UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO – UFRPE
PRÓ-REITORIA DE PÓS-GRADUAÇÃO - PRPG
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE – PPGBio

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(JESSE ELIAS MARINHO DE OLIVEIRA)

EFEITO DOS ELICITORES METIL JASMONATO E ÁCIDO SALICÍLICO NAS RESPOSTAS BIOMÉTRICAS, BIOQUÍMICAS, TEOR E PERFIL DO ÓLEO ESSENCIAL DE *Lippia alba* (VERBENACEAE) SOB RESTRIÇÃO HÍDRICA

RECIFE

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Trabalho de dissertação apresentado ao Programa de Pós-graduação em Biodiversidade (PPGBio/UFRPE) como requisito para obtenção do título de Mestre em Biodiversidade.

RECIFE

2024

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema Integrado de Bibliotecas da UFRPE
Biblioteca Central, Recife-PE, Brasil

O48e Oliveira, Jessie Marinho de
Efeitos dos elicitores metil jasmonato e ácido salicílico nas respostas biométricas, bioquímicas teor e perfil do óleo essencial de *Lippia alba* (Verbenaceae) sob restrição hídrica / Jessie Marinho de Oliveira. – 2024.
75 f.: il.

Orientador: Marcus Vinícius Loss Sperandio.
Dissertação (Mestrado) – Universidade Federal Rural de Pernambuco, Programa de Pós-Graduação em Biodiversidade Neotropical, Recife, BR-PE, 2024.
Inclui bibliografia e apêndice(s).

1. Plantas medicinais 2. Metabolismo 3. Estresse oxidativo 4. Essências e óleos essenciais I. Sperandio, Marcus Vinícius Loss, orient. II. Título

CDD 333.95

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AGRADECIMENTOS

Agradeço primeiramente a Deus, ao qual me apeguei nos momentos difíceis durante a execução deste trabalho. Agradeço também aos meus amigos e familiares que me incentivaram e apoiaram durante todo o percurso do Mestrado, em especial, minha mãe Maria da Conceição e minha amiga-irmã Juliana Oliveira, sem vocês, provavelmente eu teria desistido em algum momento da caminhada. Agradeço também ao meu companheiro de vida, Luan Villardo, por toda paciência, compreensão e suporte. Você foi essencial para que eu conseguisse finalizar este trabalho. Obrigada por acreditar no meu potencial.

Gostaria de agradecer aos meus professores da educação básica que me moldaram para tal momento e aos meus professores do ensino superior que despertaram em mim a vontade de ingressar no mundo acadêmico.

Agradeço imensamente ao meu professor Orientador Marcus Sperandio e ao meu coorientador Marcilio Moraes, pela parceria e paciência, serei eternamente grata.

Agradeço aos alunos de graduação e pós graduação do Laboratório de Fisiologia de Plantas do Departamento de Biologia da UFRPE que me auxiliaram em diversos momentos durante a pesquisa, sem vocês teria sido ainda mais difícil. Agradeço a professora Cláudia, Flávia e ao professor Marcus, ao Matheus, Larisse, Henarmmany, Francismary, Eduarda, Ana, Bruna, Esdras, Lucas, Layla, Manuela, Marina, Andressa, Sabrina e Yasmim, em especial, agradeço a Mirella Silva, pela parceria durante todo o meu projeto. Agradeço igualmente aos alunos do Laboratório de Produtos Naturais Bioativos do Departamento de Química da UFRPE por todo auxílio a mim prestado.

Agradeço ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela bolsa de estudos concedida.

Por fim, agradeço a mim mesma, por ter continuado em meio às dificuldades de saúde e instabilidades emocionais. Consegui me apegar no objetivo maior e estou orgulhosa de mim mesma.

SUMÁRIO

RESUMO

Lippia alba é uma planta de grande interesse farmacológico por possuir propriedades antimicrobianas, calmantes, antioxidantes entre outras. A restrição hídrica (RH) já conseguiu modular positivamente o Óleo Essencial desta planta. Elicitores são fatores, moléculas ou agentes capazes de estimular o sistema de defesa das plantas ao serem incorporados por elas, podendo causar modulações na produção de compostos bioativos. Este trabalho busca investigar as respostas das plantas eliciadas por Ácido Salicílico (AS) e Metil Jasmonato (MeJA), dois fitohormônios, de forma combinada, associadas a RH. Assim, em casa de vegetação, plantas de *L. alba* foram submetidas a cinco tratamentos de elicitação submetidas aos seguintes regimes hídricos: 70% (Controle) e 10% (Restrição Hídrica severa) da capacidade do vaso: T1: Controle (Água destilada); T2: Controle etanol 1%; T3: 2 mM de MeJA; T4: 4 mM de AS; T5: 2 mM de MeJA + 4 mM AS, todos os primeiros tratamentos foram regados com 70% da Capacidade de Pote. Os demais tratamentos foram submetidos à RH: T6: Controle (Água destilada); T7: Controle etanol 1%; T8: 2 mM de MeJA; T9: 4 mM de AS; T10: 2 mM de MeJA + 4 mM AS. Foram avaliados aspectos biométricos, bioquímicos, teor e perfil de óleo essencial. Observamos que os tratamentos controles não diferiram estatisticamente entre si nos parâmetros avaliados. Dos resultados encontrados os melhores desempenhos foram encontrados nos tratamentos submetidos à RH elicitados com MeJA+AS e MeJA, respectivamente. A elicitação combinada foi capaz de melhorar o teor de clorofila *a*, de carotenoides e o teor de óleo essencial de *L. alba.* Já o MeJA foi capaz de aumentar os níveis de clorofila *b*, clorofilas totais e teor relativo de água. AS não destacou-se positivamente em condições de RH. Recomendamos a utilização da elicitação combinada de AS e MeJA submetidas à RH, bem como a elicitação por MeJa, dada a eficácia no aumento dos parâmetros supracitados. Não sugerimos combinar AS com RH dado o efeito negativo nos parâmetros acima mencionados.

PALAVRAS-CHAVE: Planta medicinal. Metabolismo secundário. Déficit hídrico. Estresse antioxidante.

ABSTRACT

Lippia alba is a plant of great pharmacological interest as it has antimicrobial, calming, antioxidant properties, among others. Water restriction (WR) has already managed to positively modulate the Essential Oil of this plant. Elicitors are factors, molecules or agents capable of stimulating the defense system of plants when incorporated by them, which can cause modulations in the production of bioactive compounds. This work seeks to investigate plant responses elicited by Salicylic Acid (SA) and Methyl Jasmonate (MeJA) in combination, associated with WR. Thus, in a greenhouse, *L. alba* plants were subjected to five elicitation treatments: Control, 1% ethanol Control, 2 mM MeJA, 4 mM SA and 2 mM MeJA + 4 mM SA and subjected to the following regimes water: 70% (Control) and 10% (Severe water restriction) of the vessel capacity. Biometric, biochemical aspects and essential oil content were evaluated. We observed that the control treatments did not differ statistically from each other in any aspect evaluated. Of the results found, the best performances were found in treatments subjected to WR elicited with MeJA+SA and MeJA, respectively. The combined elicitation was able to improve the chlorophyll *a* and carotenoid content and the essential oil content of *L. alba.* MeJA was able to increase the levels of chlorophyll *b*, total chlorophylls and relative water content. SA did not stand out positively in WR conditions. We recommend the use of cross-elicitation of SA and MeJA submitted to WR, as well as elicitation by MeJA, given the effectiveness in increasing the aforementioned parameters. We do not suggest combining SA with WR given the negative effect on the aforementioned parameters.

KEYWORDS: Medicinal plant. Secondary metabolism. Water deficit. Antioxidant stress.

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OBJETIVOS

Objetivo Geral

Avaliar os efeitos da elicitação por Ácido Salicílico e Metil Jasmonato, isolada e combinada, sob estresse hídrico, nos parâmetros bioquímicos e perfil do óleo essencial de *Lippia alba*.

Objetivos específicos

- ➢ Mensurar os parâmetros biométricos e fisiológicos de crescimento de plantas de *L. alba*;
- ➢ Examinar as respostas bioquímicas de plantas de *L. alba* sob influência de elicitores e estresse hídrico;
- ➢ Analisar o estresse oxidativo em plantas de *L. alba* gerado pelo estresse induzido por meio da dosagem das enzimas antioxidantes;
- ➢ Relacionar os parâmetros fisiológicos, morfológicos e bioquímicos de plantas de *L. alba* com a restrição hídrica e elicitação usando Análise de cluster hierárquico (HCA);
- ➢ Avaliar o teor e o perfil de óleo essencial de plantas de *L. alba* buscando entender as adaptações fisiológicas e as melhores condições dentre as que foram propostas.

HIPÓTESES

1. Metil Jasmonato e Ácido Salicílico são capazes de modular o perfil do óleo de *Lippia alba* sob estresse hídrico;
2. As plantas de *L. alba* submetidas aos elicitores Metil Jasmonato e Ácido Salicílico diferem do controle em respostas bioquímicas e biométricas quando submetidas ao estresse hídrico;
3. A elicitação combinada dos elicitores Metil Jasmonato e Ácido Salicílico difere da elicitação isolada.

FUNDAMENTAÇÃO TEÓRICA

CONHECENDO A *LIPPIA ALBA*

A família botânica Verbenaceae é composta por aproximadamente 98 gêneros e 2614 espécies de ervas, arbustos e subarbustos no mundo. Destes, ocorrem no Brasil cerca de 47 gêneros e 407 espécies, distribuídas em diversos domínios fitogeográficos como: Amazônia, Caatinga, Cerrado, Mata Atlântica e Pampa (Malik et al., 2021; Salimena et al., 2013). As espécies dessa família são usadas com diferentes finalidades, como por exemplo: religiosa, ornamental e para aquisição de madeira. Além de serem utilizadas como biorremediadoras e fitoterápicas, graças à presença de tricomas glandulares secretores capazes de produzir óleos essenciais com grande desempenho medicinal (Cordeiro, 2020; Melo et al., 2010).

A *Lippia alba* (Mill.) N.E.Br. ex P. Wilson (Fig. 1), também conhecida como erva-cidreira, carmelitana, cidreira-de-arbusto, cidreira-brava, erva-cidreira-brasileira, falsa-melissa, chá-de-tabuleiro, salva limão, alecrim-do-campo, entre outros, é uma espécie nativa do Brasil com ampla distribuição ao longo de todo país, ocorrendo principalmente na região do semiárido, também ocorrendo desde o sul dos Estados Unidos, América Central e do Sul e no continente africano (Froz et al. 2024). É uma espécie fortemente aromática, arbustiva e perene, podendo atingir até 3 m de altura. Possui ramos ascendentes, alongados, que ao longo do crescimento tornam-se arqueados e, ao tocar no chão, acabam enraizando. Os caules possuem seção transversal quadrangular, são pubescentes quando novos e glabros quando velhos. Isto é, possuem tricomas quando novos e os perdem à medida que envelhecem (Malik et al, 2021). A filotaxia é oposta-cruzada, com pequenas folhas de pecíolo curto, de cor verde a verde-acinzentadas, discolores, elípticas a ovadas, com borda serrada e superfície ligeiramente escabrosa na face adaxial e levemente pilosa e com nervuras proeminentes na face abaxial. As inflorescências são axilares, capituliformes, consideradas na literatura como glomérulos, comumente um por axila, com pequenas flores de corola violácea com fundo amarelo, marcadamente zigomorfa, ou seja, com simetria bilateral. Os frutos são drupas globosas de cor róseo-arroxeada (Malik et al, 2021; Camêlo et al, 2011).

Figura 1. Inflorescências de *Lippia alba* (Mill.) N.E.Br. ex P. Wilson**.** Fonte: Autora própria (2023).

A *L. alba* é uma das plantas mais utilizadas pela medicina tradicional brasileira e pela fitoterapia. As propriedades terapêuticas se concentram nas suas folhas, que são utilizadas em diversas preparações como: chás, compressas, banhos, macerados, inalações, extratos, xaropes e tinturas (Sales, et al., 2022; Kujawska & Schmeda-hirschmann, 2022; Camêlo et al, 2011). Estudos científicos identificaram propriedades antimicrobianas, antifúngicas, analgésicas, insecticidas, sedativas, relaxantes, ansiolíticas, anestésicas, antioxidantes, espasmolíticas, emenagogas e carminativas (Kujawska & Schmeda-hirschmann, 2022). É recomendada, sobretudo, no tratamento de problemas gastrointestinais e respiratórios, mas possui dezenas de aplicações dentro da medicina popular. Em algumas localidades da região Nordeste é ainda considerada como PANC, Planta Alimentícia Não Convencional, visto que suas raízes são saboreadas como aperitivo (Cordeiro, 2020).

Todas essas possíveis aplicações da *L. alba* são possíveis graças aos compostos presentes em seu Óleo Essencial (OE) que pode sofrer influências e consequentes modulações em quantidade e qualidade quando submetida a diferentes condições ambientais naturais, como por exemplos, clima, solo, disponibilidade hídrica, ou até mesmo, em condições induzidas, como no caso das elicitações (Palhares-neto et al. 2023). Dentre os principais compostos de seu óleo estão: Geraniol, Geranial, β-cariofileno, Germacreno D e Mirceno (Silva-santos et al., 2023). O geraniol, por exemplo, é um monoterpeno e um álcool, muito usado na produção de perfumes, como repelente de insetos, e está relacionado na biossíntese de outros terpenos (Ben ammar, 2023; Silva-santos et al., 2023).

Óleo Essencial da *L. alba*

Os Óleos Essenciais (OEs) são produtos do metabolismo secundário de plantas medicinais, ricos em compostos fenólicos que garantem a esses OEs diversas aplicabilidades, podendo ser farmacológicas, cosméticas, terapêuticas e médicas. O Óleo Essencial de *L. alba* é extraído das folhas dessa planta e possui várias propriedades e benefícios que são bastante valorizados em aromaterapia e medicina natural. Aqui estão alguns dos principais benefícios do uso dos compostos presentes no EO de *L. alba*: Propriedades Relaxantes e Ansiolíticas; Ação Antibacteriana e Antifúngica; Propriedades Anti-inflamatórias; Efeitos Analgésicos; Benefícios para o Sono; Propriedades Digestivas; Ação Antioxidante (Malik et al. 2021); Ações antitumorais, como o câncer de mama (Hedhili 2024); Repelente natural como prevenção para arboviroses: dengue, zika e chikungunya (com ação sob o *Aedes aegypti*) e tratamento de Covid 19 pela inibição do SARS-CoV-2 (Yamari et al. 2024)

O EO de *L. alba* pode apresentar várias características diferentes dependendo das condições em que as plantas forem submetidas, como diferentes condições climáticas, estágios de disponibilidade hídrica, condições nutricionais, processos de herbivoria e até mesmo condições induzidas como o uso de primers e elicitores (Palhares-Neto et al. 2023; De Souza Silva et al. 2022). Essas variações quantitativas e qualitativas na composição química do óleo essencial de *L. alba* têm sido amplamente comprovadas, e levaram à classificação da espécie em diferentes quimiotipos de acordo com seus principais constituintes, sendo os mais descritos na literatura o citral, a carvona e o linalol (Júnior et al.2024). Entretanto, ocorrem também 1,8-cineol, mirceno e β-elemeno. Na Amazônia, há relato dos quimiotipos citral, carvona-limoneno, mirceno-citral, citral-carvona-limoneno, limoneno-1,8-cineol, carvona-limoneno-germacreno D e citral-germacreno D (Júnior et al. 2024). O quimiotipo usado para este trabalho foi o Geranial-geraniol, o mesmo usado por Santos-silva et al. 2023.

O Geranial e o Geraniol, por exemplo, são compostos encontrados no óleo essencial de *L. alba*, tendo propriedades principalmente antimicrobianas e antifúngicas. Ele é usado em produtos de cuidados pessoais e empregado em repelentes de insetos e em produtos farmacêuticos devido à essas qualidades antibacterianas e antifúngicas. Além de ser usado como aromatizante em alimentos e bebidas. Ele também atua como um atrativo para insetos, o que pode ajudar nas interações entre plantas e polinizadores, tendo um importante serviço ecossistêmico. Esses compostos foram descritos ativando respostas antitumorais com diversos tipos de tumores, apoptose, morte celular autofágica, citostase e necrose (Santos e Silva et al. 2022) e potencial cardioprotetor (Zou et al. 2022).

O citral, por sua vez, tem atividade anti-helmintos, tripanocida e imunomodulador, vasorrelaxante, antioxidante, antimicrobiano e repelente (Barbosa et al. 2023; Quintero et al. 2021; Borges et al. 2022; Filho et al. 2023; de Brito et al, 2021). Já o Eugenol tem ação anestésica (de Lima et al. 2021), bem como o Linalol (Postay 2021) e 1,8-cineol tem ação inseticida (Lima et al. 2021).

O EO de *L. alba* possui diversidade de compostos que pode chegar a ultrapassar 80 componentes diferentes em um único perfil (Santos-silva et al. 2023), essa variedade bioquímica confere à espécie o leque amplo de aplicabilidade supracitado, pois cada componente exerce uma função biológica diferente.

Restrição hídrica: condição capaz de modular o metabolismo primário e secundário de plantas como a *L. alba*

A água desempenha papel fundamental para a manutenção da vida, seja ela animal, vegetal, micro ou macroscópica. Ela está envolvida em processos fisiológicos essenciais para a manutenção da vida vegetal como por exemplo a fotossíntese e o transporte e absorção de nutrientes (Pokhrel et al, 2021; Wahab et al, 2022). É considerada um importante constituinte pois representa cerca de 90% da biomassa fresca dos vegetais, tornando-a importante para a manutenção da integridade funcional de células, tecidos, órgãos e organismos (De moraes campos, 2021).

O estresse por déficit hídrico pode causar múltiplas alterações morfofisiológicas e bioquímicas que afetam adversamente o desenvolvimento e a produtividade da planta (Ullah et al. 2018). As plantas podem frequentemente resistir a condições hídricas limitadas, mas ao custo de perdas substanciais na biomassa total e na produtividade (Yahaya & Shimelis, 2022; Pepe et al, 2022). A fotossíntese, o crescimento e outras atividades fisiológicas e bioquímicas críticas são interrompidas sob condições de estresse hídrico (Muhammad aslam et al, 2022; Mcdowell et al, 2022; Pepe et al, 2022). Estudos anteriores descobriram que o estresse hídrico causa estresse oxidativo, danificando membranas biológicas e macromoléculas como DNA, proteínas, lipídios e pigmentos fotossintetizantes (Zandi & Schnug et al, 2022; Sofy et al, 2021; Perveen & Hussain et al, 2021). As respostas das plantas ao estresse hídrico também foram observadas nos níveis transcriptômico e metabolômico (You et al, 2019; Bogati & Walczak, 2022).

O Ácido Abscísico (ABA) é um hormônio vegetal que desempenha um papel crucial na regulação do crescimento e desenvolvimento das plantas, especialmente em resposta a condições de estresse ambiental, como a restrição hídrica (Brookbank et al, 2021). Ele é sintetizado em várias partes das plantas, incluindo raízes, folhas verdes e frutos. O ABA desempenha vários papeis durante esses períodos, ajudando as plantas a conservar água e minimizar os efeitos da escassez hídrica. Algumas das funções do ABA em situações de estresse hídrico incluem: fechamento estomático, promoção da dormência de gemas e sementes, aumento da tolerância à desidratação e modulação do desenvolvimento radicular (Brookbank et al, 2021; Ali et al, 2022; Sano & Marion-poll, 2021).

Além do ABA, outros hormônios têm participação nas respostas que plantas desenvolvem em condições de seca. Estudos com aplicação exógena dos hormônios Ácido Salicílico (AS) e Metil Jasmonato (MeJA), de forma isolada e combinada, conferem efeitos positivos sobre os aspectos de crescimento e desenvolvimento de plantas frente à seca, como o aumento de pigmentos fotossintetizantes, teor de água, aumento da atividade antioxidante, aumento da estabilidade de membrana e em casos de plantas medicinais, aumento no teor do óleo essencial que conferem proteção aos danos causados pelo estresse oxidativo (Mohi-ud-din et al, 2021; Tayyab et al, 2020; Santos-silva et al, 2023).

A fotossíntese, por exemplo, é a principal fonte de energia para as plantas e pode ser afetada fortemente por estresses abióticos (Baslam & Sanz-saez, 2023). Os pigmentos fotossintetizantes, clorofilas *a* e *b,* desempenham papel importante na fotossíntese, sendo responsáveis pela captação de energia luminosa, destacando-se a clorofila *a* como o principal pigmento dos complexos coletores de luz para as reações fotoquímicas (Jogawat et al. 2021). A degradação das clorofilas é uma das consequências da deficiência hídrica, que pode levar à fotoinibição e diminuição da eficiência fotossintética, além de afetar outros processos como a divisão e expansão celular (Palhares neto et al, 2023; Azhar et al. 2021). Plantas submetidas a condições de seca têm duas vias de regução de condutância estomática, o controle hidráulico passivo e o controle hormonal ativo, por ABA principalmente (Chen et al. 2023). Quanto maior a condutância estomática, menor a perda de água, porém, menor é a taxa fotossintética. A diminuição da fotossíntese resulta em menor deposição dos estoques de carbono, ou seja, na aquisição de biomassa resultando em um retardo ou paralisação temporário ou não do crescimento e desenvolvimento vegetal. Além disso, em casos extremos, a redução do potencial hídrico no solo pode resultar em graves danos estruturais e até mesmo na morte da planta, já que a escassez hídrica causa aumento do estresse oxidativo, ou seja, quando o equilíbrio entre a defesa antioxidante e a produção de espécies reativas de oxigênio (EROs) entra em desequilíbrio. O acúmulo de EROs causa estresse oxidativo celular e pode gerar danos em lipídios de membrana, proteínas e outros componentes das células causando uma desorganização celular, muitas vezes incompatível com o reparo ou a manutenção da vida (Palhares neto et al, 2023; Jogawat et al. 2021).

Em outros casos de estresse hídrico, a planta sofre com redução de parte aérea, de número de folhas, de área foliar exposta, na produção de flores e frutos (Zaib et al. 2023), mas em contrapartida, aumenta o sistema radicular em busca de água no solo (Cao et al. 2023) e pode sofrer modulações a níveis de metabólitos secundários em plantas medicinais, como exemplo a *L. alba*, que já fora notificado que quando exposta a estresse hídrico aumenta o teor do seu óleo essencial e modula o perfil do mesmo (Palhares-neto et al. 2023).

Fazendo um recorte sociogeográfico, temos as regiões brasileiras de clima semiárido, como é o caso da nossa Floresta Tropical Sazonalmente Seca conhecida como Caatinga, caracterizada por altas temperaturas, baixa umidade relativa do ar e baixos índices de pluviosidade, tornando-a um ambiente desafiador para a manutenção de seres sésseis que dependem de recurso hídrico (De araújo, et al., 2022). Algumas plantas dispõem de mecanismos adaptativos às condições climáticas, como é o caso dos tricomas foliares da *L. alba*, que conferem um microclima na superfície foliar, minimizando os efeitos severos de uma clima árido (Santos-silva et al., 2023). Mas tendo em vista as últimas projeções climáticas sugeridas pelo IPCC (2022), segundo o qual o efeito estufa, a poluição e o aquecimento global resultarão no aumento da temperatura global em 1,5 Cº e todos os fatores correlacionados a este aumento, como por exemplo a mudança dos regimes pluviométricos, torna-se importante analisar para que se possa compreender as adaptações de espécies suscetíveis a tais condições (Pokhrel et al. 2021), a fim de mitigar projetos de conservação frente às mudanças climáticas.

Elicitação: Ferramenta capaz de modular aspectos do metabolismo primário e secundário de *L. alba*

Elicitores são fatores, moléculas ou agentes de origem física, química ou biológica capazes de estimular o sistema de defesa das plantas ao serem incorporados por elas, podendo causar modulações na produção de compostos bioativos (Nielsen et al., 2019). Essa técnica é usada em plantas medicinais visando o aumento dos metabólitos secundários do Óleo Essencial (De oliveira, et al., 2020), podendo promover uma maior eficiência na produção do OE de *L. alba*. Alguns dos fatores que são essenciais ao tipo de resposta que a planta-alvo terá são: o tipo do elicitor, concentração usada e tempo de exposição (Nabi et al. 2021). Os estímulos extracelulares geralmente são reconhecidos por receptores de membrana, que desencadeiam processos intracelulares capazes de alterar o crescimento, a bioquímica e até mesmo a atividade molecular da célula (Iula, et al., 2022). Elicitores também são estímulos pois tem a capacidade de se ligar a receptores celulares e modular o metabolismo vegetal. Para cada elicitor, existe um receptor específico e cada planta dispõe de um grupo seleto de receptores. Para ser capaz de desencadear uma cascata metabólica, cada receptor percebe seu elicitor em concentrações diferentes, relacionado ao nível de afinidade, quanto maior este nível, menores as concentrações necessárias para iniciar o processo sinalizador. O tempo de exposição é essencial, visto que, quando excedido, pode causar efeitos negativos na planta como visto em De Souza Silva et al., 2022.

Dentre os vários elicitores abióticos, temos o Metil Jasmonato e Ácido Salicílico. Metil Jasmonato (MeJa) é um fitormônio e regulador celular envolvido em diversos processos de desenvolvimento, como germinação de sementes, crescimento de raízes, fertilidade, amadurecimento de frutos e senescência. Além disso, é capaz de ativar os mecanismos de defesa da planta, como o aumento da atividade antioxidativa, em resposta a danos mecânicos causados por insetos, vários patógenos e estresses ambientais, como seca, baixa temperatura e salinidade (Jeyasri et al, 2023).

Semelhantemente, temos o Ácido Salicílico (AS), que forma parte de um amplo grupo de compostos sintetizados em plantas denominadas fenólicos. Os compostos fenólicos participam de muitas funções metabólicas em plantas, como a síntese de lignina, atividade alelopática, e em alguns casos na biossíntese de compostos relacionados à defesa como as fitoalexinas. O AS participa de processos como a germinação de sementes, crescimento celular, respiração, condutância estomática, expressão de genes associados à senescência e resposta a estresse abiótico, assim como na resistência a enfermidades (Rodríguez-sánchez, et al., 2022; Iula, et al., 2022; Singh & Dwivedi, 2018). Além disso, descreveu-se que em alguns casos o efeito do AS dentro do metabolismo das plantas pode agir de forma indireta na síntese e/ou sinalização de outros hormônios que incluem a via do ácido jasmônico, etileno e auxinas (Iula, et al., 2022; Singh & Dwivedi, 2018). MeJa e AS, portanto, estão relacionados com Fatores de Transcrição envolvidos na resposta à seca, como por exemplo: MYB, MYC, WRKY, DREB, bZIP e MPK. Sendo assim, capazes de modular o metabolismo secundário de plantas submetidas a essas condições, por respostas moleculares. Alguns trabalhos já testam combinações entre elicitores para avaliação de atividade cinestésica benéfica (Abdollahi et al, 2023; Chayjarung et al, 2022; Arya et al, 2022).

Os Ácidos Jasmônicos (JA), incluso o Metil Jasmonato, desempenham um papel significativo na sinalização das respostas antioxidantes induzidas pelo estresse hídrico, via metabolismo do ascorbato (Mohi-ud-din et al, 2021; Tayyab et al, 2020). A aplicação exógena de JA foi considerada eficaz no fornecimento de proteção contra o estresse oxidativo induzido pela seca no melão (*Cucumis melo*), aumentando a atividade de enzimas antioxidantes (Nafie et al, 2011), além de, conseguir aliviar os efeitos nocivos do estresse hídrico, aumentando o teor total de proteínas, prolina, carboidratos e atividades antioxidantes em sementes de milho tratadas com MeJA (Abdelgawad et al, 2014). O Ácido Salicílico (AS), regula positivamente a ação de enzimas antioxidantes, proteínas de choque térmico e vários outros produtos genéticos envolvidos no metabolismo de metabólitos secundários (Tayyab et al, 2020; Mohi-ud-din et al, 2021; Miura & Tada, 2014). Fornece proteção às plantas contra estresses abióticos pela regulação de processos fisiológicos importantes, incluindo fotossíntese, metabolismo de nitrogênio e prolina, sistema de defesa antioxidante e respostas das plantas ao déficit hídrico (Tayyab et al, 2020; Miura & Tada, 2014; Nazar et al, 2011). A aplicação exógena desses dois hormônios juntos em mostarda (Alam et al, 2013; Alam et al, 2014), gramíneas (Shyu & Brutnell, 2015), *Jatropha* (SOARES et al, 2016), *Verbascum* (Karamian et al, 2020) e milho (Tayyab et al, 2020) demonstrou proteger as plantas contra estresses abióticos, incluindo seca, por regulando importantes processos fisiológicos que vão desde a fotossíntese até o metabolismo do nitrogênio e da prolina e ativando o sistema de defesa antioxidante.

Ambos os elicitores desempenham papeis fundamentais na eliminação de espécies reativas de oxigênio (ROS), sendo capazes de melhorar a atividade das enzimas antioxidantes e regular o ajuste osmótico, resultando em uma melhor relação com a água e atributos estomáticos (Jeyasri et al, 2023; Mohi-ud-din et al. 2021; Tayyab et al. 2020), além da ação comprovada na regulação da divisão e do alongamento celular, aceleração do metabolismo, promoção da biossíntese de celulose e melhora da produção de biomassa (Quamruzzaman et al. 2021; Singh & Dwivedi, 2018) e aumento do teor e modulação do Óleo Essencial de plantas medicinais como a *L. alba* (Santos-silva et al. 2023).

Analisando as informações acima mencionadas fica claro que a aplicação isolada destes dois elicitores tem um enorme papel na defesa fisiológica das plantas. No entanto, alguns trabalhos testaram o efeito da aplicação combinada de AS e MeJA. A aplicação exógena desses dois fitohormônios juntos em *Verbascum* (Karamian, Ghasemlou & Amiri 2020) e milho (Tayyab et al, 2020) demonstrou proteger as plantas contra estresses abióticos, incluindo a seca, regulando importantes processos fisiológicos que vão da fotossíntese ao nitrogênio e metabolismo da prolina e ativação do sistema de defesa antioxidante. Também mitigou os danos oxidativos induzidos pela seca no feijão francês (*Phaseolus vulgaris* L.) (Mohi-ud-din et al. 2021). A aplicação combinada de AS e MeJA também é considerada uma nova abordagem para prolongar a vida útil de Romã Arils (El-beltagi, Al-otaibi, & Ali 2023); Esses dois elicitores melhoram sinergicamente os danos induzidos pela salinidade, mantendo o equilíbrio redox e o movimento estomático na Batata (Shekhar et al. 2023) e de acordo com Jiao et al. Em 2022, o seu sinergismo foi capaz de aumentar as emissões voláteis e as funções anti-herbívoras nas plantas medicinais.

Assim, tanto o estresse hídrico quanto a elicitação, individualmente e combinada, possuem capacidades modulatórias na fisiologia vegetal. O objetivo do presente trabalho foi avaliar se a elicitação combinada de AS e MeJA com restrição hídrica pode ser útil na produção de OE de *L. alba,* compostos ativos e garantindo a qualidade da matéria-prima produzida a partir desta espécie em experimento conduzido em casa de vegetação.

DEFESA SOCIAL

A defesa social implica na divulgação científica à comunidade extra-acadêmica, como forma de retribuição à sociedade pelo tempo de estudo realizado na educação superior pública. No ano de 2023 ocorreu a Feira de Profissões e Cursos Superiores da Universidade Federal Rural de Pernambuco realizada na Exposição de Animais de Recife. No dia 16 de novembro do mesmo ano, os integrantes do Laboratório de Fisiologia de Plantas - LFP/UFRPE ficaram responsáveis por apresentar uma técnica de produção sustentável de plantas: o Cultivo *in vitro*. A prática extensionista tinha por objetivo divulgar os estudos realizados pelo laboratório e estimular a adesão de alunos ingressantes ao curso de Ciências Biológicas da UFRPE. Durante todo o dia foram realizadas apresentações orais, com auxílio de material complementar como slides e banner, destinadas ao grande público visitante da Feira de Profissões. A produção de material, banner e marca-textos também foi realizada como forma de divulgação e explicação breve dos principais processos que envolvem o Cultivo *in vitro* de plantas. O trabalho desenvolvido foi supervisionado pelos professores responsáveis do laboratório: professor Dr. Marcus Vinicius Loss Sperandio e a professora doutora Cláudia Ulisses.

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MANUSCRIPT

Water restriction stress in *Lippia alba* (Verbenaceae) combined with salicylic acid and methyl jasmonate elicitation modulate oxidative stress and essential oil yield and composition

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Submissão para a revista: **Plant Physiology and Biochemistry**

Fator de impacto: 6.1

Print ISSN: 0981-9428 | Online ISSN: 1873-2690

Formatação:

https://www.sciencedirect.com/journal/plant-physiology-and-biochemistry/publish/guide-for-authors

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Plant Physiology and Biochemistry

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Acknowledgment

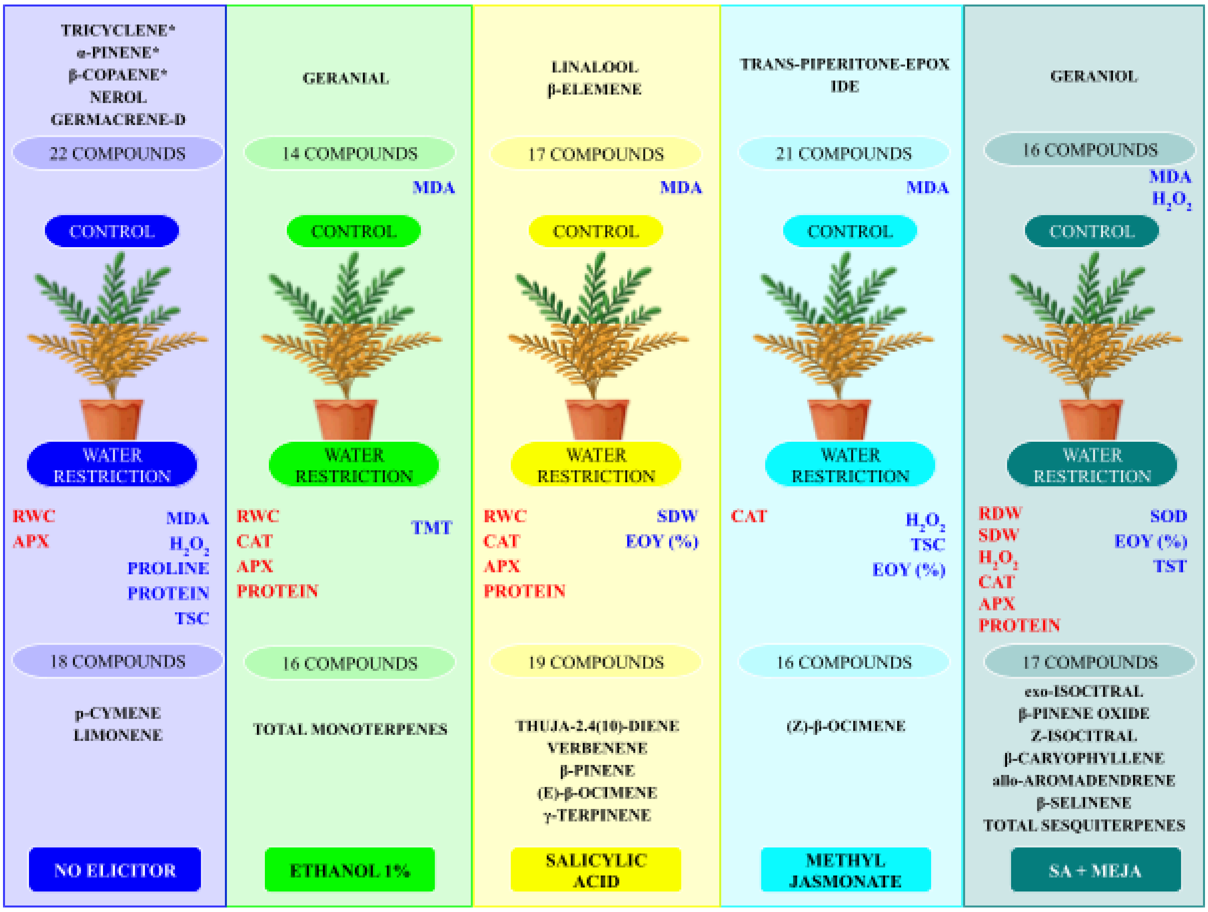
We would like to thank the National Council for Scientific and Technological Development (CNPq) for financial support.

Abstract

Water restriction (WR) is a common abiotic stress which can modulate plant growth and essential oil (EO) yield in *L. alba*. Elicitation is also an important approach to mitigate abiotic stress and modulate EO yield in *Lippia alba*, which has pharmacological interest through antimicrobial, calming, antioxidant properties, among others. This work aims to investigate the responses of *L. alba* elicited by Salicylic Acid (SA) and Methyl Jasmonate (MeJA) isolated and in combination, associated with WR, on morphological, biochemical and EO responses. *Lippia alba* plants were subjected to five elicitation treatments through foliar spray: no elicitor (water), 1% ethanol (used to dissolve SA), 2 mM of MeJA, 4 mM of SA and 2 mM MeJA + 4 mM SA. After 15 days, elicited *L. alba* were subjected to following water regimes for 15 days: 70% (control) and 10% (WR) of pot capacity. Elicitation with MeJA and combined SA and MeJA mitigated the WR effect on relative water content (RWC) compared to control. Elicitation also modulated malondialdehyde (MDA) and H_2O_2 under control and WR. By using Headspace-Gas Chromatography-Mass Spectrometry (HS/GC-MS) to analyze EO composition, the major EO compounds were Geraniol and Geraniol. The combined elicitation of SA and MeJA under WR presented the higher EO yield, increasing the sesquiterpene content. Overall, the isolated or combined elicitation with SA and MeJA had the potential to mitigate the WR effects in *L. alba*, however, the combined elicitation of SA and MeJA synergistically stimulated the secondary metabolism to increase EO yield.

Keywords: Secondary metabolism. Medicinal plant. Oxidative stress. HS/GC-MS.

Graphical Abstract - Growth, oxidative stress, biochemical parameters and essential oil yield and composition in leaves of *Lippia alba*



SA + MEJA- combined elicition with Salicylic Acid and Methyl Jasmonate; RWC- relative water content; RDW- root dry weight; LDW- leaves dry weight; SDW- stem dry weight;MDA- malondialdehyde; H_2O_2-hydrogen peroxide; CAT- catalase; APX- ascorbate peroxidase; TSC- total soluble carbohydrates; EOY (%)- essential oil yield; Parameters in red are signaling decrease in comparison to the control and parameters in blue are signaling increase also in comparison to the control. The compounds with (*) appears exclusively in its treatment. Compounds shown here are the major of their treatment.

1. Introduction

Water availability is essential for maintaining plant growth and physiology. Currently, the reduction in water availability in soils is considered one of the main abiotic stresses that affect plant growth, especially considering the acceleration of climate change (Pokhrel et al. 2021). Plants under water restriction (WR) tend to reduce their transpiration through stomatal regulation to control excessive water loss and delay the effects of osmotic stress. This mechanism negatively affects photosynthesis, reducing the assimilation of CO_2 and the production of primary metabolites important for biomass production (Azhar et al. 2021). Photosynthetic efficiency and damage caused to photosynthetic devices are also affected by excess reactive oxygen species (ROS) (Jogawat et al. 2021). Plant survival strategies under drought conditions encompass stress tolerance responses, such as increased efficiency of enzymatic and non-enzymatic antioxidant defense, accumulation of osmoregulatory molecules and anatomical changes, such as the density of stomata and trichomes (Suslov, Daminova & Egorov 2024; Ahmad et al. 2019). Furthermore, water restriction can influence the secondary metabolism of plants, affecting the production of EOs and thus playing fundamental roles in stress tolerance (Jogawat et al. 2021).

Lippia alba (Mill.) N.E.Br. ex P. Wilson, also known as lemon balm, shrub lemon balm, wild lemon balm, Brazilian lemon balm, Carmelitana and false melissa, is a species of the Verbenaceae family that occurs in the region semiarid, being native to Brazil. It occurs from the south of the United States to Argentina, comprising countries in South America, Central America and part of the African continent (Froz et al. 2024). It is an aromatic shrub and its leaves and stem are rich in glandular trichomes capable of producing and secreting essential oils (EOs) rich in secondary metabolites of economic interest widely used in the production of cosmetics and medicines such as myrcene, linalool, β-ocimene, α-guaiene, germacrene, carvone, limonene, eucalyptol, camphor, caryophyllene, neral, geranial and piperitone (Palhares-Neto et al. 2023). The EOs produced by *L. alba* have scientifically proven antimicrobial, antioxidant, analgesic and calming activities (Malik et al. 2021). It can also be involved in important disease treatment such as breast cancer (Hedhili 2024) and Covid-19 by inhibiting SARS-CoV-2 (Yamari et al. 2024). Essential oils can present different variations in their chemical composition when the plant is subjected to different environmental, geographic conditions and flowering time. These EOs are produced by the activation of plants' secondary metabolism, which can be influenced by factors associated with climate change, such as water stress; and external abiotic factors, which function as elicitors, such as methyl jasmonate and salicylic acid (Palhares-Neto et al. 2023; De Souza Silva et al. 2022).

In addition, we have elicitation, which consists of the use of substances of chemical, physical or biological origin capable of simulating a natural stressful condition, thus signaling and stimulating the biochemical cascade of plant defense against stress, being responsible also by modulating the content of these metabolites of economic interest in EOs (Maimone, 2020). Among the many elicitors, we have Salicylic Acid (SA), which is an endogenous small-molecule phenolic compound, also an important phytohormone that acts as a signal sensor to regulate plant response acting as a vital signaling molecule. It protects plant cells from the toxicity of ion accumulation and cell death by managing processes such as antioxidant defense by accumulation of antioxidant enzymes, nitrogen metabolism, proline metabolism, photosynthesis (Yang, Zhou & Chu, 2023), thus supporting the growth and development of plants under abiotic stresses such as drought, for example (Vázquez-Martínez et al. 2022).

Salicylic Acid positively regulates the action of antioxidant enzymes, heat shock proteins and several other genetic products involved in the metabolism of secondary metabolites (Tayyab et al. 2020; Mohi-Ud-Din et al. 2021). SA provides protection to plants against abiotic stresses by regulating important physiological processes, including photosynthesis, nitrogen and proline metabolism, antioxidant defense system, and plant responses to water deficit, all of those being important mechanisms that plants have to adjust their physiology (Tayyab et al, 2020). The profound impacts of SA on plant physiology suggest that it is likely to have many target proteins. Indeed, screens utilizing SA analogs in combination with protein arrays or crosslinking revealed nearly 100 SA-binding proteins (SABPs) (Spoel & Dong, 2024), which can explain the diversity of its functions.

Studies have shown that SA elicitation is also capable of increasing photosynthetic activity by increasing chlorophyll content, leaf water potential, membrane stability index, activities of antioxidant enzymes such as SOD, CAT and APX, in addition to helping to reduce electrolytes leakage and lipid peroxidation under water stress conditions (Zafar et al. 2021; El-Beltagi et al. 2022; Vázquez-Martínez et al. 2022). Its application also modulates and increases the EO compounds in *L. alba* plants (Silva-Santos et al. 2023) which also works as a tool against water stress. Therefore, the application of SA has been considered a short-term solution to reduce the negative effects of drought stress on plants.

Methyl Jasmonate (MeJA) is also used as elicitor to alleviate stress conditions, derived from jasmonic acid, which is a phytohormone involved in several plant functions, from morphophysiological to molecular level (Jeyasri et al. 2023; Kandoudi et al. 2021). Given its volatility and ability to diffuse between biological membranes, it is considered a signal capable of modulating defense responses, such as antioxidant systems in plants (Jeyasri et al. 2023; Kandoudi et al. 2021), making it a strong candidate in treatment with medicinal plants subjected to stressful situations such as water stress, playing a significant role in signaling antioxidant responses induced by water stress, via ascorbate metabolism (Mohi-Ud-Din et al, 2021; Tayyab et al, 2020). The exogenous application of MeJA was capable of influencing ethylene formation, defense systems, nutrient homeostasis and carbohydrate metabolism to alleviate arsenic-induced stress in rice (*Oryza sativa*) (Nazir et al. 2023). It was also capable of enhancing salt tolerance of okra (*Abelmoschus esculentus* L.) plants by regulating ABA signaling, osmotic adjustment substances, photosynthesis and ROS metabolism (Wang et al. 2023). Similar to SA application, MeJA increased and modulated EO of *L. alba* (Silva-Santos et al. 2023) and under drought stress conditions improved the yield and EO of fennel (*Foeniculum vulgare* Mill.) (Peymaei, Sarabi & Hashempour 2024).

Analyzing the aforementioned information it is clear that the isolated application of these two elicitors have a huge role on plant responses to abiotic stresses. However, some works tested the effect of SA and MeJA combined application. Exogenous application of these two phytohormones together in Verbascum (Karamian, Ghasemlou & Amiri 2020) and

Corn (Tayyab et al, 2020) has been shown to protect plants against abiotic stresses, including drought, by regulating important physiological processes ranging from photosynthesis to nitrogen and proline metabolism and activating the antioxidant defense system. It also mitigated drought-induced oxidative damages in French Bean (*Phaseolus vulgaris* L.) (Mohi-Ud-Din et al. 2021). The combined application of SA and MeJA is also considered a new approach for extending shelf-life of Pomegranate Arils (El-Beltagi, Al-Otaibi, & Ali 2023). These two elicitors synergistically ameliorate salinity induced damage by maintaining redox balance and stomatal movement in Potato (Shekhar et al. 2023) and accordingly to Jiao et al. 2022 their synergism was able to enhanced volatile emissions and anti-herbivore functions in tea plants.

Thus, both water stress and elicitation, individually and combined, have modulatory capabilities in plant physiology. The objective of the present work was to evaluate whether the combined elicitation of SA and MeJA mitigate water restriction stress and can modulate the yield of EO in *L. alba*, as well as EO components by using Headspace-Gas Chromatography-Mass Spectrometry (HS/GC-MS).

2. Material and Methods

2.1. Plant Material, Growth Conditions and Experiment Setup

The experiment was conducted in a greenhouse located at Federal Rural University of Pernambuco (UFRPE), at coordinates 8°00'45.6"S 34°57'03.8"W. Initially, cuttings of approximately 15 cm of *L. alba* were used to carry out this experiment. These cuttings were planted in tubes and were watered daily. This stage lasted 30 days. After acclimatization, the plants were transplanted into polyethylene bags with a capacity of 5 kg containing washed sand, commercial substrate and conditioner in a ratio of 4:1:1, respectively. This stage marked the first day of the experiment. The substrate chemical analysis was as follows: pH 6.16; P

(69.29 mg dm^{-1}); Ca, Mg, Al, Na, K, Al+H (4.70; 1.30; 0.05; 0.30; 0.78; 3.31 $cmol_c$ dm^{-3}, respectively). Organic matter was 37.53 g kg^{-1}. Daily watering and humidity were checked using a soil moisture sensor model HydroSense II CS 658 to maintain substrate humidity at 70% of pot capacity (PC). The average relative humidity and temperature recorded during the experiment were 64% and 31°C, respectively.

After the thirtieth day (30th), the establishment period, the plants were divided into treatments elicited with SA and MeJA in an isolated and combined manner. The elicitation used SA 4 mM and MeJA 2 mM, which presented the best responses in *L. alba* according Santos-Silva et al. (2023), being made solutions with 250 ml of each elicitor to standardize treatments. The elicitation with SA, MeJA or combined SA and MeJA were sprayed on the aerial part of the plants, plus Tween 20, used as an adhesive spreader, enhancing elicitor-plant contact. As SA is diluted in ethanol 1%, this treatment was added in the experiment. For the combined elicitation of salicylic acid and methyl jasmonate, the aforementioned concentration was maintained. It is worth highlighting the care protocols that were followed to avoid contamination by suspended particles, such as spatial and temporal barriers. Therefore, we have the following elicitors treatments: No elicitor (water sprayed), ethanol 1%, SA 4 mM, MeJa 2 mM, and SA 4 mM + 2 mM MeJA.

Fifteen (15) days after elicitation, half of the plants from all treatments were subjected to water restriction with 10% pot capacity (PC), and the remaining plants were maintained with watering at 70% of the PC (control). The harvest was carried out after 15 days of being subjected to water restriction. Fresh leaves were frozen using liquid nitrogen and stored in a freezer -20°C to further biochemical analysis. Biometric, Biochemic and Essential Oil analysis were carried out using these nitrogen preserved leaves. The experiment lasted 60 days and considering the total execution since the first acclimatization stage it lasted 90 days.

The experimental design was in a completely randomized with a 5x2 factorial scheme, containing five elicitation conditions (water, ethanol 1%, SA 4 mM, MeJA 2 mM and combined SA + MeJA (4 mM and 2 mM, respectively) and two water regimes (control with 70% PC and water restriction with 10% PC). The ten treatments had 6 replications each, totaling 60 plants.

2.2. Biometric Analysis and Relative Water Content

L. alba plants were evaluated at harvest by measuring number of leaves, height and number of flowers. The root, stem and leaves were dried and weighed. Fresh, turgid and dry masses of leaf discs were measured to calculate relative water content (RWC) (Barrs and Weatherley 1962; Marques et al. 2021).

2.3. Biochemical and Oxidative Analysis

Leaves of *L. alba* stored in a freezer at -20°C after the experiment harvest were macerated using a mortar and pestle. The analyzes of total chlorophyll and carotenoids were carried out by macerating 0.2 g of fresh leaves in 80% acetone and quantification using spectrophotometer at the wavelength 663 nm, 645, and 470 nm (Lichtenthaler 1987; Bezerra Neto and Barreto 2011).

The extract to determine enzymes activity (SOD, CAT and APX) and total protein content were obtained using extraction buffer (100 mM potassium phosphate buffer pH 7.5, 3 mM dithiothreitol, 1 mM ethylenediamine tetra-acetic acid polyvinylpolypyrrolidone as an antioxidant agent). After centrifugation at 10,000 x g for 30 minutes at 4°C, the supernatant were used to determine the activity of SOD (Giannopolitis and Ries 1977), CAT (Havir and McHale 1987 with adaptations from Azevedo et al. 1998) and APX (Nakano and Asada 1981). Total protein content was determined according to Bradford (1976).

The extract to determine malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were obtained using 0.1% trichloroacetic acid and polyvinylpolypyrrolidone as an antioxidant agent. The MDA content was determined according to Heath and Packer (1968) and H_2O_2 according to Alexieva et al. (2001) and Loreto and Velikova (2001).

The concentration of free proline were determined by ninhydrin method (Bates et al. 1973; Bezerra Neto and Barreto 2011) and total soluble carbohydrates (TSC) was evaluated by using the anthrone spectrophotometric method (Yemm and Willis 1954; Bezerra Neto and Barreto 2011).

2.4. Essential Oil extraction

Lippia alba leaves were subjected to the hydrodistillation process in a Clevenger-type apparatus. The oil was separated from the water by a difference in density. Remaining water residues were removed with the addition of anhydrous sodium sulfate. The oil yield obtained was calculated after two hours of extraction, based on the total mass of leaves used. The equation for calculating yield is:

EO yield = M(oil) / M(botanical material) x 100

, where EO = essential oil; M(oil)= weight of oil in g; M(botanical material) = dry weight of the botanical material in g. All oil was stored in a glass container, hermetically sealed and kept at low temperature (< -5 ºC).

2.5 Headspace-Gas Chromatography-Mass Spectrometry (HS-CG/MS)

Analysis of the chemical composition of the oils was carried out on the Shimadzu CG-EM QP 2010 SE equipment with a mass selective detector, operating with an electron impact of 70 eV with a scan interval of 0.5 s and fragments of m/z 40 to 550 Da . Apparatus equipped with a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 mm) (J & W Scientific, Folsom, CA, USA), with the following parameters: carrier gas: helium; flow: 1 mL/min-1 and 30 p.s.i. inlet pressure in splitless mode. The oven temperature was programmed from 60 to 240 °C at a rate of 3 °C/min. The injector and detector temperatures were 260 °C. Samples were injected using the Shimadzu OAC6000 automatic sampler using the following parameters: incubation time 5 min, incubation temperature 80 ºC, shaker speed 250 rpm, syringe prepurge time 60 seconds, injection flow rate 25 mL min. 1 and post-injection waiting time 60 seconds. The amount of each compound was calculated from the GC peak areas in the order of elution from the DB-5 column and expressed as a relative percentage of the total area of the chromatograms. All EO analyzes were performed in triplicate.

2.6 Identification of the constituents of essential oils

Component identification was based on GC-MS retention indices with reference to a homologous series of C8-C40 n-alkanes calculated using the Van der Dool and Kratz equation (Van den Dool and Kratz, 1963) and by comparison with the mass spectral library from the GC-MS data system (NIST 14) and co-injection with authentic standards as well as other published mass spectra (Adams, 2017). Area percentages were obtained from the GC-MS response without the use of internal standard or correction factors.

2.7. Statistical Analysis

The quantitative data from the experiment were submitted to ANOVA and the means were compared using the SNK test considering 5% probability using Software R (R Core Team, 2017). Hierarchical Cluster Analysis (HCA) was performed using the evaluated parameters and treatments with elicitation and water regimes using R Software.

3. Results

3.1. Growth parameters and relative water content

The ANOVA (Supplementary Table 1) showed that elicitation and WR differentially affected growth and RWC in *L. alba* ($p < 0.05$). Elicitation with combined SA and MeJA decreased root dry weight under WR compared to control elicited plants (32.7%), meanwhile, the other elicitors did not show significant differences under control contrition and WR ($p > 0.05$) (Fig. 1A). In addition, leaves dry weight was not significantly affected by WR and elicitation (Fig. 1B), as well as total chlorophyll and carotenoids content ($p > 0.05$) (Supplementary Table 1). Elicitation with SA increased stem dry weight under WR compared to other elicitors, as well as compared to control plants elicited with SA (31.3%) (Fig. 1C). Elicitation with combined SA and MeJA decreased stem dry weight by 26.5% compared to non-elicited plants under WR (Fig. 1C), presenting a lower root/shoot ratio compared to control plants (Fig. 1D).

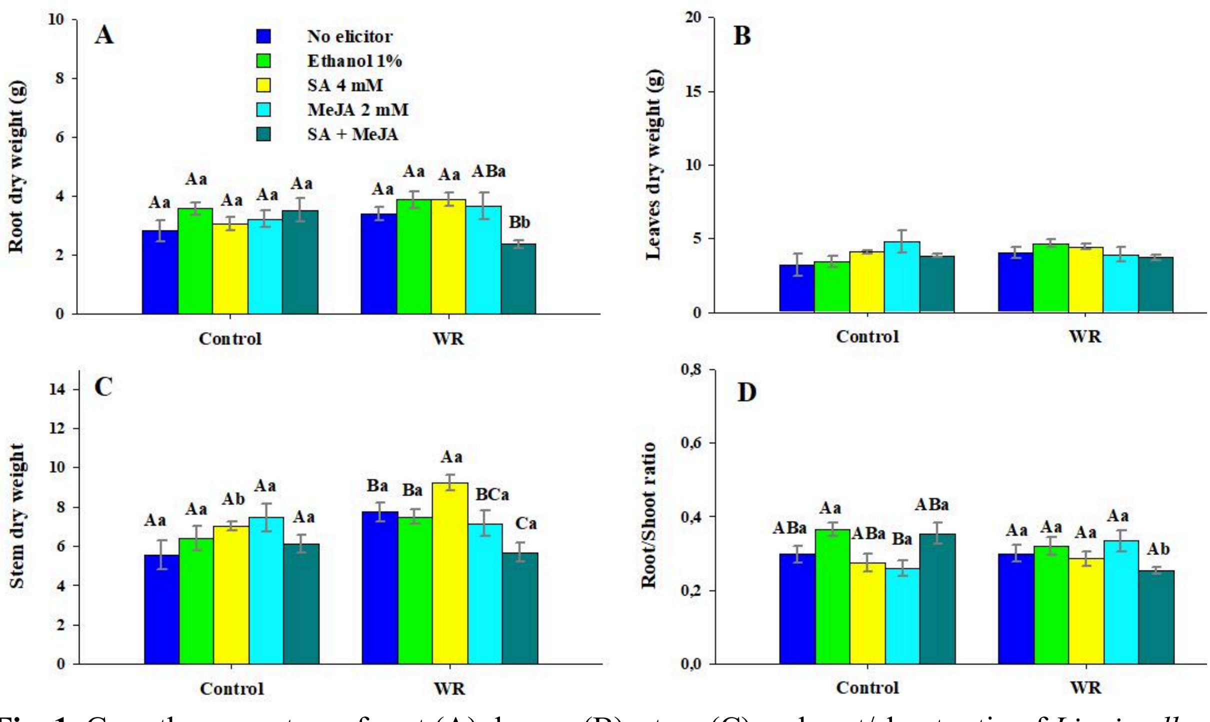


Fig. 1. Growth parameters of root (A), leaves (B), stem (C) and root/shoot ratio of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate

(SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

The number of leaves was affected by WR ($p < 0.001$) (Fig. 2A and Supplementary Table 1). Plants height were not affected by WR and elicitation (Fig. 2B), meanwhile number of flowers increased under WR and elicitation with SA, MeJA and combined SA and MeJA compared to control (Fig. 2C).

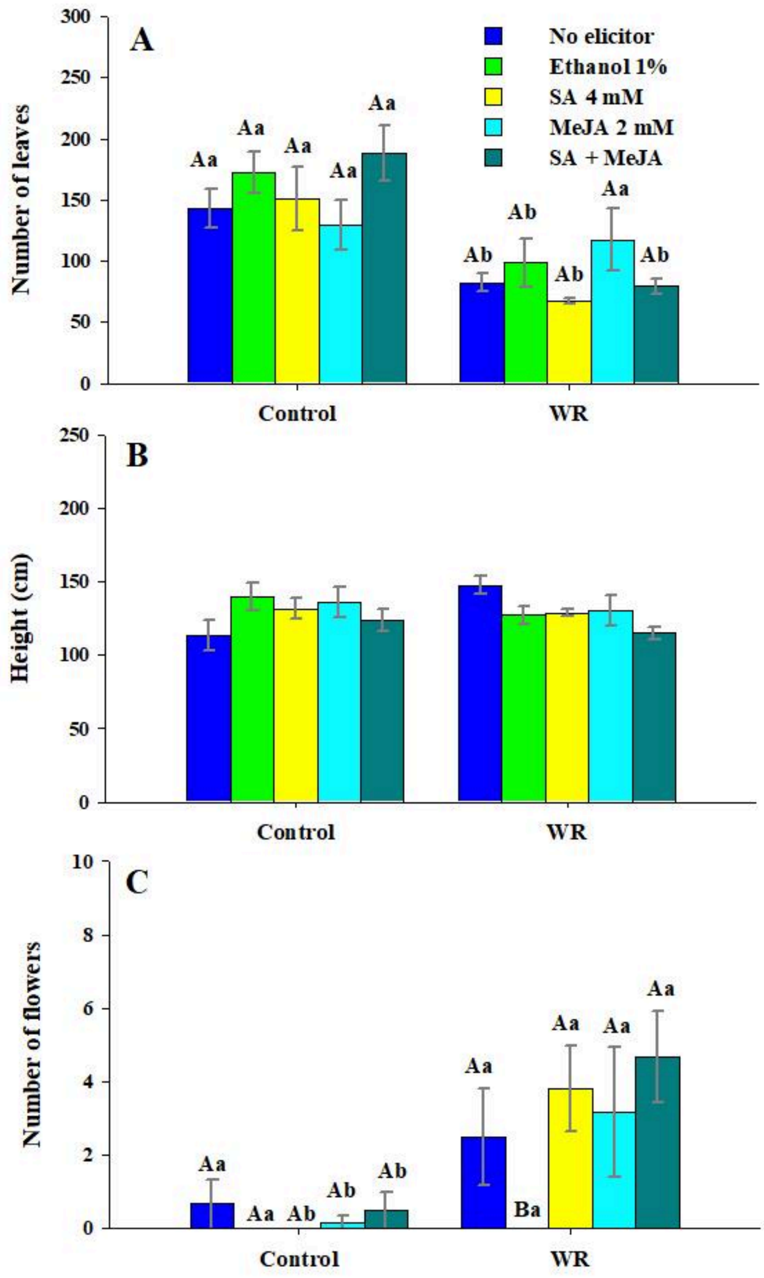


Fig. 2. Number of leaves (A), height (B) and number of flowers (C) of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

The elicitors presented different responses in RWC under WR (Fig. 3). Water restriction decreased RWC in plants with no elicitor, ethanol 1% and SA when compared to control (70% pot capacity). Conversely, elicitation with MeJA and combined SA and MeJA did not show significant difference under WR compared to *L. alba* with the same elicitors in control conditions.

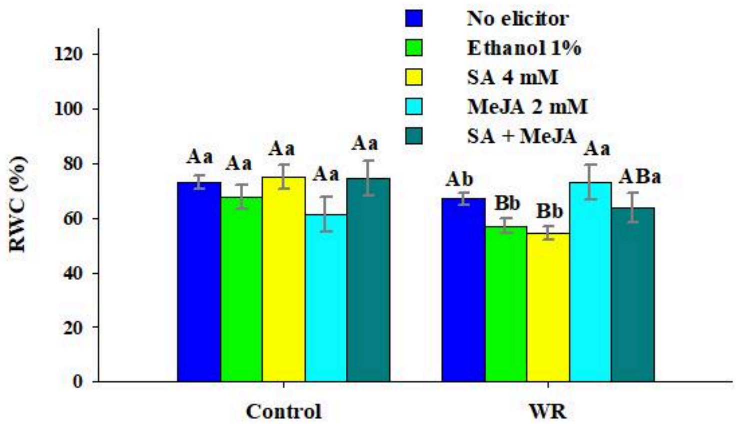


Fig. 3. Relative water content (RWC) in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

3.2. Oxidative stress

The MDA levels were significantly affected by WR ($p < 0.01$) and elicitors ($p < 0.01$) (Supplementary Table 1). *Lippia alba* sprayed with water (no elicitor) presented an increase in MDA levels under WR compared to control (207%) (Fig. 4A). However, elicited plants showed an increase in MDA compared to sprayed with water (no elicitor) under control (145%). Elicited plants under WR did not show significant differences compared to sprayed with water (no elicitor).

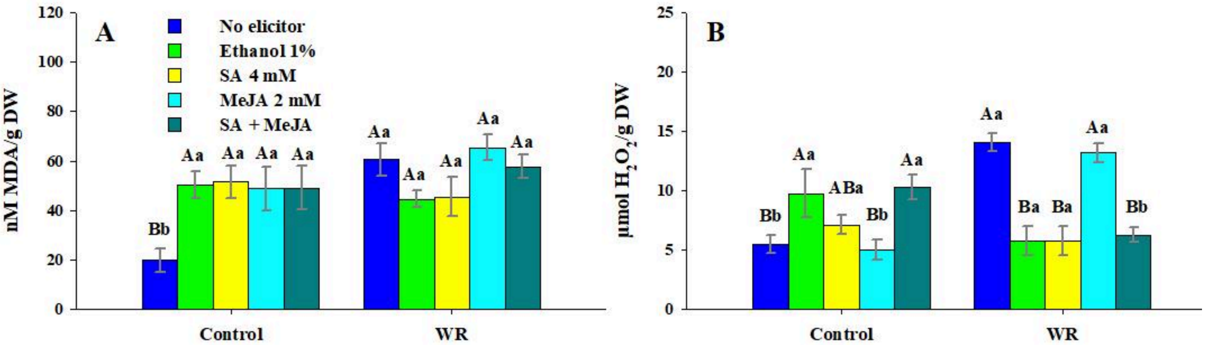


Fig. 4. Malondialdehyde (MDA) (A) and hydrogen peroxide (H_2O_2) (B) levels in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

The H_2O_2 levels were affected by WR and elicitors ($p < 0.05$) (Supplementary Table 1). Water restriction increased the H_2O_2 level in *Lippia alba* sprayed with water (no elicitor) (155%) and MeJA (161%) compared to control (Fig. 4B). However, *L. alba* sprayed with ethanol 1% and SA did not present significant differences in H_2O_2 level compared to control. Elicitation with combined SA and MeJA increased H_2O_2 levels compared to plants sprayed with water (no elicitor) under control (87.2%), however, under WR presented a decrease in H_2O_2 levels (38.5%).

The SOD activity was affected by elicitors ($p < 0.05$) (Supplementary Table 1), with combined SA and MeJA increasing the SOD activity under WR compared to plants under control (53.4%) (Fig. 5A). On the other hand, *L. alba* sprayed with water (no elicitor), ethanol 1%, SA and MeJA did not present significant differences under control and WR treatments. The CAT activity decreased under WR for all sprayed elicitors (Fig. 5B). Plants elicited with

MeJA did not present a significant difference in APX activity under WR compared to control, however, plants sprayed with water (no elicitor), ethanol 1%, SA and combined SA and MeJA decreased APX activity compared to control.

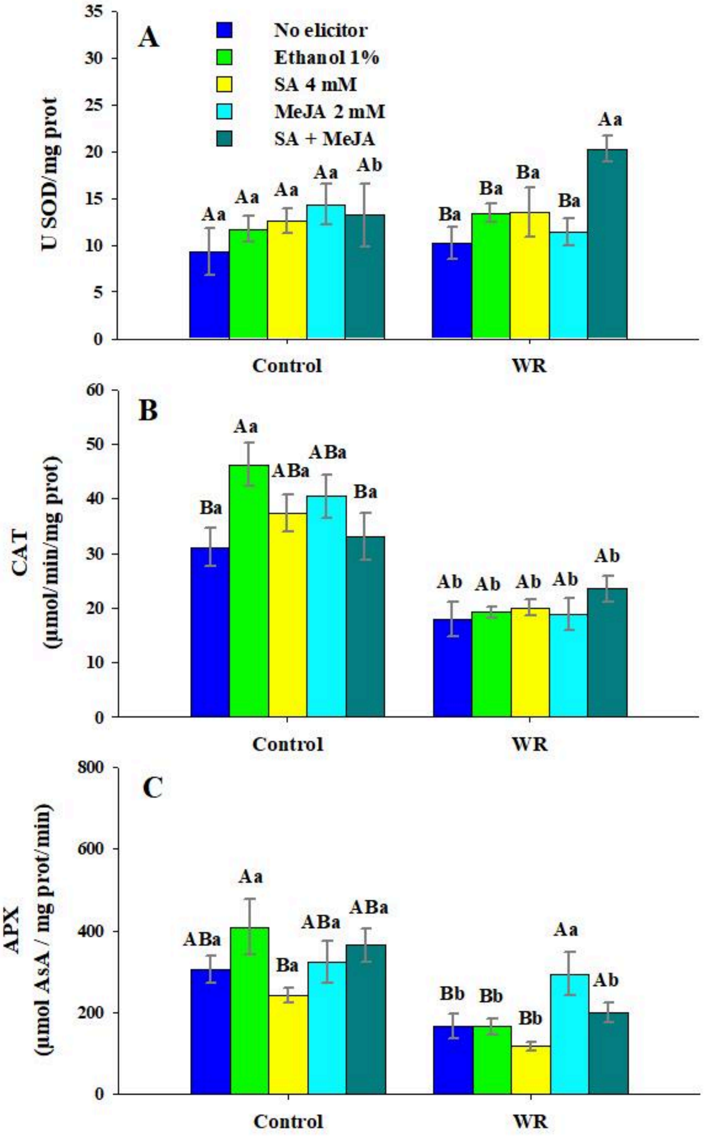


Fig. 5. Superoxide dismutase activity (SOD) (A), catalase activity (CAT) (B) and ascorbate peroxidase activity (APX) (C) in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

3.3. Proline, protein and total soluble carbohydrates content

Proline and protein levels increased in *L. alba* sprayed with water (no elicitor) under WR compared to control (81.9% and 123.5%, respectively) (Fig. 6A and 6B). However, the application of ethanol 1%, SA, MeJA and combined SA and MeJA did not significantly affect the proline levels under control and WR. Plants elicited did not show significant differences in protein levels in WR compared to control. The elicitation with ethanol 1%, SA and combined SA and MeJA showed lower protein level compared to *L. alba* sprayed with water (no elicitor) under WR.

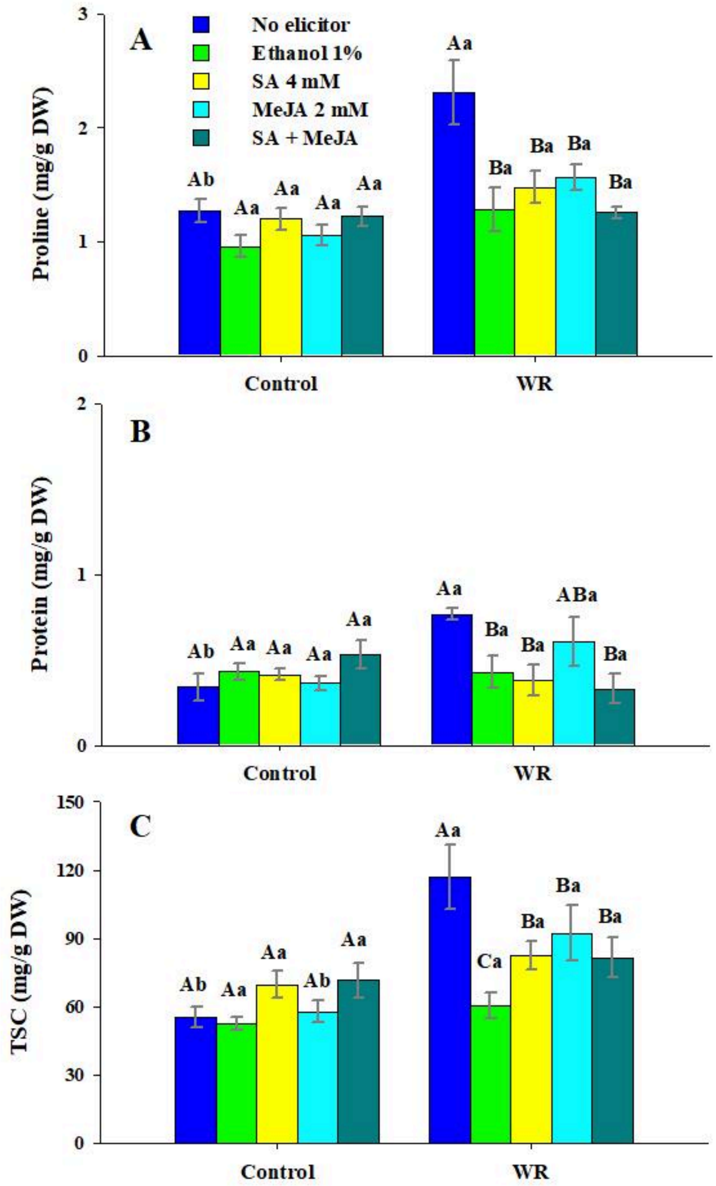


Fig. 6. Proline (A), protein (B) and total soluble carbohydrates (C) content in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

Total soluble carbohydrates (TSC) increased in plants sprayed with water (no elicitor) and MeJA when submitted to WR compared to control (111.3% and 59.2%, respectively) (Fig. 6C). However, elicitation with ethanol 1%, SA and combined SA with MeJA did not show significant differences with WR compared to control. Additionally, plants sprayed with water (no elicitor) under WR presented higher TCS compared to elicited plants.

3.4. Essential oil yield and components analysis

Essential oil yield was affected by WR ($p < 0.001$), elicitor ($p < 0.01$) and combined WR with elicitor application ($p < 0.01$) (Supplementary Table 1) (Fig. 7). The EO yield was not affected by WR when sprayed with water (no elicitor) and ethanol 1%, however, elicitation with SA, MeJA and combined SA and MeJA increased EO yield under WR compared to non elicited plants (84.2%, 80.7% and 141.5%, respectively) (Fig. 7). In addition, combined elicitation with SA and MeJA was significantly higher than elicitation with SA and MeJA isolated.

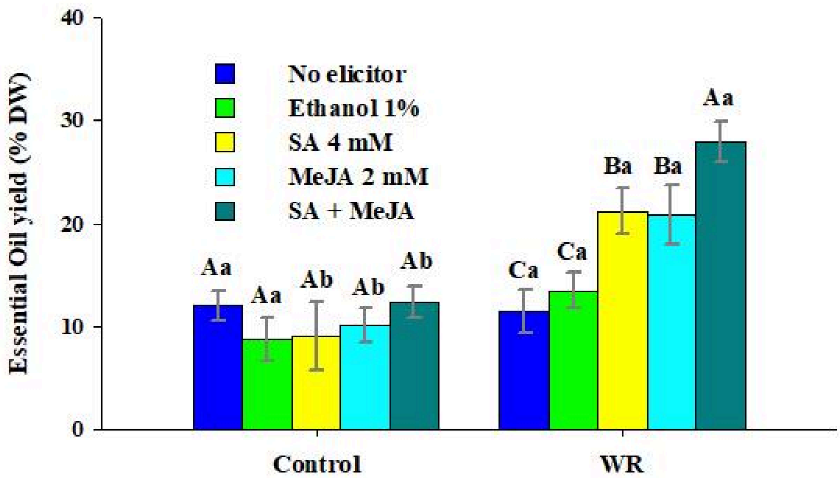


Fig. 7. Essential oil yield in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different uppercase letters represent significant differences among the elicitors with the same water treatments, meanwhile different lowercase letters represent significant differences among the water treatments with the same elicitor using SNK test ($p < 0.05$).

The elicitation and water restriction modulated the production of several compounds of EO in leaves of *L. alba* (Table 1). The Headspace-Gas Chromatography-Mass Spectrometry identified 26 compounds: 18 monoterpenes and 8 sesquiterpenes. Three compounds were exclusive to plants in control conditions without elicitors: Tricyclene, α-pinene and β-Copaene. The application of SA 4 mM induced the presence of 17 (65.3%) and 19 (73.07%) among the identified compounds, under control and WR respectively. The application of MeJA 2 mM induced the presence of 21 (80.7%) and 16 (61.5%) compounds, under control and WR respectively. The combined elicitation with SA and MeJA induced the presence of 16 (61.5%) and 17 (65.3%) compounds, under control and WR respectively. There were compound presence when elicited with SA or MeJA, but were not identified with combined SA and MeJA under control and WR: Thuja-2.4(10)-diene, (E)-β-Ocimene.

The most abundant compounds were Geranial (47.47-60.45%) and Geraniol (23.55-29.38%) (Table 1). The compound γ-terpinene was not present when elicited with SA 4 mM under control, but presented the higher concentration under WR when elicited with SA

4 mM (239% higher compared to non-elicited plants under WR). The application of combined SA and MeJA presented the lowest monoterpenes content under WR, however, the sesquiterpenes were the highest by increasing the content of β-caryophyllene, allo-Aromadendrene and β-selinene.

The control (no elicitor) presented the higher levels of the monoterpene Nerol and the sesquiterpenes Germacrene D and Caryophyllene oxide; control with 1% ethanol, presented the highest content of the monoterpene Geranial among all treatments; SA 4mM presented the highest levels of the monoterpene Linalool and the sesquiterpene β-Elemene; MeJa 2mM showed the highest content of the monoterpene Trans-piperitoneepoxide; the combined Sa+MeJA presented the highest content of the monoterpene Geraniol. In treatments subjected to water restriction, the treatment without elicitor presented the highest levels of the monoterpenes p-cymene and Limonene; 1% ethanol submitted WR showed better total monoterpene levels but no specific component. SA 4mM subjected to WR showed highest content in monoterpenes such as: Thuja-2,4(10)-diene, Verbenene (compound that gives the name to the Verbenaceae family of *Lippia alba*), β-pinene, (E)-β -Ocimene and γ-terpinene. The MejA 2mM treatment subjected to WR showed highest content only in the monoterpene (Z)-β-Ocimene. The combined treatment of SA and MeJA subjected to WR showed better results in sesquiterpene content. The monoterpenes with highest response to WR with combined SA and MeJA are as follows: Exo-isocitral, β-pimene oxide and (Z)-Isocitral; and the sesquiterpenes β-caryophyllene, allo-Aromadendrene and β -selinene. This treatment was also responsible for presenting a higher total content of sesquiterpenes when compared to the other treatments, on the other hand, it was the treatment with the lowest total content of monoterpenes.

Table 1. Analysis of essential oil composition using Headspace-Gas Chromatography-Mass Spectrometry in leaves of *Lippia alba* cultivated with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. Different letters represent significant differences among the treatments using SNK test ($p < 0.05$).

			Control					**Water restriction (WR)**				
RI[a]	**RI[b]**	**Compounds**	**No elicitor**	**Ethanol 1%**	**SA 4 mM**	**MeJA 2 mM**	**SA+MeJA**	**No elicitor**	**Ethanol 1%**	**SA 4 mM**	**MeJA 2 mM**	**SA+MeJA**
		Monoterpenes										
921	918	Tricyclene	0.12									
932	932	α-pinene	0.67									
953	951	Thuja-2.4(10)-diene***	0.22 g		2.39 b	0.74 d		0.42 f	0.61 e	**3.55 a**	1.49 c	
961	960	Verbenene**				0.37 b	0.42 b			**0.58 a**		
974	971	β-pinene***	0.58 d	1.14 c	3.63 b	1.07 c			0.25 e	**8.13 a**	0.65 d	0.56 d
1020	1019	p-cymene***	0.70 g	0.12 h		1.35 e	0.87 f	**6.13 a**	2.50 c	4.45 b	1.51 d	0.86 f
1024	1022	Limonene***	4.70 c	3.05 e	1.26 h	0.81 i	2.72 f	**15.63 a**	9.52 b	2.61 f	3.63 d	1.60 g
1032	1033	(Z)-β-Ocimene***		0.12 b	0.16 b					0.15 b	**0.58 a**	
1044	1041	(E)-β-Ocimene***		0.14 f	1.48 b	0.76 c			0.22 e	**2.16 a**	0.63 d	
1054	4152	γ-terpinene***	1.43 d			3.43 b	0.74 e	3.43 b	2.91 c	**11.63 a**	2.81 c	0.92 e
1095	1092	Linalool***	0.84 d		**1.83 a**	1.29 b	0.70 e	1.40 b	0.92 d	1.12 c	1.36 b	0.92 d

1140	1138	exo-Isocitral***	0.26 de		0.39 c	0.34 cd	0.31 cde	1.12 a	0.22 e	0.88 b		**1.14 a**
1154	1151	β-Pinene oxide***	0.77 c		0.75 c	0.43 e	0.36 f	1.03 b	0.22 g	0.80 c	0.63 d	**1.43 a**
1160	1159	(Z)-Isocitral***	0.95d		0.55 g	0.82 e	0.83 e	2.18 b	0.55 g	1.62 c	0.67 f	**2.53 a**
1227	1226	Nerol***	**1.76 a**	0.05 f	0.29 e	1.22 c	1.37 b	0.80 d	0.82 d			
1249	1245	Geraniol***	28.33 b	26.41 d	24.34 e	28.57 b	**29.38 a**	21.60 g	26.85 d	16.18 h	27.68 c	23.55 f
1252	1255	Trans-Piperitoneep oxide***	1.96 b	0.99 d	0.62 e	**4.35 a**	1.17 c	0.30 g	0.36 fg	0.12 h	0.59 e	0.42 f
1264	1261	Geranial***	50.39 cd	**60.46 a**	48.89 e	49.62 de	56.54 b	41.57 g	51.05 c	33.31 h	48.68 e	47.47 f
Total Monoterpenes (%)* **			93.68 c	92.48 c	86.58 e	95.18 b	95.41 b	95.62 b	**97.06 a**	87.28 e	90.96 d	82.49 f
		Sesquiterpenes										
1359	1355	Nerylacetate	0.14						0.15			
1389	1384	β-Elemene***	0.54 b	0.61 b	**0.87 a**	0.12 d	0.26 c	0.28 c				0.54 b
1417	1416	β-caryophyllene***	0.26 f	0.11 g	3.61 b	2.57 c		0.22 f		1.88 d	1.58 e	**5.26 a**
1430	1426	β-Copaene	0.10									
1458	1454	allo-Aromadendrene***	0.15 d					1.33 c		1.74 b		**6.21 a**
1480	1475	Germacrene D***	**3.52 a**			1.13 c	2.34 b	0.53 e	1.22 c	0.71 d		1.22 c
1489	1485	β-selinene***		0.07 d	0.27 b	0.21 c						**0.75 a**

1582	1578	Caryophyllene oxide***	**0.58 a**			0.15 b	0.55 a		0.22 b		0.23 b	
Total Sesquiterpenes (%)* **			5.30 b	0.79 h	4.75 c	4.18 d	3.15 e	2.36 f	1.59 g	4.33 d	1.82 g	**14.01 a**
		Total	98.98	93.26	91.33	99.36	98.56	97.99	98.65	91.61	92.77	99.50

RIa = Calculated retention index are based on the retention times relative to a series of n-alkanes C8–C40 on a DB-5 capillary column; RIb = Linear retention index from the literature (Adams, 2017). ** represents $p < 0.01$; *** represented $p < 0.001$.

3.5. Hierarchical cluster analysis

To analyze the overall clustering among the different treatments with elicitor and WR, HCA was performed using the experimental data of growth, oxidative stress, biochemical and EO yield (Fig. 8). One cluster was formed with *L. alba* under WR sprayed with SA 4 mM and WR sprayed with combined SA 4 mM and MeJA 2 mM. The other cluster grouped several treatments, forming subclusters. The treatments with ethanol 1% (used to dissolve the SA) and SA under control were grouped in the same subcluster, however, under WR ethanol 1% and SA 4 mM were grouped in different clusters. The elicitation with combined SA and MeJA under control and WR were also grouped in different clusters.

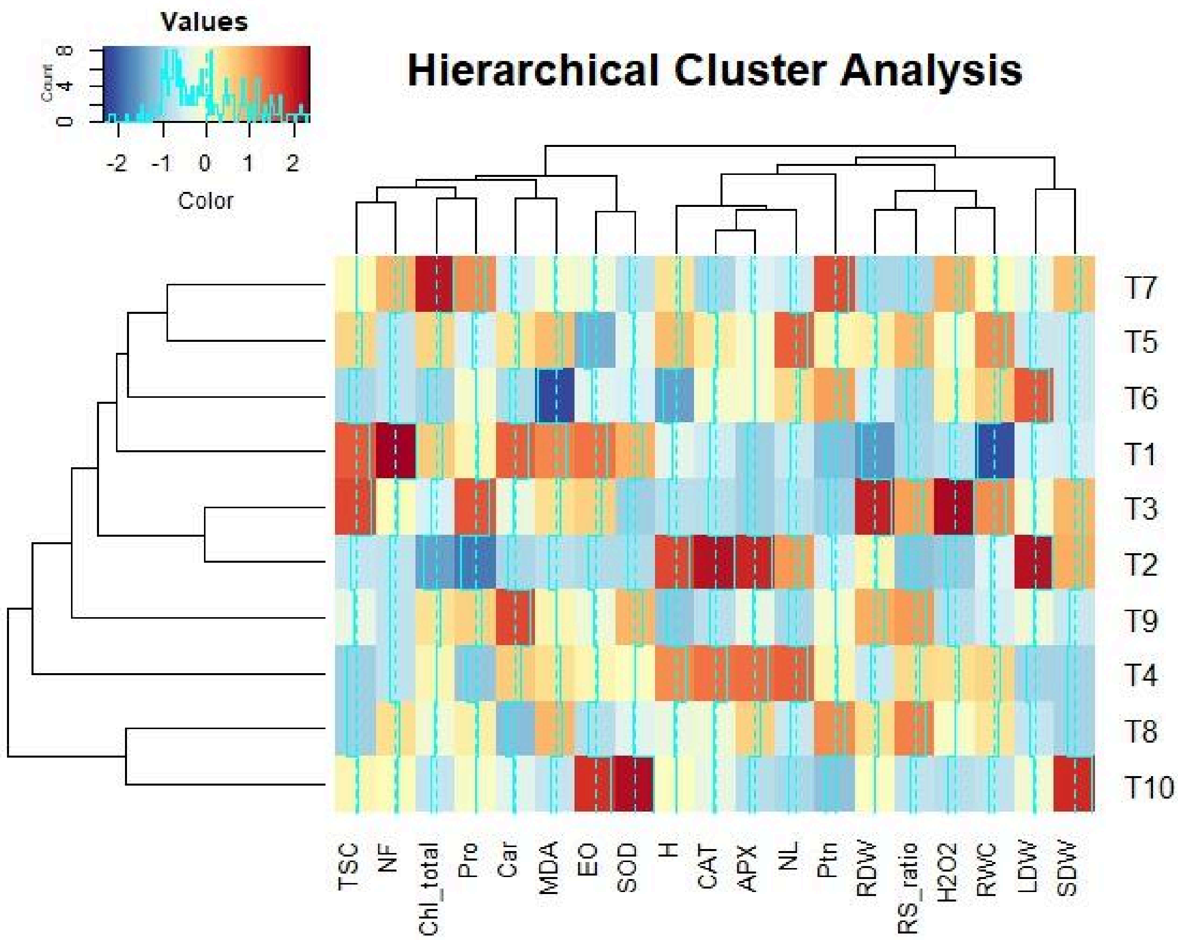


Fig. 8. Hierarchical cluster analysis (HCA) of growth, oxidative stress, biochemical and essential oil yield parameters in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2

mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. RCW: relative water content, LDW: leaves dry weight, RDW: root dry weight, SDW: stem dry weight, RS_ratio: root/shoot ratio, Chl_total: total chlorophyll content, Car: carotenoids content, NL: number of leaves, H: Height, NF: number of flowers, MDA: malondialdehyde; H_2O_2: hydrogen peroxide, Pro: proline content, Ptn: protein content, TSC: total soluble carbohydrate, SOD: Superoxide dismutase activity, CAT: Catalase activity, APX: Ascorbate peroxidase activity, EO: essential oil yield. T1: control (70% pot capacity) sprayed with water (no elicitor), T2: control sprayed with ethanol 1%, T3: control sprayed with SA 4 mM, T4: control sprayed with MeJA 2 mM, T5: control sprayed with SA 4 mM and MeJA 2 mM, T6: water restriction (WR, 70% pot capacity) sprayed with water (no elicitor), T7: WR sprayed with ethanol 1%, T8: WR sprayed with SA 4 mM, T9: WR sprayed with MeJA 2 mM, T10: WR sprayed with SA 4 mM and MeJA 2 mM.

The HCA of EO composition among the treatments applied formed two main clusters, one containing plants non elicited, SA 4 mM and combined SA and MeJA under WR (Fig. 9). In the other subcluster, non-elicited plants under control grouped separately, and ethanol 1% under control and WR were grouped in the same subcluster. The combined elicitation with SA and MeJA grouped in the same cluster with SA under control.

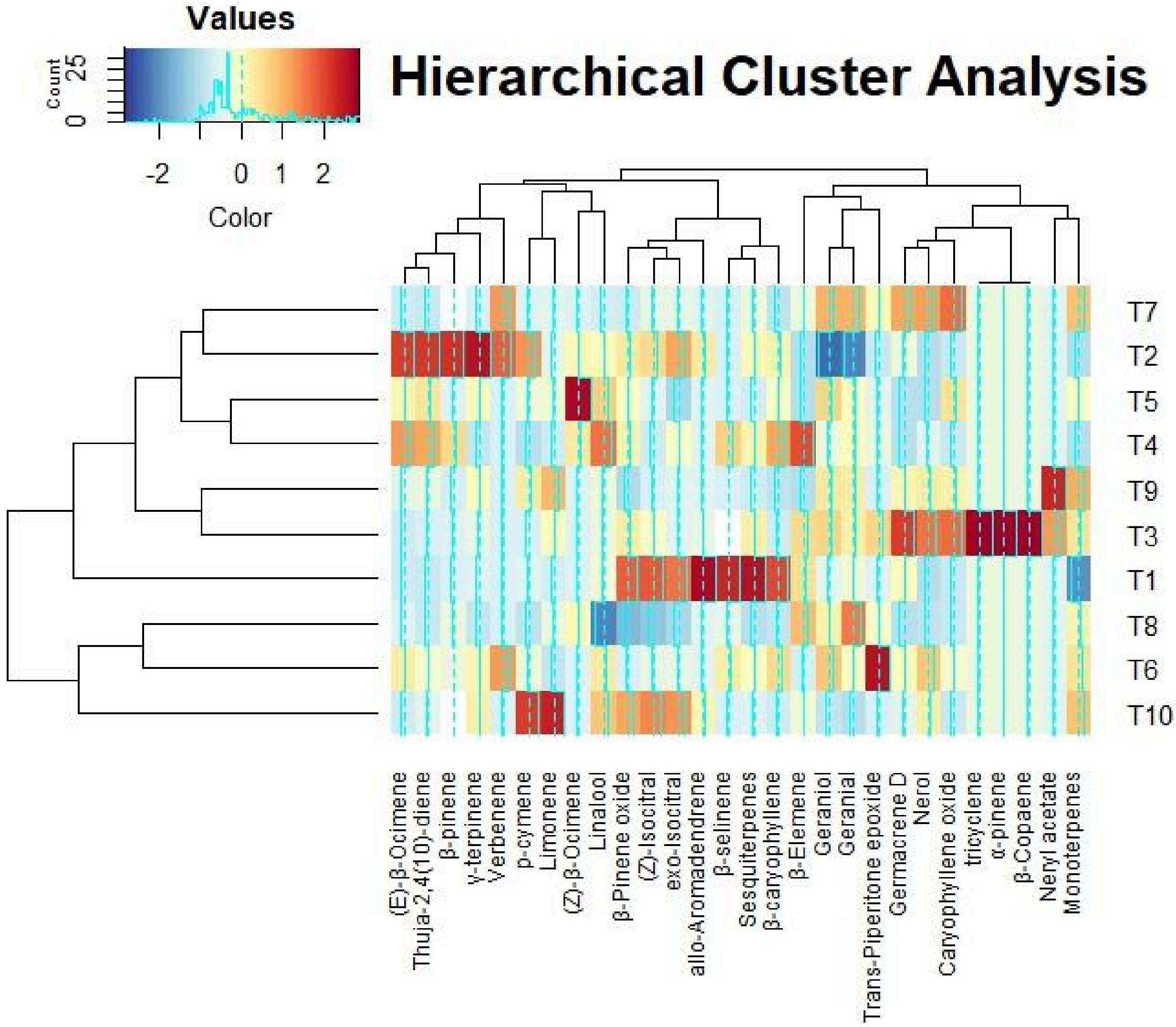


Fig. 9. Hierarchical cluster analysis (HCA) of essential oil composition using Headspace-Gas Chromatography-Mass Spectrometry in leaves of *Lippia alba* with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. T1: control (70% pot capacity) sprayed with water (no elicitor), T2: control sprayed with ethanol 1%, T3: control sprayed with SA 4 mM, T4: control sprayed with MeJA 2 mM, T5: control sprayed with SA 4 mM and MeJA 2 mM, T6: water restriction (WR, 70% pot capacity) sprayed with water (no elicitor), T7: WR sprayed with ethanol 1%, T8: WR sprayed with SA 4 mM, T9: WR sprayed with MeJA 2 mM, T10: WR sprayed with SA 4 mM and MeJA 2 mM.

4. Discussion

Elicitation is an important tool to mitigate WR stress and increase EO production in several species, as well as improve plant responses to drought stress (Shekhar et al. 2023). Most elicitation tests focus on individual elicitor application (Ilyas et al. 2021), meanwhile the use of two elicitors may synergistically enhance EO production and plant responses to WR. The elicitors SA and MeJA are widely used in foliar application improving plant growth parameters, mitigating oxidative stress and ROS damage, as well as increasing SOD, CAT and APX activities (Kazemi et al. 2024, Nyanasaigran et al. 2024, Lamnai et al. 2021, Vithana et al. 2024). The isolated elicitation of SA (4 mM) and MeJA (2 mM) in *L. alba* improved OE and modulated the biochemical and physiological responses without stress application (Silva-Santos et al. 2023). Thus, this is the first study to evaluate the isolated and combined elicitation of SA and MeJA in *L. alba* under WR on growth, biochemical, EO yield and compounds.

Since SA and MeJA are plant hormones and therefore can modulate plant growth (Jeyasri et al. 2023), the impact of elicitation on shoot and root needs to be measured. Although leaves dry weight were not affected by elicitation with SA, MeJA or combined SA and MeJA, stem dry weight was higher with SA and WR. The increase in JA and SA levels may decrease plant growth by allocating resources to defenses, inhibiting mitotic activity and affecting the production and transport of auxin (Heinrich et al. 2013, Noir et al. 2013, Farooq et al. 2016, Pasternak et al. 2019). On the other hand, elicitation may improve plant growth, such as 1.0 mM of MeJA in *Portulaca oleracea* (Nyanasaigran et al. 2024). Several studies demonstrated the positive impact of elicitation on plant growth and mitigating the damage caused by stress (Mohi-Ud-Din et al. 2021, Tayyab et al. 2020, Karamian, Ghasemlou & Amiri 2020). Overall, elicitation with SA 4 mM, MeJA 2 mM or combined SA and MeJA did not decrease the leave dry weight in our experiment, although number of leaves were affected by elicitation with SA and combined SA and MeJA under WR, suggesting the concentration indicated by Silva-Santos et al. (2023) can be used to *L. alba* under WR.

The increase in number of flowers when elicited with SA, MeJA and combined SA and MeJA occurred when *L. alba* was subjected to WR. The levels of SA and MeJA regulate flowering and may increase the number of flowers (Kaur et al. 2022, Zhao et al. 2022). Our results suggest the elicitation stimulus in the number of flowers is improved by WR, which may induce ABA production and stimulate flowering (Bader et al. 2023).

Water restriction typically decreases cell turgor and expansion, leading to a lower RWC (Wang et al. 2023, Ulloa-Inostroza et al. 2024), indicating plant water status (Pandey and Lal, 2018). Non-elicited *L. alba* under WR decreased RWC compared to control, as well as *L. alba* elicited with ethanol 1% and SA 4 mM. On the other hand, *L. alba* elicited with MeJA 2 mM and combined SA and MeJA did not present reduction in RWC under WR compared to control elicited plants. The results highlight the importance of MeJA to maintain water balance under WR, applied isolated or combined with SA. Exogenous application of MeJA (50 μM) increases JA and ABA content in *Abelmoschus esculentus* L. under salt stress (Wang et al. 2023). The elicitation with MeJA 2 mM in *L. alba* decreases stomatal conductance (*gs*) without water stress (Silva-Santos et al. 2023). The elicitation with MeJA and combined SA and MeJA improved *L. alba* RWC under WR, which may be related to MeJA influence on stomatal control and ABA content (Yan et al. 2022).

Plants subjected to WR may increase the production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), damaging membranes, chlorophyll, DNA and causing cell death (Chen et al. 2023, Huang, et al. 2023). The levels of Malondialdehyde (MDA) are commonly used as a membrane damage marker promoted by increased ROS and oxidative damage (Yu et al. 2020, Huang, et al 2023). The most common strategies to mitigate ROS production are enzymatic (SOD, CAT and APX) and non-enzymatic, leading to a decrease in MDA levels (Zulfiqar and Ashraf 2023). Additionally, the accumulation of organic osmoprotectants, such as proline and soluble carbohydrates, may mitigate ROS production and oxidative damage (Saddique et al. 2020, Yang et al. 2021). Non-elicited plants under WR presented an increase in MDA and H_2O_2 levels, indicating higher oxidative damage (Rajput et al. 2021). However elicited plants (ethanol, SA, MeJA and combined SA and MeJA) showed higher MDA levels under control conditions compared to non-elicited plants and did not present increase in MDA levels under WR. Elicitation with SA or MeJA is reported to decrease MDA levels (Wang et al. 2023, Shekhar et al. 2023, Lopes et al. 2024, Nyanasaigran et al. 2024). However, elicitation in *L. alba* with SA and MeJA may have induced ROS production by inducing NADPH oxidase in control and WR, which in turn produce ROS involved in plant responses to (a)biotic stresses (Spoel and Dong et al. 2024).

It is reported elicitation with SA and MeJA mitigate abiotic stress by triggering enzymatic responses of SOD, CAT and APX, reducing ROS and oxidative damage (Shekhar et al. 2023, Lopes et al. 2024, Nyanasaigran et al. 2024). However, in our experiment, ethanol 1%, SA 4 mM or MeJA 2 mM decreased APX and CAT activities under WR, similar to non-elicited plants. The combined elicitation of SA and MeJA increased SOD activity under WR, being the highest SOD activity in our experiment. In addition, only non-elicited plants showed an increase of osmoprotectants proline, soluble protein and total soluble carbohydrates under WR. The accumulation of osmoprotectants mitigate abiotic stress (Saddique et al. 2020, Yang et al. 2021). We hypothesize the induction of ROS production by elicitation in *L. alba* may mitigate the effects of WR non-enzymatically, decreasing osmoprotectants production under WR compared to non-elicited.

Lippia alba is a plant that produces EO rich in metabolites with anti-inflammatory, antimicrobial, anxiolytic, calming and other actions. The modulation of the content of this EO can occur for several reasons, such as conditions of WR and induction of responses through elicitation (de Castro et al. 2020). In the present work, the highest EO yield (%) was found in the treatment that combines WR, SA and MeJA, followed by the treatment with SA and MeJA under WR. As reported by Silva-Santos et al. 2023, the two elicitors, in isolation, were able to increase the EO content in properly watered *L. alba* plants 22 days after elicitation. Under conditions of WR added to the combination of these elicitors, a considerable increase in the OE content was observed, thus pointing to a synergy between the action of the elicitors and submission to WR, as had already been projected in a volatile emissions model by Jiao et al. 2022. Synergistic action occurs when two elicitors combined result in a response greater than their individual responses (Thaler et al, 2012), which was highlighted in the HCA analysis showing the different grouping in plants with stress elicited individually and jointly with SA and MeJA.

The results indicated that applying combined elicitation with SA and MeJA there is an amplification in the plant's biochemical responses. This may happen because the elicitors act at different points in the signaling pathways or because one elicitor enhances the response induced by the other elicitor. The increase in EO content with MeJA is attributed to the regulation of the activity of the PAL enzyme, genes related to terpene synthesis and the density of EO-producing trichomes (Khalvandi et al, 2019). SA has an effect attributed to stimulating the expression of enzymes that regulate terpene biosynthesis, probably enzymes other than PAL (Es-sbihi et al, 2020).

Analyzing the EO profile two major monoterpenes were found: Geranial and geraniol. This result was found in all treatments of *Lippia alba* used in this work and converging with the results also found by Santos-Silva et al. 2023, Palhares-Neto et al. 2023, Sá Filho et al. 2022 and Barros et al. 2022. These monoterpenoids are of great importance for the pharmaceutical industry, due to the properties of their molecules which have antineoplastic activity in animal and cell models in several types of cancers. These compounds have been found to activate multiple antitumoral responses, like apoptosis, autophagic cell death, cytostasis and necrosis (Santos and Silva et al. 2022) and cardioprotective potential (Zou et al. 2022).

The terpenoids biosynthesis occurs in two separated pathways: methylerythritol phosphate pathway (MEP) take place in plastids contributes to monoterpenes and mevalonic acid pathway (MVA) take place in cytoplasm and contributes to sesquiterpenes (Huang et al. 2021; Humbal and Pathak, 2023; Wei et al., 2023). *Lippia alba* under WR without elicitor increased Limonene, p-cymene and total monoterpenes compared to plants with control treatment, showing a positive regulation of terpenes to WR stress, highlighting the stimulus of secondary metabolites biosynthesis by WR (Boncan et al. 2020). Abiotic stresses modulate the *Terpene Synthase* (*TPS*) genes in *Camellia sinensis* (Zhou et al. 2020). Elicitation with SA under WR increased the content of several EO components, specially Thuja-2.4(10)-diene, β-pinene and γ-terpinene, compared to the other treatments. However, combined elicitation with SA and MeJA under WR increased total sesquiterpenes content (β-caryophyllene, allo-Aromadendrene and β-selinene), indicating the activation of MVA in *L. alba*. The elicitation of SA and MeJA strongly affect the content of secondary metabolites in plants (Jeyasri et al. 2021). Our results indicate strong association combining SA and MeJA with WR to increase EO yield and sesquiterpenes in *L. alba*, modulating the MEP and MVA.

In this sense, the combined elicitation of SA and MeJA in *L. alba* plants subjected to WR were able to modulate not only physiologically but was also able to increase the EO yiled (150.2%) and modify the profile of *L. alba* EO inhibiting the production of monoterpenes and stimulating the production of sesquiterpenes.

5. Conclusion

In summary, elicitation and WR were able to modulate EO yield and compounds and other physiological aspects of *L. alba*. Treatment MeJA and combined SA and MeJA mitigated the WR effect on RWC compared to control. Elicitation also modulated malondialdehyde and H_2O_2 under control and WR. By using Headspace-Gas Chromatography-Mass Spectrometry (HS/GC-MS) to analyze EO composition, the major EO compounds were Geraniol and Geraniol. The combined elicitation of SA and MeJA under WR presented the higher essential oil yield, increasing sesquiterpene content. Overall, the isolated or combined elicitation with SA and MeJA had the potential to mitigate the WR effects in *L. alba*, however, the combined elicitation of SA and MeJA synergistically stimulated the secondary metabolism to increase EO yield (150.2%) and modify the its profile inhibiting the production of monoterpenes and stimulating the production of sesquiterpenes.

Conflict of interest

The authors declare no competing interests exist.

Author contribution

JMO: conceptualization, experiment, analyses, writing the article. MLLS and MEMP: experiment and analyses. MMM and CAGC: essential oil analyses and compounds identification. MVLS: conceptualization, analyses, writing the article.

Data Availability

Data will be made available on request.

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Electronic Supplementary Material

Title: Water stress in *Lippia alba* combined with salicylic acid and methyl jasmonate elicitation modulate oxidative stress and synergistically enhance essential oil yield

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Variables	Water (W)	Elicitor (E)	W*E
RWC	**	ns	*
LDW	ns	ns	ns
RDW	ns	ns	*
SDW	*	**	*
RS_ratio	ns	ns	**
Chl_total	ns	ns	ns
Car	ns	ns	ns
NL	***	ns	ns
H	ns	ns	ns
NF	***	ns	ns
MDA	**	ns	**
H_2O_2	*	*	***
Pro	***	***	*
Ptn	ns	ns	**
TSC	***	*	**
SOD	ns	*	ns
CAT	***	ns	ns
APX	***	*	ns
EO	***	**	**

Supplementary Table 1. Analysis of variance (ANOVA) of variables with 70% pot capacity (control) or water restriction with 10% pot capacity (WR), subjected to elicitation through foliar spray with water (no elicitor), ethanol 1%, salicylic acid (SA 4 mM), methyl jasmonate (MeJA 2 mM) and combined elicitation of salicylic acid methyl jasmonate (SA 4 mM and MeJA 2mM), harvested after 15 days of water restriction. RCW: relative water content, LDW: leaves dry weight, RDW: root dry weight, SDW: stem dry weight, RS_ratio: root/shoot ratio, Chl_total: total chlorophyll content, Car: carotenoids content, NL: number of leaves, H: Height, NF: number of flowers, MDA: malondialdehyde; H_2O_2: hydrogen peroxide, Pro: proline content, Ptn: protein content, TSC: total soluble carbohydrate, SOD: Superoxide dismutase activity, CAT: Catalase activity, APX: Ascorbate peroxidase activity, EO: essential oil yield.