chewing sound and lowercase letters from the forest sound. For variables with heterogeneous variance, the Welch's t test was applied.

Flavones and flavonols biosynthesis was the most impacted pathway by the ecological sounds

Secondary metabolites have been observed as responsive to biotic and abiotic environmental stresses. In plant insect interactions, phenolic compounds may reduce the palatability of the tissues, acting as repellents or by reducing the insect survival. Some classes, such as flavonoid, have been detected in higher concentrations in the soybean genotypes during infestations by *A. gemmatalis* (Gómez et al., 2018, 2020). Thus, we evaluated the abundance of phenolic compounds and flavonoids in response to ecological sounds and the most affected secondary metabolism pathways were characterized. An enrichment analysis was performed to rank the most responsive and significative metabolites ($\alpha < 0{,}05$), and a pathway topology analysis was performed to rank the most significant and impacted pathways by ecological sounds. The significance of the pathway is related to the level and abundance of significant metabolites mapped in the pathway; the impact is related to the distribution of metabolites in the pathway - the closer the proximity of metabolites and the more favorable their positions within the pathway, the greater the impact by the pathway.

As observed for the phytohormones, the phenolic compound and flavonoid profiles were distinct for soybean under to chewing and forest sound after 12- and (**Fig. 7A**),18-hour treatments (**Fig. 7B**). Likewise, the number and specifically dysregulated compounds were also different for each sound type, as well as the significantly responsive metabolites (**Table 1)**

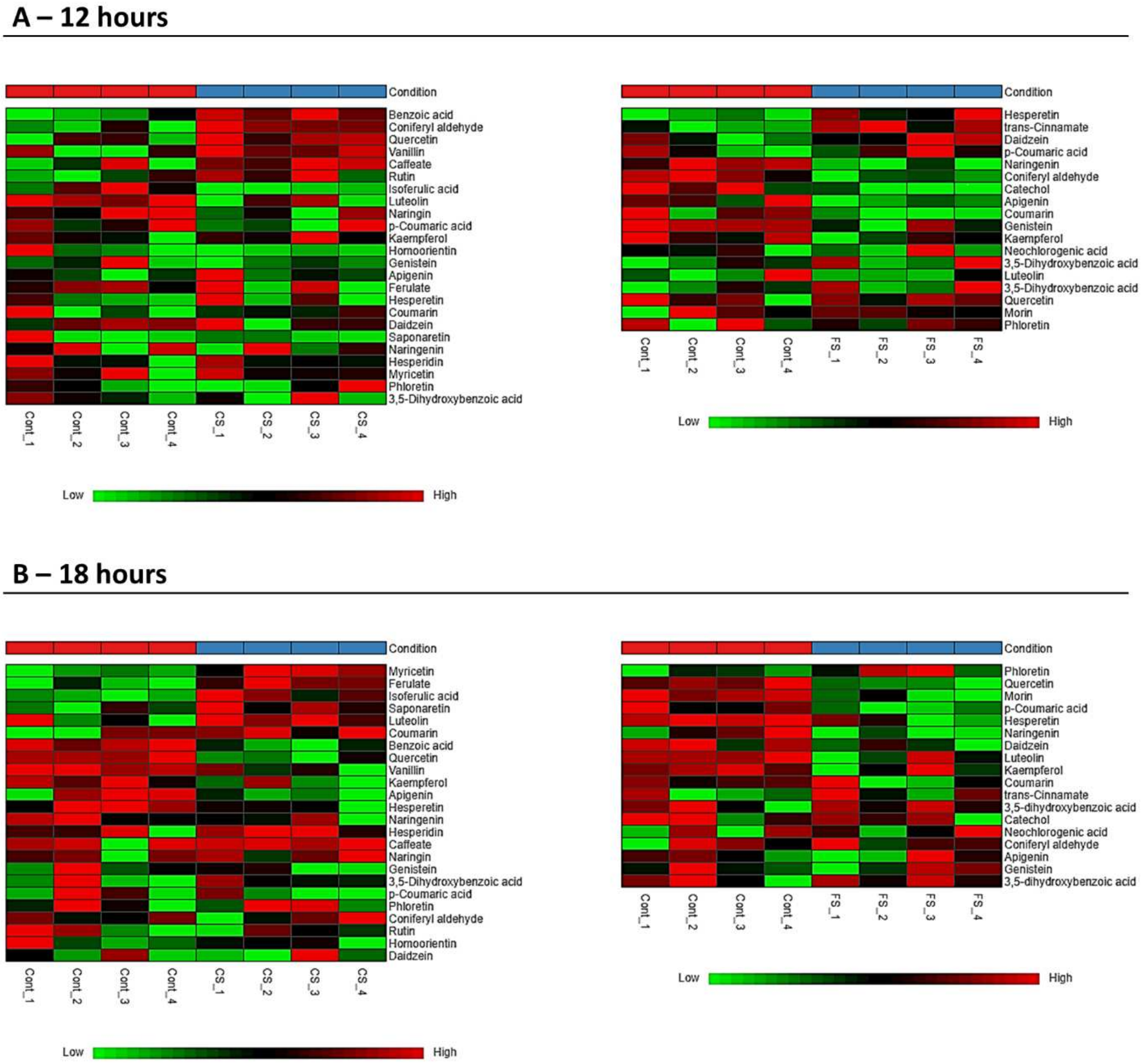


Figure 7. Heatmap of the characterized phenolic compounds and flavonoids. **A**: 12-hour after the chewing and forest sound treatments. **B**: 18-hour post chewing and forest sound treatments. **CS_1 to CS_4**: biological repetitions of the chewing sound treatment. **FS_1 to FS_4**: biological repetitions of the forest sound treatment. **Cont_1 to Cont_4**: biological repetitions of the soundless control treatment.

Table 1. Significant phenolic compounds and flavonoids in sound treatments. **t. stat**: Negative values indicate greater relative abundance for the Sound Treatment and positive values greater abundance for the Soundless Control. The higher the $|t.stat|$, the greater the variable significance. **p-value**: Important features selected by t-tests with threshold 0.05. Note the *p values* are transformed by **-log10** so that the more significant features (with smaller p values) will be plotted higher on the graph. **FDR**: False Discovery Rate

Chewing sound - 12H				
	t.stat	**p.value**	**-log10(p)**	**FDR**
Benzoic acid	-4.9573	0.002559	2.5919	0.061419
Coniferyl aldehyde	-4.0664	0.006603	2.1803	0.079233
Isoferulic acid	3.6302	0.010963	1.9601	0.087701
Quercetin	-2.6382	0.038633	1.413	0.2318
Forest sound - 12H				
	t.stat	**p.value**	**-log10(p)**	**FDR**
Naringenin	5.8637	0.001088	2.9635	0.01958
Coniferyl aldehyde	4.5845	0.003753	2.4256	0.032816
Hesperetin	-3.9865	0.00723	2.1409	0.032816
Trans-Cinnamic acid	-3.9789	0.007293	2.1371	0.032816
Catechol	3.7217	0.009832	2.0074	0.035396
Apigenin	3.2212	0.01811	1.7421	0.054331
Coumarin	2.9195	0.026648	1.5743	0.063596
Genistein	2.8744	0.028265	1.5488	0.063596
Chewing sound - 18H				
	t.stat	**p.value**	**-log10(p)**	**FDR**
Myricetin	-5.5149	0.001494	2.8257	0.012419
Benzoic acid	5.2334	0.001951	2.7098	0.012419
Ferulate	-5.2067	0.002002	2.6986	0.012419
Quercetin	5.1723	0.00207	2.6841	0.012419
Isoferulic acid	-4.6439	0.003526	2.4527	0.016926
Vanillin	2.9722	0.024885	1.6041	0.099542
Saponaretin	-2.512	0.045779	1.3393	0.15105
Forest sound - 18H				
	t.stat	**p.value**	**-log10(p)**	**FDR**
Quercetin	6.7101	0.000532	3.2739	0.00958
Morin	5.1811	0.002052	2.6878	0.01847
p-Coumaric acid	3.7348	0.009682	2.014	0.058092
Hesperetin	3.3168	0.016069	1.794	0.072311
Naringenin	2.5633	0.042716	1.3694	0.14796
Daidzein	2.457	0.049319	1.307	0.14796

In the analysis of the pathways after 12-hour chewing sound treatment, the phenylpropanoid biosynthesis pathway was the only one significantly affected. ($\alpha < 0,05$). The flavones and flavonols biosynthesis pathway was the most impacted by the sound treatment, but it was not significant ($\alpha > 0,05$). The flavonoid biosynthesis pathway was the least impacted and with the lowest significance of the three most relevant pathways (**Table 2**). In the 12-hour forest sound treatment, all three pathways most affected by ecological sounds were significant. The flavonoid biosynthesis pathway was the most significant and the second most impacted by the sound. The phenylpropanoid biosynthesis pathway was the second most significant and the least impacted pathways, and the flavones and flavonols biosynthesis pathway was the most impacted pathway and the least significant pathway (**Table 3).** In the treatment with the 18-hour chewing sound, the phenylpropanoid biosynthesis pathway was the only one not showing statistical significance but it was the second most impacted, and the flavonoid biosynthesis pathway was the most significant and the least impacted. The flavone and flavonol biosynthesis pathway were the second most significant and the most impacted by sound (**Table 4**). In the 18-hour forest sound treatment, the three pathways classified as most important were significantly affected by the sound. The flavonoid biosynthesis pathway was the most significant and the second most impacted. The phenylpropanoid biosynthesis pathway was the second most significant and least impacted, and the flavones and flavonols biosynthesis pathway was the least significant and most impacted by sound (**Table 5**). Flavonoid, flavone and flavonols and phenylpropanoid biosynthesis were the most affected pathways, and the flavone and flavonols biosynthesis was the most impacted pathway by the ecological sounds. Flavones and flavonol biosynthesis was the most impacted pathway in all sound treatments and it was a more significant for the chewing sound than forest sound.

Table 2. Metabolic pathways more responsive to 12-hour chewing sound treatment. **Total cmpd**: Number of pathway intermediaries; **Hits**: Number of characterized metabolites mapped in the pathway; **Raw p**: pathway significance level with threshold 0.05; FDR: False Discovery Rate; **Impact**: Level of impact caused in the pathway by the sound treatment.

	Total cmpd	**Hits**	**Raw p**	**FDR**	**Impact**
Phenylpropanoid biosynthesis	46	4	0.038195	0.1698	0.18319
Flavone and flavonol biosynthesis	10	4	0.067919	0.1698	0.7
Flavonoid biosynthesis	47	7	0.15759	0.23526	0.10758

Table 3. Metabolic pathways more responsive to 12-hour forest sound treatment**. Total cmpd**: Number of pathway intermediaries; **Hits**: Number of characterized metabolites mapped in the pathway; **Raw p**: pathway significance level with threshold 0.05; FDR: False Discovery Rate; **Impact**: Level of impact caused in the pathway by the sound treatment.

	Total cmpd	**Hits**	**Raw p**	**FDR**	**Impact**
Flavonoid biosynthesis	47	6	0.00414	0.02484	0.10758
Phenylpropanoid biosynthesis	46	3	0.022802	0.04507	0.09194
Flavone and flavonol biosynthesis	10	4	0.027004	0.04507	0.7

Table 4. Metabolic pathways more responsive to 18-hour chewing sound treatment**. Total cmpd**: Number of pathway intermediaries; **Hits**: Number of characterized metabolites mapped in the pathway; **Raw p**: pathway significance level with threshold 0.05; FDR: False Discovery Rate; **Impact**: Level of impact caused in the pathway by the sound treatment.

	Total cmpd	**Hits**	**Raw p**	**FDR**	**Impact**
Flavonoid biosynthesis	47	7	0.005012	0.02501	0.10758
Flavone and flavonol biosynthesis	10	4	0.029923	0.07487	0.7
Phenylpropanoid biosynthesis	46	4	0.092038	0.1534	0.18319

Table 5. Metabolic pathways more responsive to 18-hour forest sound treatment**. Total cmpd**: Number of pathway intermediaries; **Hits**: Number of characterized metabolites mapped in the pathway; **Raw p**: pathway significance level with threshold 0.05; **FDR**: False Discovery Rate; **Impact**: Level of impact caused in the pathway by the sound treatment.

	Total cmpd	**Hits**	**Raw p**	**FDR**	**Impact**
Flavonoid biosynthesis	47	6	0.005498	0.02594	0.10758
Phenylpropanoid biosynthesis	46	3	0.016519	0.02594	0.09194
Flavone and flavonol biosynthesis	10	4	0.029811	0.03573	0.7

The mapping of the most responsive metabolic pathways indicates relevant differences in the biosynthesis of flavones and flavonols for the ecological sounds

Changes in the abundance of the phenolic compounds were also examined in their respective metabolic pathways aiming to indicate those specifically responsive to sound treatments (**Fig. 8** and **9**). However, the statistical tool to find the most significant and impacted pathways does not allow us to provide a detailed dynamics of the metabolic adjustment as well as the main regions of a pathway where the changes occurred (**Fig.7**). Thus, the mapping of the metabolites in the pathways was carried out to verify in more detail the main fluctuations of the pathways most affected by sounds. Referring to the KEGG platform (https://www.genome.jp/kegg/), the Biosynthesis pathways of flavonoids, flavones and flavonols and phenylpropanoids were recreated and the responsive metabolites were mapped (**Figure 8** and **9**). This mapping provided important evidence that different types of ecological sounds can drive distinct patterns of metabolic responses. The mapping of responsive metabolites was able to provide strong evidence that in each type of ecological sound, a specific behavior was adopted by the plant in the metabolic readjustment. Although the flavone and flavonol biosynthesis pathway was the most impacted by ecological sounds, the mapping showed that the metabolic adjustment in this pathway was specific for each type of sound.

For plants after 12 hours of chewing sound, in the phenylpropanoid pathway significant changes were observed in the accumulation of caffeic acid, coniferyl aldehyde, benzoic acid, and for Flavonol pathways, the levels of quercetin and rutin were changed (**Fig. 8A**). In contrast, for plants after 12 hours of forest sound treatments (**Fig. 8B**), it was possible to verify different stretches for those biosynthetic pathways, such as cinnamic acid (UP), coumarin (down), naringenin (down), apigenin (down), hesperetin (UP) and genistein (Down). Interestingly, after 12 hours, the chewing sound signals appeared to induce the flavonol pathway while forest sound induced the isoflavonoids and flavanone pathway. Accordingly, increased levels of quercetin and rutin (flavonol class) were observed in response to chewing sounds. In the 12-hour forest sound treatment, the flavonol biosynthesis was down-regulated (low abundance for kaempferol and no adjustments for the other flavonols).

After 18 hours of sound treatments, the phenolic compound and flavonoid abundances were changed related to 12 hours (**Fig. 9**). It was highlighted by a decrease in the quercetin levels and a concomitant increase in the myricetin flavonol and isovitexin and luteolin flavone levels when the plants were elicited by chewing sounds. Likewise, ferulic acid, vanillin, and benzoic acid levels were statistically modified (**Fig. 9A**). The reductions in the levels of the benzoic acid may be related to the increased levels of SA (**Fig.2** and **3**), because benzoic acid can be converted to SA (LEFEVERE; BAUTERS; GHEYSEN, 2020). Again, flavonol biosynthesis was up-regulated (up-myricetin) by the chewing sound, and flavone biosynthesis was also up-regulated by the chewing sound because isovitexin and luteolin were more abundant in the sound treatment. For the forest sound treatment, the changes were diverse from those by the chewing sounds, highlighting reductions of relative abundances to p-coumaric acid, naringenin, daidzein, quercetin, and hesperetin. Phloretin was the only compound to be up-regulated and again, flavonol biosynthesis was down-regulated by the forest sound (**Fig. 9B**).

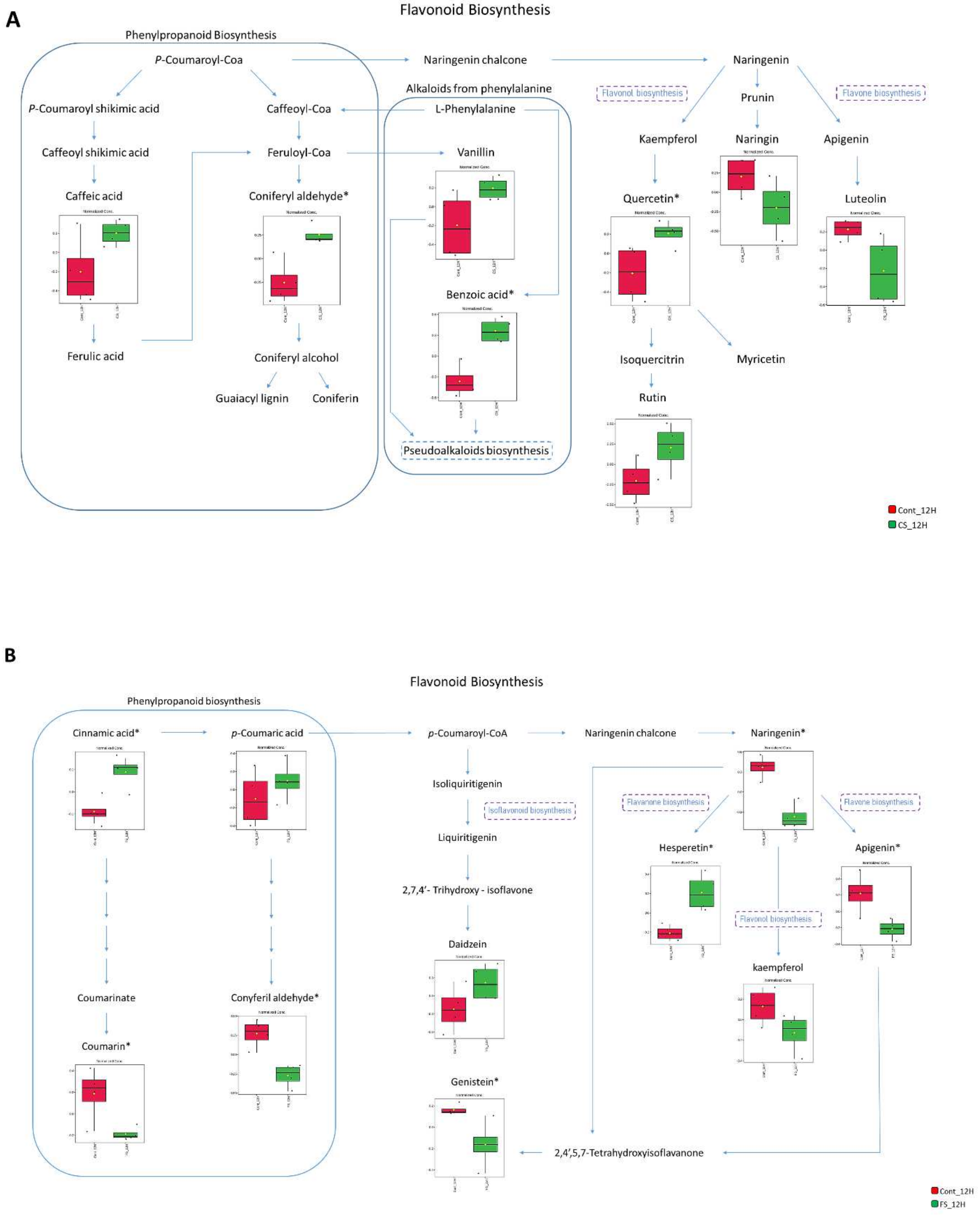


Figure 8. Mapping the most responsive metabolites to 12-hour sound treatments. **(A)** Chewing sound and **(B)** Forest sound. *Significant features

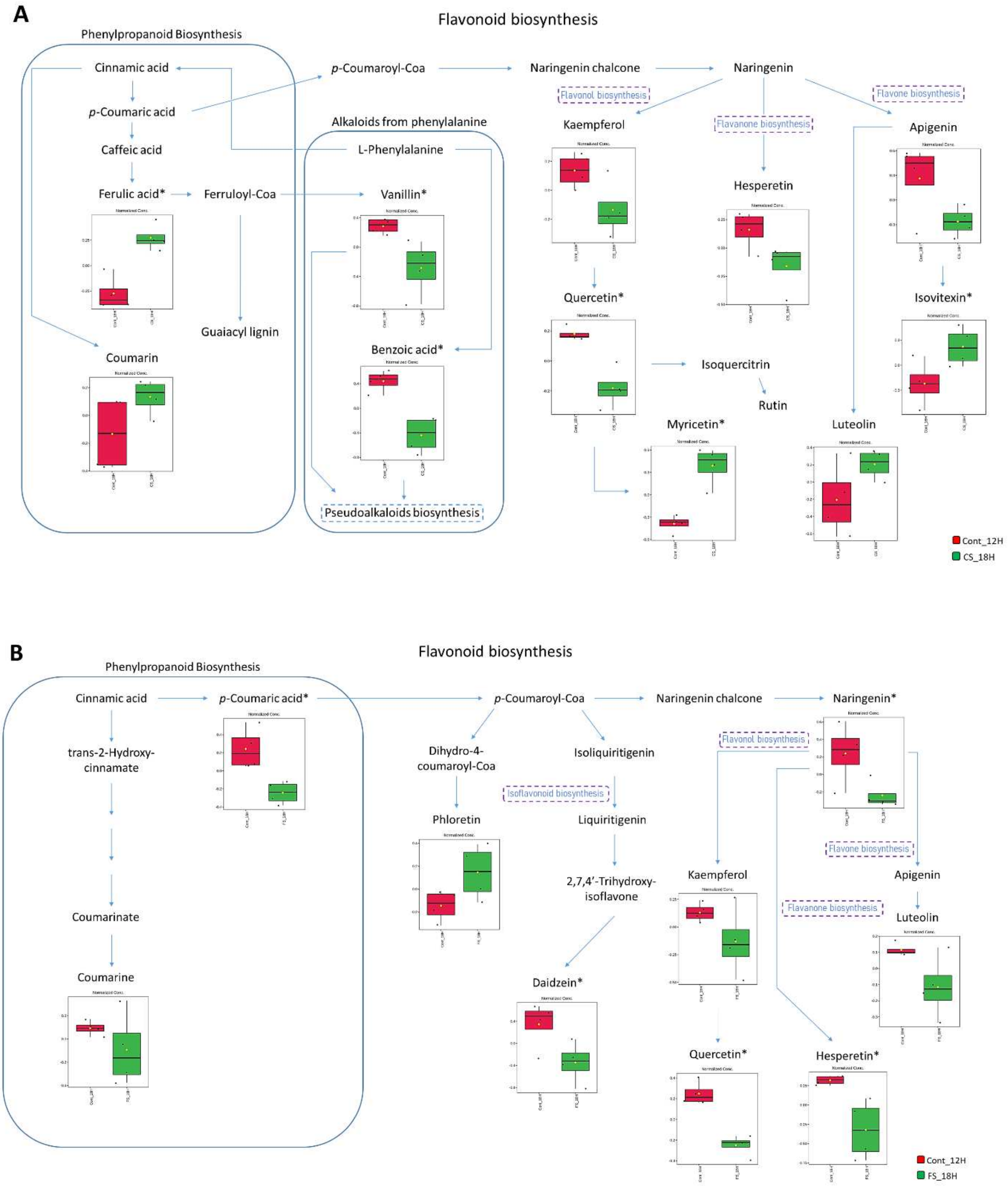


Figure 9. Mapping the most responsive metabolites to 18-hour sound treatments. **(A)** Chewing sound and **(B)** Forest sound. *Significant features

Genes involved in the Flavonol biosynthesis are responsive to chewing sound

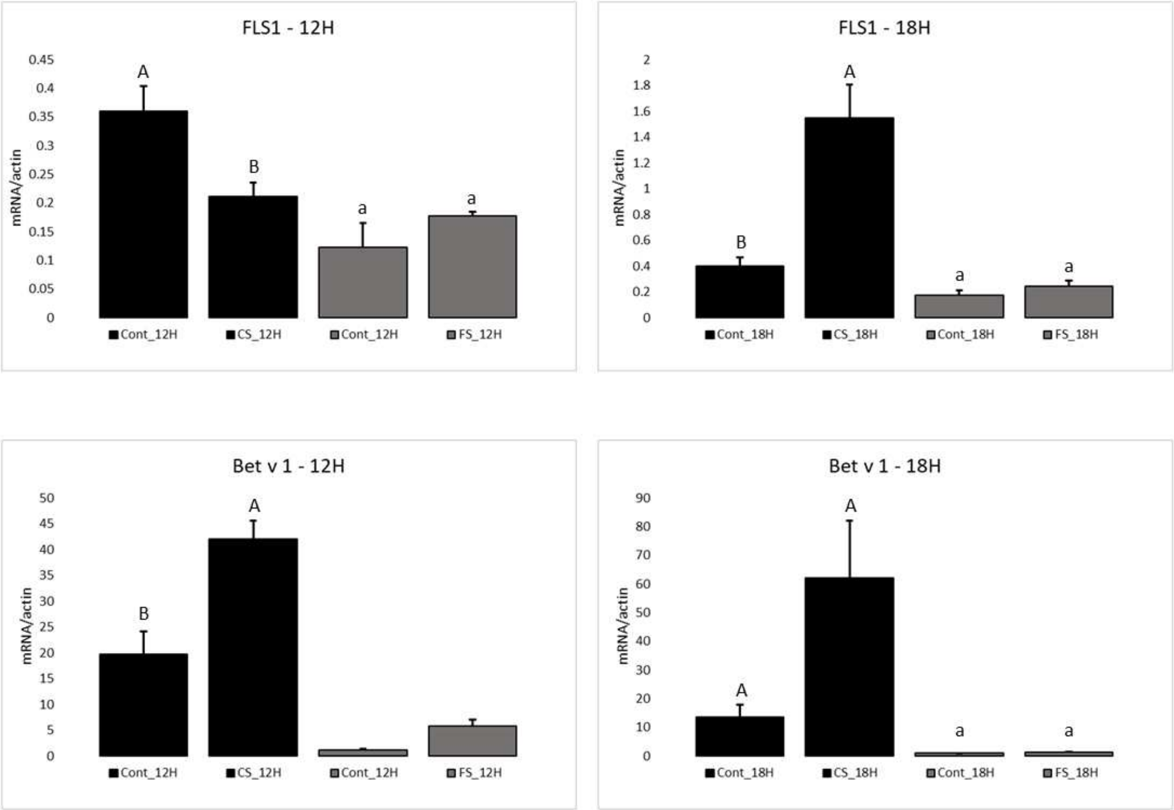
As the previous experiments indicated that cascades were differently triggered by chewing sounds, we evaluated the expression of some genes involved in the insect-plant interactions by qRT-PCR. The results also indicated that the chewing sound may be acting as inducing the herbivory-responsive cascades (**Fig. 10**). In contrast, forest sound appeared not to induce the cascades associated with herbivory attack.

LOX expression and activity have been indicated as a key player in the herbivory signaling cascade. However, both chewing sound and forest sounds did not promote significant changes in its expression levels, in accordance with the JA levels (**Fig. 2** and **3**) and LOX activity (**Fig. 6**). Thus, reinforcing the hypothesis that the chewing sound was not able to activate the JA-dependent defense pathway. However, the expression of PI genes, which are induced by JA, were significantly responsive to chewing.

The expression of the *SKTI* gene encoding another important protease inhibitor responsive to herbivore infestation was also significantly increased ($p = 0{,}0151$) after 12-hour chewing sound treatment (**Fig. 10**). Although not statistically significant ($p = 0.1204$), after 18-hour treatment the *SKTI* transcript level was higher under chewing sound treatment compared with the control. *SKTI* and *BBI* expression levels did not changed significantly in response to forest sound treatments. Although *BBI* and *SKTI* were responsive to the chewing sound, the protease inhibition activities did not alter (**Fig. 6**) at least for the evaluated time periods.

As the flavone and flavonol biosynthesis pathways were the most impacted by sound treatments, we also evaluated some *A. gemmatalis*-responsive genes (Pinheiro et al., 2021). *FLS1* expression has been induced under attack by herbivores in soybeans (GÓMEZ et al., 2020). *FLS1* encodes flavonol synthase (FLS) a key enzyme in the flavonol biosynthesis, which catalyzes the conversion of dihydroflavonols to flavonols such as kaempferol, quercetin and myricetin. The *FLS1* expression profiles at 12 hours and 18 hours after the chewing sound treatment (**Fig. 10**) were distinct from those by the forest sound. After 12-hour of the chewing sound, the *FLS1* expression level in the sound treatment was significantly lower than the control ($p = 0{,}039$). However, after 18- hour of the chewing sound, the *FLS1* expression levels were highly induced in response to chewing sound signals ($p = 0{,}0129$). In contrast, the expression levels of the *FLS1* gene were not responsive to forest sound signals (**Fig. 10**). These results are in accordance with the changes in the flavonols biosynthesis in response to the chewing sound (**Fig. 8** and **9**). Furthermore, the increases in the myricetin abundances verified after 18-hour chewing sound treatment may have been due to the increased expression of the *FLS1* gene (**Fig. 9A**).

PR10/Bet v1 is involved in the synthesis of phenolic compounds and flavonoids, as well as in the regulation of flavonol biosynthesis (DASTMALCHI, 2021). Furthermore, *PR10/Bet v1* is also highly responsive to infestations by in *A. gemmatalis* in soybean leaves (Pinheiro et al., 2021). After 12-hour of the chewing sound treatment, *Bet v-1* expression was significantly (p = 0,0173) induced (**Fig. 10**) and remained higher than the control plants even after 18 hours (p = 0,0752). These results are very interesting because they correlated with the greater abundance of flavonols in plants treated with the chewing sound (**Fig. 8** and **9**). In contrast, after 12-hour and 18-hour of the forest sound treatments, flavonol biosynthesis was down-regulated (**Fig. 8** and **9**), which was consistent with the expression levels of *Bet v-1* and *FLS1* genes (**Fig. 10**), which did not alter under forest sound signals.



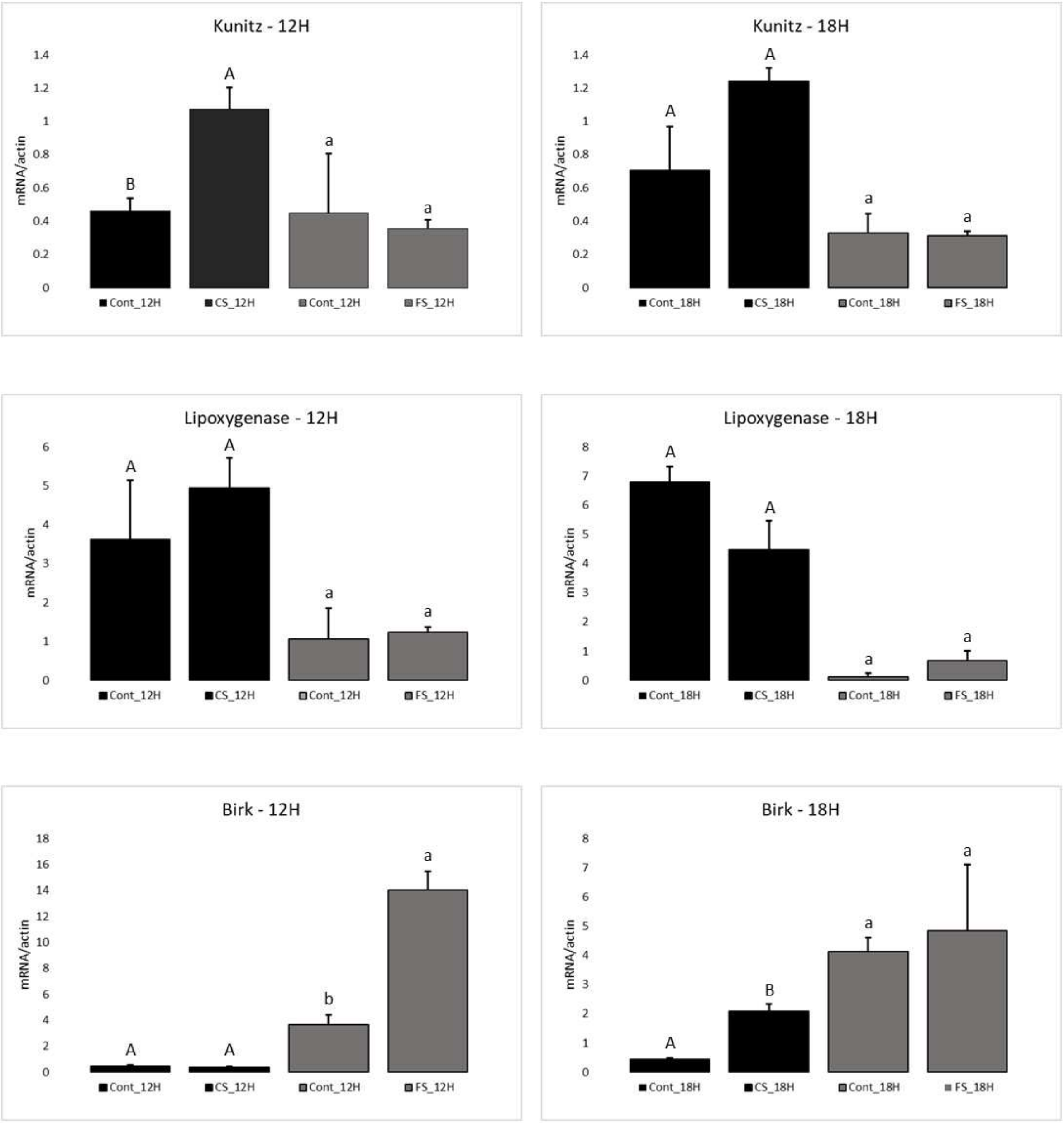


Figura 10: Bar graph for the gene expression levels in the 12- and 18-hour sound treatments. Significant variables (*p*-value < 0,05) are represented by different letters and non-significant variables by the same letters. As the experiments are independent, the comparisons took place within each sound treatment. Uppercase letters are for comparisons within the chewing sound and lowercase letters from the forest sound. For variables with heterogeneous variance, the Welch's t test was applied.

DISCUSSION

Recent evidence has indicated that sound vibrations (SVs), naturally occurring and artificially generated, contribute to plant robustness. Thus, acting beyond the chemical triggers may improve plant health by enhancing plant growth and resistance (JUNG et al., 2018). Otherwise, plant membranes are equipped with an enormous number of mechanosensitive channels that are believed to be responsive to mechanical vibrations (HASWELL; PHILLIPS; REES, 2011). Others studies have suggested that SVs increase the transcription of specific genes (MISHRA; GHOSH; BAE, 2016), as the up-regulation of some kinases (e.g. MPK11) and transcription factors (MYB77, DREB26, and RAV1) in Arabidopsis. Furthermore, Zhao et al., (2002) suggested that cells treated with SVs exhibit enhanced protein kinase activity. Thus, we evaluated whether soybean plants can recognize chewing sounds as signals that specifically trigger cascades involved in the plant-insect interactions. For this, gene expression and metabolic profiles were compared in response to treatment with sound signals containing distinct frequencies from chewing and forest.

One of first response of the soybean plants to insect attach is the increase of the JA levels due to the LOX pathway activation (GÓMEZ et al., 2020) after recognizing the chemical signals from insect salivary glands. However, the levels of JA, LOX expression and activities did not change after chewing sound treatment. While a trend for increasing the SA levels was observed after 12-and 18-hour chewing sound treatments, the levels for ACC levels were decreased under the same conditions. SA have been observed to act positively during plant-insect interactions, whereas ACC negatively modulates plant response to insect attack. Accordingly, the insect resistant genotype IAC 17 has been shown to accumulate higher SA levels than control lines (FAUSTINO et al., 2021; GÓMEZ et al., 2020). Thus, chewing sound may activate SA-dependent signaling cascades. Ghosh et al., 2016 also investigated the global gene expression and phytohormonal changes in *Arabidopsis thaliana* upon treatment with sound vibrations of 500Hz treatment during 6, 24 and 48 hours. Significant changes have been observed for the levels of all the phytohormones, except ABA, while JA levels was increased only 48 hours. These authors also observed SA levels higher at all time points under sound treatment. As also observed in our profiles, sound treatments may produce general changes in phytohormonal profiles, however these modulations may depend of the applied time and frequencies (GHOSH et al., 2016). When soybean plants were exposed to different ecological sounds, we observed distinct phytohormonal profiles after 12 and 18 hours, specifically for SA levels under chewing sound signals. Furthermore, the levels of some stress responsive metabolites were also distinct such as proline, both 12 and 18 hours after chewing sound treatment, and spermidine upon the forest sound.

Environmental stresses, including drought, salt stress, extreme temperatures, wounding, UV light, cause oxidative conditions that stimulate photoinhibition of the photosynthesis, production of reactive oxygen species (ROS) and antioxidant defense. Several enzymes are indicative of the oxidative status as well as in the defensive responses to biotic and abiotic stresses, including catalase (CAT), and polyphenol oxidase (PPO). The enzymatic activities of both CAT and PPO were changed by sound treatments. CAT activity significantly decreased after 12-hour chewing sound treatment ($p = 0,0201$) and 18-hour forest sound treatment ($p = 0,0021$). On the other hand, within 18 hours of treatment, PPO showed a strong trend towards increased activity by the chewing sound ($p = 0,051$), and a significant decreased activity by the forest sound (0,0351) (**Fig. 6**). This responsiveness to sound treatments may be due to these enzymes are activated by several environmental stresses that produce ROS, thus indicating that sound treatments may act such as a general or specific stress. However, ROS strengthens cell walls through oxidative crosslinking of glycoproteins, and acts as a second messenger in some cell signaling pathways (LAMB; DIXON, 1997), indicating that the forest sound and the chewing sound may be triggering different signaling cascades. The increase in the PPO activity may also be a specific response to the chewing sound treatment, because it is also highly responsive to insect attack (BHONWONG et al., 2009; FELTON; SUMMERS; MUELLER, 1994). (**Fig. 6**). This hypothesis is consistent with our results and those observed by Ghosh et al. (2016) for phytohormones profiles. In corroboration with this, protein kinases involved in ROS scavenging also display enhanced activities in response to sound, and so some protective enzymes, including superoxide dismutase (SOD), peroxide dismutase (POD), and ascorbate dismutase (APX) (MISHRA; GHOSH; BAE, 2016).

Other common plant response to environmental signals are the activation of the phenylpropanoid and flavonoid pathways, which have an important role in plant adaptation to different conditions (BIDART-BOUZAT; KLIEBENSTEIN, 2008; KLIEBENSTEIN, 2004; WINKEL-SHIRLEY, 2001a, 2001b), predominantly to the biotic stresses (GUTIÉRREZ-ALBANCHEZ et al., 2020). Both chewing and forest sound induced significative changes in the abundances of phenolic compounds and flavonoids; however, the dysregulated branches of the phenylpropanoid and flavonoid pathways were remarkable distinct (**Fig. 8** and **9**). These specific changes in the plant secondary metabolisms also are consistent with the hypothesis that sound with different frequencies may act as a general or specific signal. For example, a tendency to activate the flovonol (12- and 18-hours treatments) and flavone (18-hours treatment) biosynthesis was observed in response to the chewing sound, whereas isoflavonoid and flavanone biosyntheses (12-hours treatment) were changed in response to forest sounds. Phenolic compounds may be the main metabolites that affect caterpillar survival (Gómez et al., 2020). The addition of rutin (Flavonol class) in caterpillar diets favors a decrease in the survival rate (KRISCHIK; GOTH; BARBOSA, 1991; ONKOKESUNG et al., 2014; PIUBELLI et al., 2005; SILVA et al., 2016). Accordingly, the IAC 17 soybean resistant genotype displayed higher abundance levels of four quercetin conjugates, including rutin (GOMEZ et al., 2018). The increased levels of rutin and quercetin in the 12-hour treatment and myricetin in the 18-hour treatment in the chewing sound, and the likely reduction in flavonols in the 12- and 18-hour treatment of forest sounds, suggest that the acoustic chewing signals may trigger herbivory signaling cascades responsible for the up-regulation of flavonols. This is an indication that the aglycone substrate is being funneled to produce active glycosylated forms. Kim et al., (2021) also observed alterations on flavonoid contents (25–88% increase) of the sprouts of radish, lettuce, and cabbage in response to acoustic waves, which also vary according to the sound frequency, exposure time and species. Likewise, *A. thaliana* rosettes pre-treated with the sound vibrations caused by caterpillar feeding have been showed higher levels of glucosinolate and anthocyanin, when subsequently fed by *Pieris rapae* caterpillars than did untreated plants. These plants also discriminated between the vibrations caused by chewing and those caused by wind or insect song (APPEL; COCROFT, 2014). Other evidence for a possible specific regulatory cascade activated by the chewing sounds was suggested by an increase of the benzoic acid levels after 12h of the treatment (**Fig. 8A**), which acts as a substrate for increasing the SA levels (**Fig. 2** and **3**).

Expression gene regulations also were activated specifically for the chewing sound signals. During the response to the insect attack, the soybean plants produces molecules and toxins that interact directly with the insects, causing damage to their development, called antibiosis (GÓMEZ et al., 2020; MAFFEI; MITHÖFER; BOLAND, 2007). These include Kunitz family protease inhibitors (SKTI) and Bowman-Birk (BBI) serine protease inhibitor, which are JA-induced trypsin inhibitors (PI), which reduce the absorption of amino acids in the insect gut. Accordingly, both *BBI* and *SKT*I genes were responsive specifically to chewing sound signals (**Fig. 10**); however, the variation in the *BBI* and *SKTI* expression did not correlate with the unchanged. JA levels have been shown to be increased under infestation; yet both resistant and susceptible soybean genotypes display similar JA levels in response to infestation and thus the insect resistance of this genotype is JA-independent (GÓMEZ et al., 2020). Despite this observation, the *BBI* gene was highly up-regulated in the IAC 17 (Pinheiro et al., 2021), which indicated that induction of PI biosynthesis may also be induced by other phytohormones such as SA and ethylene (ET) acting in synergism (NGUYEN et al., 2016). Absence of changes in the LOX and protease inhibition activities and JA levels in the soybean leaves under chewing sounds may be attributed to the reduced exposure times. In fact, Ghosh et al., 2016 observed that the levels of JA from *A. thaliana* leaves were increased only after 48 hours of sound treatment.

Pathogenesis-related (PR) proteins have been well defined as plant proteins that are induced not only during pathogen infection but also in response to abiotic stresses, including wounding, drought and high salinity. PR10/Bet v 1-like proteins have been involved in the production of plant phenolics including flavonoids, which have also been implicated as determinant of the soybean resistance to insect attack (GÓMEZ et al., 2020). Accordingly, *gmPR10/Bet v-1* gene expression was significantly induced in soybean plants under chewing sound signals. Takeuchi et al. (2011) have indicated a complex cascade regulation of *gmPR10/Bet v-1* expression involving JA, ET and SA. In fact, SA and ACC were the most responsive phytohormones in the soybean leaves under chewing sounds treatment.

Genes involved in the glucoconjugate flavonol biosynthesis, such as Flavonol Synthase (*FLS1)* and methyltransferase (*SOMT-9* and *Flavonol 3-O-methyltransferase*), heve been shown to be highly induced by insect attack (KIM et al., 2021). These authors also verified in resistant genotypes higher levels of flavonol glycosylated, indicating that FLS activity can intensely regulate the metabolic flow towards flavonols or anthocyanin biosynthesis. In fact, expression levels of *gmFLS1* gene were highly induced in response to chewing sound signals and did not responsive to forest sound signals (**Fig. 10**). Thus, these results are in accordance with metabolomic data, indicating a specific signaling cascades for chewing sounds inducing differently the flavone and flavonol pathway.

Plants has evolved to perceive a myriad of external stimuli which have been essential to their successful survival. Plants responses to environmental factors such as light, temperature, moisture, and mechanical perturbations have been broadly characterized physiologically as well as at the genetic and molecular levels. Despite of SVs as a stimulus have only started receiving attention relatively recently (JUNG et al., 2018; MISHRA; GHOSH; BAE, 2016), it is evident that the plants trigger cascades affecting the metabolites profiles and gene expression. Here, we also observed these changes and indicated that the cascades may be specific for the applied frequencies. Furthermore, we observed that physiological and metabolic response may also be used to induce specific defense mechanisms according to environmental stress generating by the sound waves. Soybean plants under chewing sound signals showed some responses similar to the caterpillar attack. As the cascades responsive to chewing sounds appear did not involve LOX and JA, other point was used to converge both signals of chewing with the molecular elicitors produced by herbivory during insect attack. In fact, the overlapping between biotic and abiotic signaling cascades have been potentialized some similar response of the plant to environmental stresses (DE MELO et al., 2020; FAUSTINO et al., 2021), such as drought and herbivory.

It has been well-known that mechanical stimulus, which alters the physiology of plants at various levels, is touch and repetitive touch treatment may alter the plant defense against fungal pathogen and herbivore (CHEHAB et al., 2012). Otherwise, SV and touch share some common mechanosensitive (MS) signaling events, as many of the touch-regulated genes were induced by SV (GHOSH et al., 2017). These genes including transcription factors and receptors kinases that were common in touch- and SV-mediated induction; thereby, reinforcing the hypothesis of the existence of crosstalk between the signaling cascades triggered by chewing sounds and the regulation of the responses of defense against insect attack.

Because of the positive effects it produces in several growth parameters of plants, SV treatments have been extensively used in biotechnology and agriculture (MISHRA; GHOSH; BAE, 2016). In the present study, we also verified significative changes in the relative abundances of phenolic compounds including flavonoids, when the plants were grown under both chewing and forest sound treatment. Flavonoids are an important class of antioxidants that play a crucial role in eliminating toxic ROS, protecting both animal and plant cells (CHOI et al., 2013). Increase in the flavonoid over 88% of the sprouts has been be considered an additional advantage, because the increase in flavonoids, as an antioxidant, is expected to improve the overall antioxidative capacities of plants used as food (KIM et al., 2021).

Studies that decipher the ecological relevance of responses triggered by sound vibrations have only recently made some advances (MISHRA; GHOSH; BAE, 2016). As suggested by De Luca et al., (2016), buzz pollination has evolved to allow plants to distinguish between pollen thieves and true pollinators through the appropriate/selective buzz frequency produced by bees (DE LUCA; VALLEJO-MARÍN, 2013); and plants can respond to the chewing sound by producing molecules that are stimulated when plants are attacked by herbivores(APPEL; COCROFT, 2014). Therefore, the divergences in metabolic and gene expression adjustments between chewing sound and forest sound treatments, along with the consistency of the chewing sound response with herbivore attack stimuli reinforce the hypothesis that ecological sounds can induce specific signaling pathways used by plants to process information from the environment.

Finally, based on our results we propose that acoustic chewing signals may be acting as a first line of defense against herbivory. Although the chewing sound and the forest sound shared similarities in the expression of molecules responsive to biotic and abiotic stresses, the differences in metabolic adjustments between the two sound signals were more striking and accentuated. These observations are consistent with the proposition that the type of SV-triggered signaling cascade is dependent on the frequencies that characterize the sound. It was demonstrated that the chewing sound induced regulatory cascades similar to those triggered by an herbivore, such as: SA-dependent herbivory signaling pathway, increase in the PPO activity, up regulation in the biosynthesis of flavonols and genes involved in the biosynthesis and regulation of flavonoids and flavonols. In contrast these metabolic adjustments were not induced by the forest sound, showing that ecological sounds can serve as an important long-range signaling capable of transmitting alerts and habitat information. However, studies on the acoustics of plant-herbivore interactions need to be expanded to other plant-insect systems to reveal their wide applicability, magnitude and ecological relevance.

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SUPPLEMENTAL FIGURES AND TABLES

Figure S1. Acoustic treatment boxes equipped with a lighting and audio reproduction system.

Table S1. Metaboanalyst input: XIC area values of phytohormones, polyamines and proline for the 12- and 18-hour sound treatments biological repetitions. Metabolites with undetermined relative abundance and low area values (threshold signal) were excluded and not used in the normalization and statistical modeling procedures.

Sample	C1T1	C2T1	C3T1	C4T1	S1T1	S2T1	S3T1	S4T1
Label	Cont_12H	Cont_12H	Cont_12H	Cont_12H	CS_12H	CS_12H	CS_12H	CS_12H
Jasmonic Acid	43	18	27	23	19	18	23	26
ABA	106	100	100	93	116	99	75	80
Salicylic acid	16856	12941	19875	19113	20704	11252	18835	22106
ACC	69	137	91	157	137	72	139	61
trans-Zeatin	140	131	126	122	109	174	155	152
Methyl jasmonate	83	74	82	87	65	74	80	80
Proline	3995	4580	5259	5148	3842	3348	3797	4130
Spermine	229101	230697	233818	239198	203397	224086	201916	224748
Spermidine	1375	2038	1000	1826	1475	1884	1546	1617
Putrescine	26	33	55	63	50	29	14	26

Forest sound - 12H

Sample	C1T1	C2T1	C3T1	C4T1	S1T1	S2T1	S3T1	S4T1
Label	Cont_12H	Cont_12H	Cont_12H	Cont_12H	FS_12H	FS_12H	FS_12H	FS_12H
Jasmonic acid	162	240	183	109	127	106	104	131
ABA	813	739	707	895	921	822	969	1197
Salicylic acid	73704	64658	57548	32846	39860	38482	50890	49709
IAA	41	52	41	33	49	48	62	56
ACC	219	293	117	245	427	501	148	292
trans-Zeatin	930	955	1229	288	1007	980	1302	1040
Proline	3306	4516	3673	4908	4141	5298	3976	3023
Spermine	255580	210000	198013	249407	250625	231921	239393	246079
Spermidine	749	1106	1047	1083	765	327	500	388

Chewing sound - 18H

Sample	C1T2	C2T2	C3T2	C4T2	S1T2	S2T2	S3T2	S4T2
Label	Cont_18H	Cont_18H	Cont_18H	Cont_18H	CS_18H	CS_18H	CS_18H	CS_18H
Jasmonic Acid	21	44	26	29	26	17	11	24
Abscisic acid	99	104	100	96	100	87	105	91
Salicylic acid	13115	18155	19311	16919	21914	19375	18701	19455
ACC	76	66	64	92	54	45	37	72
trans-Zeatin	133	92	103	95	117	140	138	134
Methyl jasmonate	69	69	79	77	70	68	79	74
Proline	3588	3932	3876	3833	4205	4406	3970	4223
Spermine	257832	200895	240082	240634	223637	261246	276420	233290
Spermidine	1636	1560	1540	1867	1980	2030	1209	2110
Putrescine	39	53	40	17	20	33	26	29

Forest sound - 18H

Sample	C1T2	C2T2	C3T2	C4T2	S1T2	S2T2	S3T2	S4T2
Label	Cont_18H	Cont_18H	Cont_18H	Cont_18H	FS_18H	FS_18H	FS_18H	FS_18H
Jasmonic acid	88	280	135	196	147	236	429	162
ABA	940	1163	1120	745	1439	729	1252	1127
Salicylic acid	24747	48435	69864	58798	41248	39438	46562	41161

AIA	47	29	51	67	64	50	53	56
ACC	135	163	295	67	512	332	177	684
trans-Zeatin	1238	1262	346	905	1164	1364	1169	1103
Proline	3674	3429	3769	3531	4652	5104	3071	3418
Spermine	301643	218643	334571	210519	227287	290271	229928	198916
Spermidine	801	457	676	641	811	1156	1400	1052

Table S2. Metaboanalyst input: XIC area values of phenolic compounds and flavonoids for the 12- and 18-hours sound treatments biological repetitions. Metabolites with undetermined relative abundance and low area values (threshold signal) were excluded and not used in the normalization and statistical modeling procedures.

Chewing sound - 12H

Sample	Cont_1	Cont_2	Cont_3	Cont_4	CS_1	CS_2	CS_3	CS_4
Label	Cont_12H	Cont_12H	Cont_12H	Cont_12H	CS_12H	CS_12H	CS_12H	CS_12H
Coniferyl aldehyde	253	231	319	220	409	355	353	349
Benzoic acid	8	9	10	14	23	18	25	18
Caffeate	398	481	693	380	614	574	716	680
Coumarin	4862	2734	3318	2774	3441	3538	3496	4000
Ferulate	73	82	85	70	93	54	89	51
Genistein	3385	3477	3894	3243	3198	3353	3455	3336
Isoferulic acid	232	278	318	261	208	208	214	219
p-Coumaric acid	1042	633	795	1312	535	610	396	1262
Vanillin	18	11	11	16	20	17	17	19
3,5-Dihydroxybenzoic acid	94	82	74	61	82	56	110	60
Hesperidin	159	101	102	71	142	101	107	102
Rutin	1823	1629	2165	2608	3134	2657	3540	2055
Naringin	154	136	203	205	110	139	87	173
Homoorientin	3198	919	831	569	602	653	719	671
Saponaretin	1193	276	258	295	372	381	296	278
Myricetin	74	60	87	39	82	61	61	63
Hesperetin	1106	998	971	952	1214	961	1119	932
Kaempferol	1625	1517	1463	1238	1551	1527	1795	1506
Luteolin	7017	6402	5878	7079	3491	5467	6329	3581
Phloretin	253	239	211	201	199	204	238	292
Naringenin	2021	2121	1885	2108	1896	2132	1942	2033
Apigenin	133653	110714	77241	112070	211386	100087	122113	110007
Daidzein	21868	27299	30467	28958	33579	16621	25533	25631
Quercetin	679	998	971	728	1214	975	1119	1138

Forest sound - 12H

Sample	Cont_1	Cont_2	Cont_3	Cont_4	FS_1	FS_2	FS_3	FS_4
Label	Cont_12H	Cont_12H	Cont_12H	Cont_12H	FS_12H	FS_12H	FS_12H	FS_12H
Coniferyl aldehyde	514	553	471	398	272	342	349	310
Benzoic acid	30	43	55	37	77	20	74	45
Catechol	69	68	69	67	67	66	66	66

Coumarin	47145	33180	41359	43171	34488	32091	31743	32343
Genistein	2484	2374	2350	2362	2019	1750	2324	2016
Neochlorogenic acid	65	64	66	59	62	60	71	61
p-Coumaric acid	1466	1262	1021	960	1118	1316	1577	1292
trans-Cinnamate	92	89	90	90	95	96	92	95
3,5-Dihydroxybenzoic acid	17	21	30	25	39	19	22	45
Morin	105	247	190	162	194	187	168	157
Hesperetin	171	183	190	175	244	207	214	268
Quercetin	283	247	257	197	262	231	268	256
Kaempferol	246004	214007	201924	236929	173477	195354	213953	207510
Luteolin	1040886	989453	1022794	1158545	1013164	1010743	1007516	1072212
Phloretin	4754	3788	4880	4102	4388	4149	4579	4442
Naringenin	334	500	420	436	199	176	263	182
Apigenin	45040	43257	35694	53401	29511	31972	35718	33650
Daidzein	3280	2374	1470	1912	2566	2553	4281	3823
	Chewing sound - 18H							
Sample	Cont_1	Cont_2	Cont_3	Cont_4	CS_1	CS_2	CS_3	CS_4
Label	Cont_18H	Cont_18H	Cont_18H	Cont_18H	CS_18H	CS_18H	CS_18H	CS_18H
Coniferyl aldehyde	351	320	324	350	280	320	345	377
Benzoic acid	19	13	17	21	8	5	4	8
Caffeate	576	605	319	620	602	617	568	635
Coumarin	3695	3737	4778	4788	4835	5115	4436	5192
Ferulate	101	131	108	101	150	189	161	162
Genistein	3186	3979	3270	3468	3513	3537	3005	3059
Isoferulic acid	258	242	219	243	463	392	299	368
p-Coumaric acid	1271	3400	2273	1147	2514	1390	1127	1090
Vanillin	17	17	14	15	13	9	11	6
3,5-Dihydroxybenzoic acid	87	156	80	73	137	107	103	100
Hesperidin	93	91	110	66	101	112	112	90
Rutin	3397	3086	2335	2089	2146	2955	2686	2531
Naringin	148	156	97	156	168	125	154	183
Homoorientin	841	718	686	706	736	747	756	661
Saponaretin	261	212	346	277	463	316	411	338
Myricetin	45	55	60	54	82	149	141	121
Hesperetin	945	1148	1164	1075	962	968	929	762
Kaempferol	2083	1959	2145	1893	1749	2028	1717	1608
Luteolin	7889	4213	5375	3492	7832	6725	8051	5934
Phloretin	259	294	266	234	252	296	292	247
Naringenin	2276	2380	2029	2015	2014	1984	2240	1695
Apigenin	94901	209944	253648	240567	138403	104011	117741	90134
Daidzein	29413	25822	34045	24864	24976	23840	36771	27076
Quercetin	1150	1148	1142	1197	962	968	904	1058
	Forest sound - 18H							
Sample	Cont_1	Cont_2	Cont_3	Cont_4	FS_1	FS_2	FS_3	FS_4

Label	Cont_18H	Cont_18H	Cont_18H	Cont_18H	FS_18H	FS_18H	FS_18H	FS_18H
Coniferyl aldehyde	306	394	378	348	399	337	365	363
Benzoic acid	46	27	29	36	28	17	56	50
Catechol	69	69	65	67	68	67	68	64
Coumarin	46913	43223	44766	45177	51035	35016	36817	41807
Daidzein	2280	2618	841	1968	736	1283	856	435
Genistein	2282	2618	2296	2209	2047	2258	2550	2453
Naringenin	820	1166	1294	1600	739	965	753	769
Neochlorogenic acid	62	69	61	69	67	62	66	71
p-Coumaric acid	1449	1028	1040	1235	911	754	791	882
trans-Cinnamate	93	87	88	89	94	90	88	92
3,5-dihydroxybenzoic acid	24	32	18	10	27	19	29	20
Morin	240	192	209	222	132	156	106	103
Hesperetin	217	242	225	244	184	156	84	97
Quercetin	240	254	242	281	194	186	187	167
Kaempferol	218010	226020	237477	210929	157095	192823	241014	184131
Luteolin	1165321	1164928	1159633	1202017	976177	1051767	1180285	1073910
Phloretin	4051	4777	4685	4340	4848	5679	5980	4528
Apigenin	49164	50559	45786	41321	38590	40465	55524	47758