SUBSTÂNCIAS HÚMICAS MODULAM A RESISTÊNCIA SISTÊMICA VEGETAL: UMA ABORDAGEM BIOQUÍMICA E MOLECULAR

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RESUMO

SILVA; Rakiely Martins; D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Janeiro de 2026; SUBSTÂNCIAS HÚMICAS MODULAM A RESISTÊNCIA SISTÊMICA VEGETAL: UMA ABORDAGEM BIOQUÍMICA E MOLECULAR; Orientador: Prof.ª. D.Sc. Luciano Pasqualoto Canellas.

A intensificação da agricultura moderna tem sido acompanhada pelo uso crescente de agroquímicos para o controle de doenças, impondo desafios ambientais, econômicos e à saúde humana e reforçando a necessidade de estratégias mais sustentáveis para o manejo fitossanitário. Nesse contexto, esta tese foi desenvolvida com o objetivo de avaliar o potencial das substâncias húmicas (SH) como indutoras de resistência em plantas, bem como compreender os mecanismos fisiológicos, bioquímicos e moleculares associados a essa resposta. Inicialmente, foi demonstrado que a aplicação de SH promove a ativação sistêmica de marcadores clássicos de defesa vegetal, como a atividade das enzimas fenilalanina amônia-liase (PAL), peroxidase (POX) e beta 1,3-glucanase (GLUC), em diferentes culturas agrícolas, evidenciando a capacidade de estimular respostas de resistência de forma ampla. Em seguida, verificou-se que SH provenientes de diferentes fontes de matéria orgânica modulam de maneira distinta as respostas de defesa em tomateiro sob estresse biótico, com redução da severidade de doenças e ativação de genes reguladores e efetores associados às vias hormonais de defesa. A análise integrada das respostas fisiológicas, transcricionais e proteômicas revelou que as diferenças estruturais das SH direcionam ajustes específicos no metabolismo vegetal, resultando tanto na manutenção da homeostase quanto em uma reprogramação metabólica mais dinâmica frente à infecção por patógenos. De forma geral, os resultados obtidos demonstraram que as SH atuam como moduladoras da imunidade vegetal, sendo capazes de induzir respostas de defesa eficientes e coordenadas, dependentes de sua origem e do contexto de estresse. Esses achados reforçam o potencial das SH para uso direto nas plantas como insumos biológicos estratégicos para o manejo sustentável de doenças em sistemas agrícolas, contribuindo para a redução da dependência de agroquímicos e para o desenvolvimento de uma agricultura mais resiliente e ambientalmente responsável.

Palavras-chave: elicitores naturais, imunidade vegetal, reprogramação metabólica, sinalização hormonal, respostas antioxidantes, manejo sustentável.

ABSTRACT

SILVA, Rakiely Martins da; D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Janeiro de 2026; HUMIC SUBSTANCES MODULATE SYSTEMIC PLANT RESISTANCE: A BIOCHEMICAL AND MOLECULAR APPROACH; Adviser: Prof.ª. D.Sc. Luciano Pasqualoto Canellas.

The intensification of modern agriculture has been accompanied by the increasing use of agrochemicals for disease control, which poses environmental, economic, and human health challenges, reinforcing the need for more sustainable strategies for plant health management. In this context, this thesis was developed with the objective of evaluating the potential of humic substances (HS) as plant resistance inducers, as well as understanding the physiological, biochemical, and molecular mechanisms associated with this response. Initially, it was demonstrated that HS application promotes the systemic activation of classic plant defense markers, such as phenylalanine ammonia-lyase (PAL), peroxidase (POX), and beta-1,3-glucanase (GLUC) in different agricultural crops, highlighting their ability to broadly stimulate resistance responses. Subsequently, HS from different structural sources were found to distinctly modulate defense responses in tomato plants under biotic stress, reducing disease severity and activating regulatory and effector genes associated with hormonal defense pathways. The integrated analysis of physiological, transcriptional, and proteomic responses revealed that HS structural differences drive specific metabolic adjustments in plants, resulting in both the maintenance of homeostasis and a more dynamic metabolic reprogramming upon pathogen infection. Overall, the results demonstrate that HS act as modulators of plant immunity, capable of inducing efficient and coordinated defense responses, depending on their structural origin and the stress context. These findings reinforce the potential of HS as strategic biological inputs for sustainable disease management in agricultural systems, contributing to the reduction of agrochemical dependence and to the development of a more resilient and environmentally responsible agriculture.

Keywords: Natural elicitors, plant immunity, metabolic reprogramming, hormonal signaling, antioxidant responses, sustainable management.

INTRODUÇÃO GERAL

A produção agrícola moderna enfrenta desafios crescentes relacionados à incidência de doenças em plantas, as quais comprometem de forma significativa a produtividade, a qualidade dos produtos e a sustentabilidade dos sistemas agrícolas. Culturas de elevada importância econômica, como milho, café, citros, soja e tomate, encontram-se continuamente expostas a fitopatógenos com distintas estratégias de colonização, o que exige abordagens de manejo cada vez mais eficientes e ambientalmente responsáveis (Lopes-Ferreira et al., 2022). Paralelamente, a crescente pressão por redução do uso de defensivos químicos e pela adoção de práticas agrícolas sustentáveis tem impulsionado a busca por alternativas capazes de fortalecer os mecanismos naturais de defesa das plantas. Nesse contexto, o sistema de defesa vegetal assume papel central no enfrentamento dos estresses bióticos.

As plantas apresentam um sistema de defesa altamente organizado, capaz de reconhecer sinais de ataque por patógenos e ativar respostas locais e sistêmicas que podem limitar o desenvolvimento de patógenos (Jones, 2024). A regulação dessas respostas depende de redes de sinalização altamente integradas, nas quais hormônios vegetais atuam como moduladores centrais na priorização e na intensidade das defesas ativadas (Kaur, 2023; Jones, 2024). Entre os principais agentes de estresse biótico, destacam-se os patógenos biotróficos, que se alimentam de tecidos vivos; os hemibiotróficos, que alternam entre colonização de tecidos vivos e mortos; e os necrotróficos, que colonizam e degradam tecidos mortos (Wang et al., 2014).

Nesse contexto, o ácido salicílico (AS) está amplamente associado à resistência contra patógenos biotróficos e hemibiotróficos e à ativação da resistência adquirida sistêmica (SAR), enquanto o ácido jasmônico (AJ) desempenha papel central na resistência contra necrotróficos e na resistência sistêmica induzida (ISR) (Yuan et al., 2019). A ativação coordenada e frequentemente antagônica dessas vias permite que as plantas ajustem suas respostas defensivas ao estilo de vida do patógeno e ao tipo de dano tecidual, resultando em estratégias de defesa mais eficientes, específicas e metabolicamente ajustadas ao custo fisiológico do estresse (Peng et al., 2018; Kaur, 2023). Esse ajuste dinâmico confere maior precisão às respostas de defesa das plantas, maximizando a efetividade do controle do patógeno conforme a natureza da agressão sofrida (Kaur, 2023).

Apesar dos avanços no entendimento conceitual do sistema de defesa vegetal, a transposição desse conhecimento para estratégias de manejo de doenças eficientes e consistentes em condições de campo ainda representa um desafio. Entre as principais limitações está a necessidade de abordagens que sejam simultaneamente eficazes, economicamente viáveis e alinhadas à integridade ambiental, à viabilidade econômica de longo prazo e à resiliência dos sistemas agrícolas, princípios que fundamentam a sustentabilidade no manejo fitossanitário. Nesse cenário, compostos naturais capazes de modular respostas fisiológicas e de defesa em plantas têm recebido crescente atenção da comunidade científica como alternativas ao uso intensivo de insumos químicos (Ali et al., 2024; Zhang et al., 2024). Bioestimulantes à base de substâncias húmicas (SH) podem ser recomendados para induzir a defesa sistêmica em plantas (Silva & Canellas, 2022).

As SH compõem a maior parte da matéria orgânica do solo, águas e sedimentos, resultantes da decomposição de resíduos vegetais e animais e destacam-se pela ampla distribuição no ambiente e pelos efeitos benéficos já bem descritos sobre o crescimento e o desenvolvimento vegetal (Nardi et al. 2002; 2021; Rose et al., 2014; Canellas & Olivares, 2014; Berbara & García, 2014). Tradicionalmente reconhecidas por influenciar a nutrição mineral, a arquitetura radicular e a resistência aos estresses abióticos (García et al., 2014; 2016; Jindo et al., 2020; Canellas et al., 2020; Nardi et al., 2021; Muscolo et al., 2022; Canellas et al., 2024; Zandonadi et al., 2025), essas substâncias passaram a ser investigadas também quanto ao seu papel potencial na indução de resistência a patógenos (Giovanardi et al., 2016; Silva et al., 2021; Silva & Canellas, 2022; Silva et al., 2023a; 2023b). Evidências acumuladas indicam que a aplicação de SH pode alterar o estado fisiológico das plantas, promovendo um estado de maior prontidão defensiva frente a desafios bióticos (Silva et al., 2025a; Silva et al., 2025b). Estudos recentes demonstraram que as SH podem ativar tanto a via SAR dependente de AS como a via ISR mediada por AJ/ET (Hita et al., 2020; Silva et al., 2023b; Silva et al., 2025b). Ainda assim, permanece pouco compreendido como essas respostas se organizam de forma integrada em diferentes culturas e quais vias de sinalização são predominantemente moduladas.

Diante dessas lacunas, o desafio central que motivou esta tese foi esclarecer se as SH são capazes de promover respostas defensivas consistentes e biologicamente relevantes em plantas, bem como identificar as principais rotas de

sinalização envolvidas nesse processo. Para abordar essa questão, adotou-se uma estratégia experimental integrada e progressiva. Inicialmente, avaliou-se a recorrência e a consistência das respostas associadas à defesa vegetal em diferentes culturas agrícolas de importância econômica. Em seguida, a investigação foi aprofundada em um sistema modelo, permitindo explorar de forma direcionada os mecanismos moleculares e hormonais subjacentes à indução de resistência sistêmica.

O tomateiro (*Solanum lycopersicum* L.) cv. Micro-Tom foi selecionado como planta modelo para os estudos mecanísticos por reunir vantagens experimentais, ampla caracterização prévia em estudos de imunidade vegetal e elevada relevância agronômica. A utilização de patógenos com diferentes estilos de vida possibilitou analisar, em um mesmo hospedeiro, respostas defensivas associadas a vias hormonais distintas. Nesse contexto, a análise das vias mediadas pelo AS e AJ constituiu o eixo conceitual para compreender como as SH modulam respostas de defesa específicas, associadas tanto à resistência contra patógenos biotróficos quanto necrotróficos ou hemibiotróficos.

As respostas de defesa vegetal não são universais, sendo fortemente dependentes do estado fisiológico da planta, do genótipo, do estilo de vida do patógeno e das condições ambientais (Bruce, 2014; Brzozowski et al., 2025). Quando se trata da indução mediada por SH, essa complexidade é ampliada por sua natureza intrinsecamente heterogênea e por sua diversidade estrutural e funcional (Silva et al., 2025b), fatores que podem direcionar respostas contrastantes no metabolismo (Muscolo et al., 2022) e na imunidade vegetal. Assim, distinguir entre efeitos generalizáveis e respostas condicionadas ao sistema de estudo torna-se essencial, tanto para a compreensão mecanística quanto para a proposição de recomendações agronômicas tecnicamente robustas e alinhadas aos princípios de sustentabilidade.

Nesse cenário, a indução de resistência sistêmica desponta como uma estratégia promissora para o manejo fitossanitário. Diferentemente de abordagens baseadas no controle direto do patógeno, essa estratégia atua no fortalecimento ou no preparo do sistema defensivo da própria planta, favorecendo respostas mais rápidas e metabolicamente coordenadas diante de infecções subsequentes. Além de mitigar a severidade das doenças, a indução de resistência pode contribuir para reduzir a pressão seletiva sobre populações de fitopatógenos, preservando a eficácia de ferramentas de manejo ao longo do tempo e favorecendo sistemas agrícolas mais resilientes, equilibrados e ambientalmente responsáveis (Giovanardi et al., 2016; Afifi

et al., 2017; Faccin & Piero, 2022; Silva et al., 2025b).

Diante das lacunas existentes sobre a organização integrada das respostas de defesa desencadeadas pelas SH em diferentes culturas agrícolas e sob distintos estilos de vida de patógenos, o objetivo central desta tese foi de investigar o papel das SH como indutoras de resistência e moduladoras de respostas de defesa bioquímicas, moleculares e proteômicas em plantas cultivadas. Como objetivos específicos, buscou-se: (i) avaliar a consistência e a recorrência das respostas defensivas associadas à aplicação de SH em culturas agrícolas de importância econômica; (ii) investigar como SH isoladas de diferentes fontes de matéria orgânica modulam vias hormonais e efetores associados à resistência adquirida sistêmica (SAR) em um sistema vegetal modelo; e (iii) compreender como as substâncias húmicas (SH) modulam e reconfiguram os mecanismos bioquímicos e moleculares de defesa do tomateiro durante a infecção por *Xanthomonas euvesicatoria* (Xe), um patógeno hemibiotrófico modelo, permitindo elucidar a integração e a dinâmica das vias de resistência sistêmica nas fases biotrófica inicial e necrotrófica tardia.

De forma integrada, esta tese visa contribuir para o avanço do conhecimento sobre o uso de SH como ferramentas biológicas estratégicas no fortalecimento da imunidade vegetal, na regulação da homeostase metabólica durante a infecção e na construção de estados de prontidão defensiva que conciliem crescimento, defesa e resiliência. Os resultados aqui apresentados buscam não apenas aprofundar a compreensão mecanística da indução de resistência mediada por SH, mas também fornecer bases científicas para sua aplicação como insumos biológicos no manejo sustentável de doenças, contribuindo para reduzir a dependência de agroquímicos e apoiar a transição para uma agricultura mais resiliente e ambientalmente responsável.

REVISÃO DE LITERATURA

Substâncias húmicas: natureza química e efeitos fisiológicos em plantas

As substâncias húmicas (SH) constituem a principal fração da matéria orgânica do solo, sendo formadas a partir da decomposição e transformação de resíduos orgânicos vegetais, animais e microbianos (Maffia et al., 2025). Essas substâncias estão amplamente distribuídas em solos, sedimentos e materiais orgânicos compostados, desempenhando papel fundamental na fertilidade do solo e na sustentabilidade dos sistemas agrícolas (Piccolo & Drosos, 2025).

Do ponto de vista químico, as SH apresentam elevada heterogeneidade química, contendo diferentes proporções de compostos aromáticos e alifáticos, grupos funcionais oxigenados ácidos (carbonilas, carboxilas, hidroxilas enólicas e fenólicas), resíduos de carboidratos complexos (celulose, hemicelulose e ligninas) e diferentes compostos nitrogenados (Maffia et al., 2025). Atualmente, é amplamente aceito que as SH não constituem macromoléculas poliméricas definidas, mas sim associações supramoleculares dinâmicas de moléculas orgânicas relativamente pequenas, estabilizadas por interações fracas, como ligações de hidrogênio e forças hidrofóbicas (Piccolo, 2002; Piccolo & Drosos, 2025). Essa organização em meio aquoso confere elevada plasticidade conformacional e funcional, permitindo múltiplas interações com sistemas biológicos.

Tradicionalmente, os efeitos das SH sobre as plantas têm sido associados a modificações na eficiência da absorção e uso de nutrientes, ao estímulo do crescimento radicular e a alterações na eficiência metabólica. Diversos estudos demonstram que a aplicação de SH, especialmente em baixas concentrações, promove alterações na arquitetura do sistema radicular, ativa a H^+-ATPase da membrana plasmática e favorece a absorção de nutrientes, resultando em maior crescimento e vigor das plantas (Nardi et al., 2002; Canellas et al., 2009; Zandonadi et al., 2010; Canellas & Olivares, 2014; Jindo et al., 2020; Nardi et al., 2021). Esses efeitos são frequentemente mediados por interações das SH com vias hormonais, particularmente aquelas relacionadas às auxinas (Nardi et al., 2016).

Entretanto, evidências mais recentes indicam que a atuação das SH não se restringe aos efeitos clássicos sobre crescimento e nutrição. Estudos transcriptômicos, proteômicos e bioquímicos demonstram que essas substâncias são capazes de modular a expressão de genes envolvidos no metabolismo, na sinalização hormonal, na homeostase redox e nas respostas ao estresse, sugerindo que sua ação

envolve mecanismos regulatórios compatíveis com processos de sinalização celular (García et al., 2016; Jindo et al., 2020; Olaetxea et al., 2018; Souza et al., 2022; Zandonadi et al., 2025).

A resposta das plantas às SH é fortemente dependente da fonte, da composição química, da concentração aplicada e do genótipo vegetal (Nardi et al., 2002; Canellas et al., 2015), reforçando a ideia de que diferentes materiais húmicos podem desencadear respostas fisiológicas e moleculares distintas. Nesse contexto, o reconhecimento das SH como moduladoras de processos fisiológicos e moleculares abre caminho para sua compreensão como potenciais indutoras de respostas adaptativas, incluindo aquelas associadas à imunidade vegetal.

Defesa vegetal: vias hormonais, fatores de transcrição e reguladores da resposta de defesa

As plantas desenvolveram um sistema de defesa sistêmica altamente sofisticado para reconhecer e responder a ataques de patógenos com diferentes estilos de vida. Esse sistema baseia-se na percepção de sinais de perigo e na ativação coordenada de respostas locais e sistêmicas, envolvendo alterações fisiológicas, bioquímicas e moleculares que visam limitar a colonização e a progressão da doença (Jones & Dangl, 2006).

A defesa vegetal é regulada por uma complexa rede de sinalização hormonal, na qual o ácido salicílico (AS), o ácido jasmônico (AJ) e o etileno (ET) desempenham papéis centrais. O AS está predominantemente associado à resistência contra patógenos biotróficos e hemibiotróficos e ao estabelecimento da resistência adquirida sistêmica (SAR), enquanto o AJ, frequentemente em interação com o ET, está relacionado à resistência contra patógenos necrotróficos e herbívoros, caracterizando a resistência sistêmica induzida (ISR) (Kumar et al., 2022; Pieterse et al., 2012).

A ativação dessas vias hormonais culmina em extensas reprogramações transcricionais, mediadas por fatores de transcrição específicos. Na via dependente de AS, a proteína não-expressor de genes relacionados à patogênese 1 (NPR1) atua como regulador central da SAR (Zavaliev & Dong, 2024; Liu et al., 2025). Em condições basais, a NPR1 encontra-se no citoplasma na forma oligomérica; o aumento dos níveis de AS e alterações no *status* redox celular promovem sua monomerização e translocação para o núcleo, onde interage com fatores de transcrição da família TGA, ativando a expressão de genes relacionados à patogênese (PR) (Cao et al., 1997; Liu et al., 2025). A relevância funcional da NPR1

é evidenciada pelo comprometimento severo da SAR em mutantes deficientes e pelo aumento de resistência observado em plantas que superexpressam esse regulador (Kumar et al., 2022).

Na via mediada por AJ, a regulação transcricional é controlada principalmente pelo sistema CORONATINE INSENSITIVE 1 - JASMONATE ZIM-DOMAIN (COI1–JAZ). Em níveis basais, proteínas repressoras da família JAZ inibem fatores de transcrição como MYC2. O acúmulo de jasmonoíl-isoleucina (JA-Ile) promove a degradação dessas proteínas via proteassoma, permitindo a ativação de genes associados à defesa. Além do ramo MYC (fator de transcrição), a via AJ/ET envolve fatores de transcrição da família ERF (Fator de Resposta ao Etileno), como ERF1 e ORA59 (Octadecanoid-Responsive AP2/ERF 59), importantes para a resistência contra patógenos necrotróficos (Heyman et al., 2018; Zhang et al., 2020).

A reprogramação transcricional é dependente do Complexo Mediador (CM), um coativador multissubunidades que atua como ponte entre fatores de transcrição ligados ao DNA e a maquinaria transcricional da RNA polimerase II (Pol II) (Soutourina, 2018; Zhai & Li, 2019). Em plantas, o CM contém 29 subunidades conservadas e seis específicas do reino vegetal, organizadas em quatro módulos: cabeça, meio, cauda e o módulo quinase dependente de ciclina (CDK) (Bäckström et al., 2007; Bourbon, 2008; Mathur et al., 2011). Estudos funcionais demonstraram que subunidades como MED8, MED14, MED15, MED16, MED18, MED19, MED21, MED25 e CDK8 participam da regulação de resistência sistêmica e do controle transcricional das vias de defesa mediadas por AS e AJ/ET (An & Mou, 2013; Zhang et al., 2013; Wang et al., 2015; Zhai et al., 2020; Khan et al., 2022).

A MED25 interage fisicamente com MYC2, ORA59 e ERF1, posicionando-se como um componente-chave no crosstalk hormonal AJ/ET, enquanto MED16 atua a jusante do AS, regulando a expressão de genes PR e funcionando como ponto de convergência entre as vias AS e AJ/ET (Kazan, 2017; Kidd et al., 2009; Chen et al., 2012; Zheng et al., 2006; Zhang et al., 2012). A subunidade mediadora MED14 é um importante regulador mestre na sinalização de AS. Plantas mutantes *med14* apresentam uma diminuição na expressão do gene *NPR1*. Além disso, em comparação com *MED16*, as alterações transcricionais em resposta a *Pseudomonas sp*. em plantas mutantes *med14-1* revelaram expressão diferencial de vários genes associados à SAR e ao NPR1, mostrando que MED14 e MED16 usam mecanismos distintos para regular a sinalização de AS e da SAR, o que possivelmente explica o

fato de MED16 estar envolvida no crosstalk hormonal AS e AJ/ET e MED14 somente na regulação da biossíntese e sinalização de AS (Zhang et al., 2013).

Outro grupo relevante na modulação da defesa vegetal são as Nudix hidrolases, enzimas que hidrolisam derivados de nucleosídeo difosfato e metabólitos sinalizadores, atuando tanto na homeostase fisiológica quanto no ajuste da amplitude das respostas imunes. Em Arabidopsis, a família Nudix é composta por 29 membros (AtNUDTs), dos quais AtNUDX6 e AtNUDX7 possuem papéis comprovados na imunidade (Dong & Wang, 2016). AtNUDT7 atua como regulador negativo da defesa: sua perda de função resulta em plantas hiper-responsivas ao estresse biótico e com maior proporção GSSG/GSH, sugerindo desequilíbrio redox e predisposição à ativação imune exacerbada (Dong & Wang, 2016). Já AtNUDX8, identificado como regulador positivo da defesa, participa da sinalização de AS, modulando a expressão de *ICS1, NPR1 e PR1*, e sua mutação aumenta a suscetibilidade a oomicetos (Hpa) e a bactérias (*Pseudomonas syringae*) (Zhang et al., 2012). Em tomateiro, membros da família Nudix também apresentam conservação funcional, incluindo a atuação de NUDX8 na regulação da resposta de defesa. No presente trabalho, a expressão do gene *NUDX8* foi avaliada em plantas de tomateiro, reforçando a relevância desse regulador em culturas agrícolas além do sistema modelo (Liu et al., 2019).

Em conjunto, o recrutamento coordenado das vias hormonais, FTs, CM e enzimas Nudix confere elevada plasticidade regulatória à imunidade vegetal, permitindo respostas sistêmicas ajustadas em intensidade, especificidade e custo fisiológico, sem ação direta sobre o patógeno, mas por modulação endógena da transcrição e da homeostase molecular das plantas (Conrath et al., 2006; Walters et al., 2013).

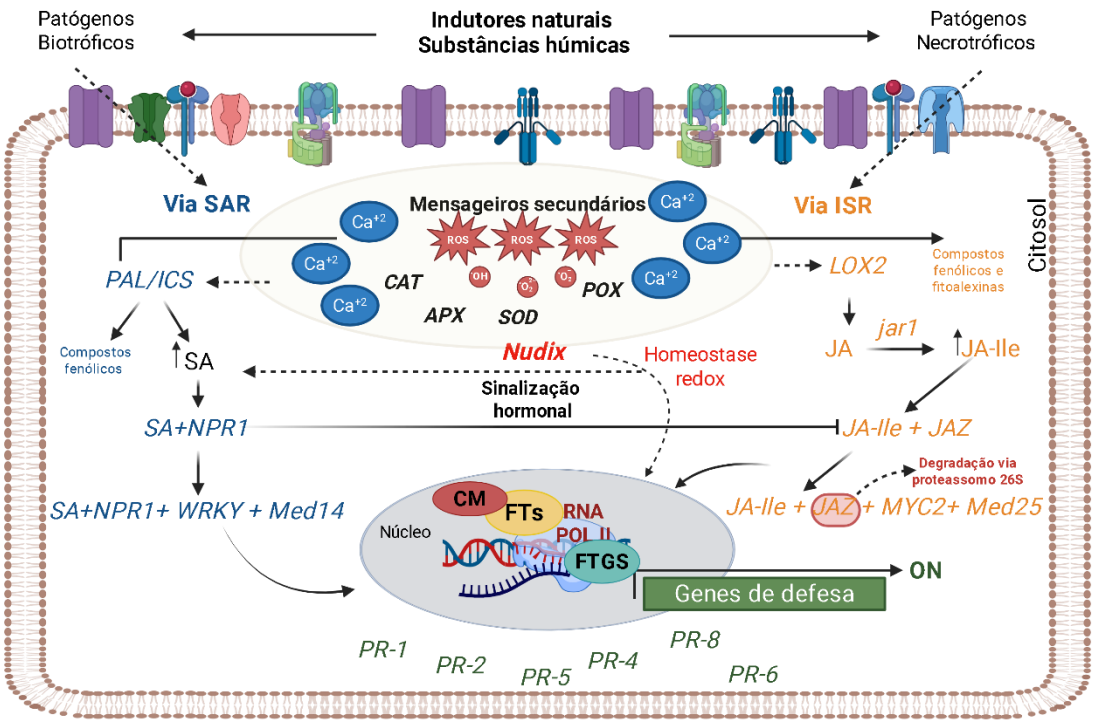

Figura 1. Visão esquemática da ativação da defesa vegetal em plantas sob ataque de patógenos biotróficos, necrotróficos, hemibiotróficos e ou indutores de resistência sistêmica (como substâncias húmicas). A Resistência Sistêmica Adquirida (SAR) é ativada após a infecção por patógenos biotróficos e hemibiotróficos e tem como principal mediador o ácido salicílico (SA), desencadeando a ativação de NPR1, fatores de transcrição WRKY, acúmulo de compostos fenólicos através da via da fenilalanina amônia-liase PAL, e aumento da atividade de enzimas do metabolismo redox/antioxidante como catalase (CAT), peroxidase (POX), ascorbato peroxidase (APX) e superóxido dismutase (SOD) e indução de genes de defesa do tipo PR (pathogenesis-related). A Resistência Sistêmica Induzida (ISR) é mediada pelo ácido jasmônico (JA) e sua forma bioativa conjugado a uma isoleucina (JA-Ile), iniciando com a lipoxigenase (LOX2), que desencadeia a biosíntese do JA. A expressão dos genes relacionados à defesa dessa via é regulada pelo Domínio ZIM do Jasmonate (JAZ), e a ativação do fator de transcrição de MYC2 e a síntese de fitoalexinas é dependente da degradação de JAZ via proteassoma 26S, culminando na expressão de genes de defesa proteínas relacionadas à patogênese (PR). A enzima Nudix é apresentada no centro de sinalização redox/ROS como um regulador da homeostase redox e da sinalização de defesa derivada de espécies reativas de oxigênio (ROS), atuando no equilíbrio e na prontidão das respostas de defesa antes da reprogramação transcricional nuclear.

Bioestimulantes na agricultura: indução de resistência e sustentabilidade dos sistemas produtivos

A intensificação da agricultura nas últimas décadas tem sido acompanhada pelo aumento da pressão de pragas e doenças, resultando em elevada dependência de agrotóxicos para garantir a produtividade das culturas. De eficácia controversa, mesmo em curto prazo, esses insumos químicos apresentam limitações importantes,

incluindo impactos ambientais, riscos à saúde humana e a seleção de populações de patógenos resistentes. Nesse contexto, estratégias que fortaleçam os mecanismos naturais de defesa das plantas têm sido amplamente discutidas como alternativas sustentáveis para o manejo fitossanitário (Walters et al., 2013; Meena et al., 2022).

No Brasil, o debate sobre a redução da dependência de insumos químicos ocorre em paralelo a mudanças regulatórias e programas governamentais que coexistem no cenário institucional. O Decreto nº 12.538/2025 instituiu o Programa Nacional de Redução de Agrotóxicos (PRONARA), com diretrizes para a redução gradual do uso de agrotóxicos e o estímulo à adoção de bioinsumos no manejo agrícola (BRASIL, 2025). Simultaneamente, novos registros de produtos agrotóxicos mantiveram-se elevados no período 2024–2025, incluindo recordes de aprovações pelo Ministério da Agricultura (BRASIL, 2025). Essa sobreposição de tendências evidencia os desafios institucionais associados à transição para modelos agrícolas menos dependentes de insumos químicos.

Entre as estratégias baseadas em insumos biológicos, destacam-se produtos e microrganismos com efeitos bioestimulantes. Embora o termo bioestimulante não tenha sido originalmente definido como categoria regulatória na legislação de fertilizantes (IN SDA nº 61/2020), substâncias húmicas, extratos vegetais, extratos de algas, aminoácidos e hidrolisados proteicos eram enquadrados como biofertilizantes, enquanto microrganismos benéficos eram regulamentados como inoculantes, sob normas específicas do MAPA (BRASIL, 2020). A sanção da Lei nº 15.070/2024 estabeleceu um novo marco legal para bioinsumos, incluindo bioestimulantes, biofertilizantes e inoculantes como categorias reconhecidas no país (BRASIL, 2024). Do ponto de vista científico, compostos e microrganismos com efeitos bioestimulantes são definidos como agentes capazes de estimular processos naturais das plantas, influenciando a eficiência nutricional, a tolerância a estresses e atributos de qualidade dos produtos agrícolas, independentemente do fornecimento direto de nutrientes (Du Jardin, 2015). Diferentemente de fertilizantes e agrotóxicos convencionais, esses insumos atuam predominantemente por meio de ajustes fisiológicos e moleculares nas plantas, incluindo a modulação de vias hormonais e redes regulatórias associadas à adaptação ao estresse.

Entre os efeitos associados aos bioinsumos com atividade bioestimulante, a modulação de respostas sistêmicas de defesa tem recebido crescente atenção. Essa abordagem fundamenta-se no conceito de priming de defesa, no qual plantas

expostas previamente a indutores apresentam ativação mais rápida e de maior amplitude de vias endógenas de resistência quando submetidas a ataques subsequentes de patógenos. A resistência induzida, incluindo a SAR e a ISR, não atua pelo controle direto do patógeno como alvo primário, mas sim pela regulação coordenada de redes hormonais e vias de defesa da própria planta, influenciando a severidade da doença e a dinâmica de seleção de populações fitopatogênicas (Conrath et al., 2006; Walters et al., 2013). Esse processo reduz a progressão dos sintomas e a severidade das doenças, além de atenuar a pressão seletiva para a evolução de resistência em fitopatógenos (Conrath et al., 2006; Walters et al., 2013).

Diversos compostos naturais têm sido descritos como indutores de resistência, incluindo oligossacarídeos, extratos vegetais, microrganismos benéficos e componentes da matéria orgânica do solo. Os bioestimulantes à base de SH destacam-se por sua ampla disponibilidade, baixa toxicidade e histórico consolidado de uso na agricultura. Evidências experimentais indicam que esses produtos podem ativar respostas associadas à defesa vegetal, incluindo o aumento da atividade de enzimas marcadoras de defesa, a modulação da expressão gênica e a redução da severidade de doenças em diferentes culturas (Giovanardi et al., 2016; Afifi et al., 2017; Silva & Canellas, 2022).

O interesse crescente por bioestimulantes também se reflete em sua expansão comercial, indicando a consolidação dessa tecnologia como componente estratégico da agricultura moderna. O mercado global de bioestimulantes atingiu aproximadamente US$ 4,3 bilhões em 2024, com projeções de crescimento contínuo, podendo alcançar cerca de US$ 7,6 bilhões em 2029, o que corresponde a uma taxa média de crescimento anual em torno de 12% entre 2024 e 2029 (Markets & Markets, 2024). Esse cenário evidencia não apenas a aceitação crescente desses insumos pelos sistemas produtivos, mas também a necessidade de embasamento científico sólido que sustente sua aplicação racional e eficiente.

No caso específico das SH, o interesse como biofertilizante está associado à sua capacidade de atuar em múltiplos níveis da organização vegetal. Além dos efeitos clássicos sobre crescimento e nutrição, essas substâncias podem influenciar vias hormonais centrais da defesa vegetal, como aquelas mediadas por AS e AJ, conforme discutido nos tópicos anteriores. Essa atuação multifacetada é particularmente relevante para a agricultura sustentável, pois permite integrar ganhos de produtividade com maior resiliência das plantas a estresses bióticos.

Outro aspecto importante diz respeito à variabilidade de respostas associadas ao uso de bioestimulantes. A eficácia desses produtos depende de fatores como a composição química do bioestimulante, a concentração aplicada, o genótipo vegetal e as condições ambientais (Nardi et al., 2002). A heterogeneidade química e a origem diversa das SH podem resultar em efeitos fisiológicos e defensivos distintos, reforçando a necessidade de estudos que considerem diferentes fontes e contextos de aplicação (Canellas et al., 2015; Rouphael & Colla, 2020).

Assim, a incorporação de bioestimulantes à base de SH em sistemas agrícolas representa uma estratégia promissora para reduzir a dependência de agrotóxicos e promover o manejo integrado de doenças. Contudo, para que seu uso seja tecnicamente consistente e amplamente adotado, é fundamental compreender os mecanismos pelos quais esses produtos modulam a imunidade vegetal e como essas respostas se traduzem em maior resistência a patógenos em diferentes sistemas de cultivo. Diante dessa lacuna de conhecimento, foram desenvolvidos os estudos apresentados nesta tese, com o objetivo de ampliar a compreensão sobre o tema.

ARTIGO 1: HUMIC SUBSTANCES PROMOTE THE ACTIVITY OF ENZYMES RELATED TO PLANT RESISTANCE

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Article

Humic substances promote the activity of enzymes related to plant resistance

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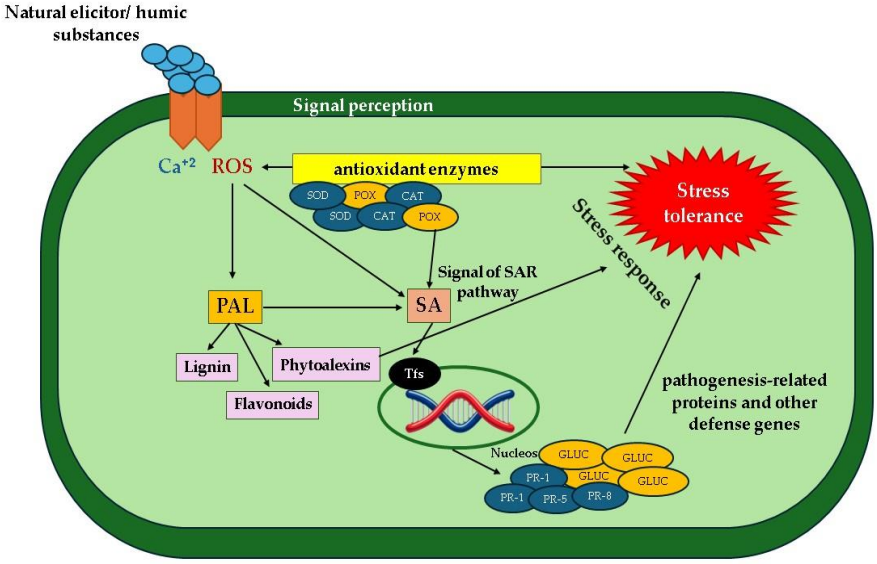
Abstract: The extensive use of pesticides has significant implications for public health and the environment. Breeding crop plants is the most effective and environmentally friendly approach to improve plant resistance. However, it is time-consuming and costly, and it is sometimes difficult to achieve satisfactory results. Plants induce defense responses to natural elicitor through the expression of multiple genes that encode proteins, including enzymes, secondary metabolites, and pathogenic proteins (PR). These responses characterize systemic acquired resistance. Humic substances trigger positive local and systemic physiological responses through a complex network of hormone-like signaling pathways and can be used to induce biotic and abiotic stress resistance. This study aimed to assess the effect of humic substances on the activity of phenylalanine ammonia-lyase (PAL), peroxidase (POX), and β-1,3-glucanase (GLU) used as a resistance marker in various plant species, including orange, coffee, sugarcane, soybeans, maize, and tomato. Seedlings were treated with a dilute aqueous suspension of humic substances (4 mM C L^{-1}) as a foliar spray or left untreated (control). Leaf tissues were collected for enzyme assessment two days later. Humic substances significantly promoted the systemic acquired resistance markers activities compared to the control in all independent assays. Overall, all enzymes studied in this work, PAL, GLUC, and POX, showed an increase in activity by 133%, 181%, and 149%, respectively. Among the crops studied, citrus and coffee achieved the highest activity increase in all enzymes, except for POX in coffee, which showed a decrease of 29% compared to the control. GLUC exhibited the highest response to HS treatment, the enzyme most prominently involved in increasing enzymatic activity in all crops. This suggests the possibility of using this natural organic matter component to trigger the plant defense system

as a component of a disease management program due to its proven ability to activate systemic acquired resistance.

Keywords: β-1,3 glucanase; phenylalanine ammonia-lyase; peroxidase; elicitors; defense response; sustainable agriculture.

Graphical abstract



1. Introduction

The cultivation and farming production of crops for conversion into commodities and agroenergy has led to the widespread use of pesticides. In Brazil, soybean, corn, coffee, orange, and sugarcane account for 80% of all pesticides used [1]. FAO states that Brazil consumes approximately 20% of all pesticides sold globally [2]. Although mancozeb, atrazine, acephate, chlorothalonil, and chlorpyrifos are banned in Europe, they are still indiscriminately used as pesticides, posing risks to the environment and public health. These pesticides have been found to have teratogenic, carcinogenic, or mutagenic characteristics, which can cause hormone disorders, damage to the reproductive system, and neurotoxicity, among other effects [3]. Significant amounts of agricultural pesticide residues were found in sediments from two protected Brazilian National Parks located in mountainous regions far from intense agricultural activity [4]. This finding reveals the vulnerability of long-distance transport of contaminants.

Adopting the agroecological management model, based on biodiversity, differentiation, efficiency, and recycling, is recommended to eliminate the risks of human and environmental toxicity from pesticides. This model balances the externalities of pests and disease control with an economically viable level of productivity. The first step towards this transition is the progressive substitution of synthetic agrochemicals with biological inputs [5]. It is widely recognized that a higher organic matter content and better-quality organic input lead to a healthier agroecosystem, increasing plant productivity and improving crop quality [6].

Various natural compounds have been reported to protect plants from invaders through inducing a priming state leading to enhanced resistance against subsequent infection in a process named induced resistance [7]. Pathogen-related proteins (PR-proteins) belongs to a systemic acquired resistance (SAR) response to salicylic acids as a cell signaling molecule. Induced systemic resistance (ISR) is characterized by the production of several secondary metabolites, such as anthocyanins, flavonoids and galactolipids, as well as activating signaling pathways, such as ethylene and jasmonates, resulting in minimal changes to plant morphology [8, 9]. Humic substances (HS) can activate both the salicylic acid (SA)-dependent SAR pathway and the SA-independent ISR pathway [10, 11].

Phenylalanine ammonia-lyase (PAL), peroxidases (POX), and 1,3 β-glucanase (GLU) are markers for the induction of the mechanism involved in SAR response. Phenolic compounds, such as flavonoids, phenylpropanoids, pterocarpans, isoflavans, prenylated isoflavonoids, stilbenes, psoralens, coumarins, 3-deoxy anthocyanidins, flavonols, and aurones, protect plants against a wide range of biotic stresses [12, 13]. PAL catalyzes the initial step in the biosynthesis of phenolics by converting phenylalanine to trans-cinnamic acid and tyrosine to *p*-coumaric acid. According to Schiavon et al. [14], the activity of PAL was significantly higher in maize seedlings treated with HS than in the control group. The increase in PAL activities was followed by an enhancement of PAL expression and the content of total phenolic acids and flavonoids. Phenylpropanoid metabolism produces antimicrobial compounds that are synthesized in response to pathogen attacks. Salicylic acid (SA) levels increase in response to infection, but instead of having antimicrobial activity, it is believed to be part of a signaling process that results in SAR [15].

Peroxidases are oxidoreductive enzymes that contribute to restoring and maintaining cell wall polysaccharide functionalities. They are involved in phenol oxidation, suberization, auxin metabolism, phytoalexin synthesis, cross-linking of cell wall components, and host plant cell lignification during the defensive response against pathogens [16]. In plant cell protection, POX plays a crucial role in inducing defensive responses or resolving the harmful effects of oxidative stress. An enhanced activity of POX has been detected in various plants, including oat, tomato, sugarcane, patchouli, and shallots, when infected by different pathogens such as *Colletotricum falcatum*, *Fusarium graminearum*, and *Ralstonia solanacearum, Pseudomonas fluorescens, Alternaria solani, Septoria lycopersici, Xanthomonas axonopodis, Fusarium avenaceum,* and *Tobacco Mosaic Virus S. pactum* Act12 [17].

β-1,3-glucanases protect plants against fungal pathogens alone or with chitinases and other antifungal proteins from the PR-2 family [18]. Additionally, β-1,3-glucanases are responsible for mobilizing callose, which regulates symplastic trafficking by degrading mixed linkage glucan through plasmodesmata [19]. Plant β-1,3--glucanases play a crucial role in regulating the outcome of hostile plant-microbe interactions by degrading non-self glucan structures and hydrolyzing β-glucans found in the cell walls of microbes. This activity includes supporting a local antimicrobial defense barrier and generating signaling glucans that activate a global response [19].

HS are the major component of natural organic matter in soil, water, and sediments [20]. They can be used as an effective alternative to alleviate symptoms and improve tolerance to various plant diseases [21]. HS also stimulates plant growth and enhances their resistance to abiotic stress, reducing the need for chemical inputs in crop production [22]. In addition, it is suggested that HS may trigger positive local and systemic physiological responses through a complex network of hormone-like signaling pathways, including nutrient transporters, plasma membrane H^+-ATPases, hormone routes, genes/enzymes involved in nitrogen assimilation, cell division, and plant development [22]. This stimulation framework promotes plant growth and yield [23, 24, 25].

This study aimed to assess the effectiveness of HS as a natural elicitor for SAR in plants. Enzymatic activities associated with the SAR response, i.e. PAL, POX, and β-1,3-glucanase, were analyzed. HS was extracted from peat and applied to various crops, including coffee, maize, orange, sugarcane, soybeans, and tomato.

2. Materials and Methods

The study was conducted to monitor the activity level of SAR marker enzymes (PAL, POX, and GLUC) in leaf extracts from various plants (maize, coffee, citrus, soybean, sugarcane, and tomato) two days after the application of a suspension of HS via foliar spray.

2.1. Humic substances

Commercial HS from peat (Iridium®, Ourinhos, São Paulo, Brazil) with 30 g L^{-1} of organic carbon were diluted in water until a concentration of 48 mg of Total Organic Carbon (C)L^{-1} for plant leaves application. HS used in the study was analyzed by solid State NMR spectroscopy using Cross-Polarization Magic Angle Spinning Carbon-13 Nuclear Magnetic Resonance (CPMAS ^{13}CNMR) performed on a Bruker AMX 400 operating at 100.625 MHz on carbon-13. The rotor spin rate was set at 4500 Hz. A recycle time of 1 s and an acquisition time of 13 ms were used with Variable Contact Time (VCT) pulse sequence and Optimum Contact Time (OCT) of 1 ms. A line broadening of 50 Hz was used to transform the free induction decay (FID). The overall chemical shift range was split into six regions, related to the main organic functional groups: 0-45 ppm (aliphatic-C): 50.2%, 45-60 ppm (methoxyl-C and N-alkyl-C): 3.7%, 60-110 ppm (O-alkyl-C): 9.8%, 110-145 ppm (aromatic-C): 22.7%, 145-160 ppm (O-aryl-C):3.5%, 160– 190 ppm (carboxyl-C): 10%. The CPMAS ^{13}C NMR spectrum of HS used in the study is shown in Figure 1.

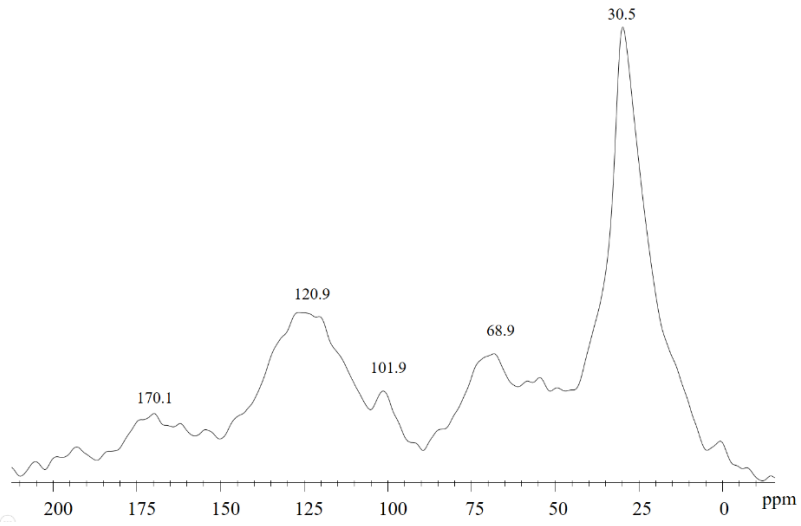


Figure 1. CP/MAS ^{13}C NMR spectrum of HS used in this study

2.2. Enzymes activity

Phenylalanine Ammonia-lyase Assay (PAL, EC 4.3.1.5): The extraction of the PAL enzyme was carried out following the method described by Pascholati et al. [25], with modifications. 100 mg of leaves were macerated in liquid N_2 and homogenized with 10 mL of extracting solution of 0.1 M L^{-1} sodium borate buffer, pH 8.8 (added with 5% w/v of polyvinylpyrrolidone (PVP) and 1.2 mL L^{-1} of β-mercaptoethanol) and subsequently centrifuged for 25 minutes at 10,000 *g* at 4 °C. After centrifugation and separation of the extract, the enzymatic reaction medium was composed of 1 mL of crude enzymatic extract + 1 mL of 0.1 M phenylalanine solution + 1 mL of 0.2 M L^{-1} sodium borate solution (without PVP and β-mercaptoethanol). The samples were incubated in a water bath (36 °C) in the dark for 60 minutes, after which the reaction was stopped with 200 µL of 6 M L^{-1} HCl and the absorbance was measured with spectrophotometer at 290 nm in quartz cuvettes. The enzyme activity (EA) was expressed in µmol transcinnamic acid min^{-1} fresh weight (FW^{-1}) using the equation: $EA = (A/60) \times (V/v \times d \times C\varepsilon 290)$, where A= absorbance of sample – absorbance of blank) – blank of solution; 60: time of reaction = 60 min; V= 3 mL; v= 1 mL; d= 1 cm; Cε290 (molar extinction coefficient at 290 nm) = 10^4.

Peroxidase (POX, E.C.1.11.1.7): Leaves extract: 1 g of fine powder using liquid N_2 was transferred to a 15 mL falcon tube with 1% (v/v) polyvinylpyrrolidone (PVP) and 5 mL of sodium acetate buffer (0.1 M L^{-1}, pH 5) and 1 mL of EDTA (1 mM L^{-1}, pH 5). The extracts were centrifuged at 10,000 *g* for 10 minutes at 4 °C, and the 5 mL of supernatant was transferred to 1.5 mL microfuge tubes and stored at -20 °C. The supernatant was used to evaluate both β-1,3-glucanase and POX activities, and soluble protein content using the Bradford method. POX activity was determined at 30 °C according to the method described by Hammerschmidt et al. [26]. The reaction medium was composed of 50 µL of guaiacol (0.02 M L^{-1}), 0.5 mL of hydrogen peroxide (0.38 M L^{-1}) and 2.0 mL of phosphate buffer (0.2 M, pH 5.8). 50 µL of the enzyme extract was added and read at a wavelength of 470 nm. The results were expressed in µmol of H_2O_2 decomposition min^{-1} g^{-1} FW using the following equation

EA = [(A x TV)/(Cε470 x t x v)]/FW, where EA; enzyme activity; TV (Total reaction volume) = 2.65 mL; Cε470 (molar extinction coefficient at 470 nm) = 26.6; t = time of reaction (5 min); v= volume of sample (100 μL)

1,3 β -glucanase (GLUC, E.C.3.2.1.29): The β-1,3 -glucanase activity in the samples was determined by the colorimetric quantification of glucose released from laminarin using p-hydroxybenzoic acid hydrazide (HAPHB) [27]. The reaction medium consisted of 150 μL of enzymatic extract and 150 μL of laminarin (2.0 mg/mL) incubated at 40 °C for 60 min. After this time, 50 μL of reaction medium was mixed with 1.5 mL of *p*-hydroxybenzoic acid hydrazide (1 g dissolved in 20 mL of 0.5 M HCl plus 80 mL of 0.5 M NaOH) and heated at 100 °C for 10 min. Afterward, the reaction was cooled to 30 °C on ice, and the absorbance was determined at 410 nm against the blank (50 μL extraction buffer + p-hydroxybenzoic acid hydrazide heated at 100 °C for 10 min). Finally, each sample result was subtracted from the control value (corresponding to a mixture identical to that of the sample, but without prior incubation). The final results were plotted on a standard curve for glucose, and the results expressed in μg glucose min^{-1} mg^{-1} protein.

2.3. Plant assays

The experiments with the different crop plants were carried out independently throughout 2024 using a completely randomised experimental design with four biological replicates and two treatments: application of the SH suspension at a concentration of 48 mg C L^{-1} via foliar spray to the dripping point or water application (control). The leaves were collected 48 hours after the application of the treatments. The following section details the varieties and growing conditions employed in each experiment.

Maize - Maize seeds (*Zea mays* Dekalb VT PRO 3) were surface sterilized by soaking in 0.5% (w/v) NaClO for 30 min, rinsing, and soaking in water for 6 h. Afterward, the seeds were sown on wet filter paper and germinated in the dark at 28 °C. Four-day-old maize seedlings with roots approximately 5 cm long were transferred into a solution containing 2 mM L^{-1} $CaCl_2$. A minimal medium ($CaCl_2$ 2 mM) has been used in this work to avoid any interference from nutrient constituents that could function synergistically along with HS on plant growth and metabolism. After 48 h of treatment, leaves were collected, ground in N_2, and after obtaining the enzymatic extract as described in the previous section, the material was stored in the freezer (-20 °C) until analysis.

Coffee: Coffee seedlings (*Coffea arabica* var red Acauã) purchased from a commercial nursery were transplanted into 7 L pots filled with a surface layer (0-20 cm) of Oxisol. The plants received irrigation and 3 fertilizations of NPK 10-10-10 for 18 months until the first treatment was applied with an aqueous suspension of peat HS at 4 mM C L^{-1} via leaf spray. After 48 h of application of the aqueous suspension, the leaves were collected and crushed in N_2 and after obtaining the enzymatic extract, the material was stored in a freezer (-20 °C) until analysis.

Citrus: Assay 1: Two sweet oranges (*Citrus sinensis*) cultivars Baia Seleta were planted in the locality of Lagoa de Cima, Campos dos Goytacazes - State of Rio de Janeiro, Brazil, 21°44'24.6 "S 41°32'07.8 "W in an Oxisol, according to US Soil Survey Classification System. The experiment was entirely randomized, with five plants per treatment spaced in 3.5 m x 5 m. The both cultivars with one year old were planted in October 2021 and

October 2022, respectively. The aqueous suspension of HS with 48 mg C L^{-1} at pH 6.8 was applied one month after planting to the leaf's surface using a backpack sprayer at 500 mL per plant. The leaves were collected 48 h after application, ground in liquid N_2 and after obtaining the enzymatic extract, the material was stored in the freezer (-20 °C) until analysis.

Assay 2: Commercial orchards located at Santa Cruz do Rio Pardo municipality in São Paulo State, Brazil, were treated with HS using a tractor sprayer with 12 L of commercial humic liquid product diluted in 2,400 L ha^{-1}, corresponding 48 mg C L^{-1}. The orchards are composed of orange cv Natal, cv Pera and cv Valencia, with 16, 7 and 3 years old, respectively .Leaf samples from both orchards were randomly taken from leaflets near the fruit on ten different trees per cultivar treated or not treated (control). The leaves were collected 48 h after the treatments and stored in liquid N_2 for transport. After grounding in liquid N_2 and obtaining the enzymatic extract, the material was stored in the freezer (-20 °C) until analysis.

Soybeans: The studies were conducted on soybean seedlings of cv NS6700, 63i64, 68i66. Soybean seeds were washed with water and sown in a pot (700 mL) filled with a superficial layer (0-20 cm) of Oxisol. Four replicates were used in a randomized statistical design. One week after germination, thinning was performed, and two seedlings were maintained per pot. Irrigation was done daily, with 200 mL of water. At the vegetative stage with three true leaves (V3 stage), an application of 80 mL per pot with HS at 48 mg C L^{-1} at pH 6.0. The leaves were collected after 48 h of application, ground in liquid N_2, and after obtaining the enzymatic extract, the material was stored in the freezer (-20 °C) until analysis was performed.

Sugarcane: sugarcane pre-sprouted seedlings RB966928, RB 855155, CTC7515 BT and CTC4 were transferred to a pot (700 mL) filled with commercial growth substrate (Basaplant). After one week of transplanting, 100 mL of aqueous suspension of HS with 48 mg C L^{-1}, pH 6.0 or with water (control) was applied to the seedlings using a hand sprayer. The leaves were collected after 48 h of treatment, ground in liquid N_2, and after obtaining the enzymatic extract, the material was stored in the freezer (-20 °C) until analysis.

Tomato: *Solanum lycopersicum* L. cv. Micro-Tom (MT) was used. MT seeds were previously disinfested and germinated in commercial organic substrate (Basaplant)/vermiculite 2:1 (v:v). After germination, the seedlings were transplanted into individual 300 mL pots, and 15 days after transplanting, the 50 mL of HS at 48 mg C L^{-1} at pH 6.0 or water (control) was added to the pots; four replicates were submitted to each treatment in a completely randomized design. The leaves were collected 48 h after the treatments, ground in N_2, homogenized with extraction buffer, and stored in a freezer (-20 °C) until analysis.

2.4. Data analysis

To evaluate the effect of HS across the different experiments conducted on various plant species, statistical analysis was first performed for each plant species. A Student t-test was applied to compare enzymatic activity between control and humic treated groups. This allowed for an initial assessment of treatment effects within each individual experiment. Subsequently, a meta-analysis was conducted

using the metafor package [30] in R software (version 4.0.3; R Development Core Team, 2020).

Initially, the enzymatic activity data from the humic-treated groups were corrected relative to their respective controls. From these corrected values, the mean, maximum, and minimum values were calculated. For standardized comparison across experiments, control group values were used as a reference and normalized to zero. Since the experiments included in the analysis differed in their methodologies, plant species, and environmental conditions, it was assumed that the true effect size might vary between studies. Therefore, a random-effects model was adopted, which assumes that each study estimates a different true effect, drawn from a distribution of effects. The model is represented by the equation: $\theta_i = \mu + \mu_i$, where $\mu_i \sim N(0, \tau^2)$. In this model, θ_i represents the true effect in study *i*, μ is the overall average effect, and τ^2 is the between-study variance (heterogeneity). The effect size was calculated using the standardized mean difference (with bias correction, Hedges' *g*) between the treated and control groups. This was done using the escalc ( ) function in the metafor package, with the argument measure = "SMD". The function requires, for each study, the mean (b_i) and standard deviation (a_i) of the treated group, the mean (d_i) and standard deviation (c_i) of the control group, and the number of replicates (n). The function outputs two main variables: $\mathbf{y_i}$ (effect size) and $\mathbf{v_i}$ (sampling variance of the effect). The random-effects model was fitted using the rma ( ) function with the restricted maximum likelihood (REML) method. The heterogeneity among studies was assessed through the estimate of τ^2. Finally, a forest plot was generated using the forest ( ) function, showing the individual effect estimates (y_i) and their 95% confidence intervals, as well as the overall pooled effect (μ). This approach allowed for the integration of results from different experiments, providing a more robust and reliable estimate of the impact of humic substances on the enzymatic activities evaluated.

3. Results

This study measured the enzymatic activity of three proteins that are known to be involved in the SAR. In general, PAL activity was increased by 76% compared to the control, regardless of the crop plants. In addition, GLUC and POX were increased by 191% and 255%, respectively, compared to the control. The activity of enzymes used as markers of SAR was significantly affected by HS when the experiments were analyzed independently (Figure 1 and 2). The PAL activity promotion in the citrus treated with HS was higher than the overall average (128% vs 76%) (Figures 1 a,d,g,j and m). In Rio de Janeiro, PAL activity increased by 57% and 108% for plants aged one and two years, respectively, compared to the control (Figures 1 a and d). In São Paulo, the trees aged three years exhibited a 109% increase, while the 7-year-old and 16-year-old citrus trees showed enhancements of 157% and 160%, respectively, compared to the control (Figures 1 g, j and m). The PAL activity increase caused by HS in coffee was similar to that observed in citrus, resulting in a 122% enhancement compared to the control (Figure 1 v). However, unlike citrus, which was evaluated in four different experiments, the PAL activity in coffee was only observed in one experiment with four repetitions (n=4). In maize seedlings, PAL activity was 260% higher than in the control (Figure 1 n). In soybeans (Figures 2 a, d and g) and tomatoes

(Figure 1 s), PAL activity was 48% and 53% higher, respectively while in sugarcane was 21% higher than in the control. Although significant differences were observed among varieties, all responded positively compared to untreated plants.

The activity of β-1,3 Glucanase (GLUC) was also promoted by HS, resulting in a general increase of 191% compared to untreated plants. In citrus, the promotion of GLUC activity was even higher, with a 316% increase compared to the control (Figures 1 e, h, k and n). Coffee plants treated with HS showed a 273% increase in GLUC activity compared to the control plants (Figure 1 x). Similarly, maize seedlings treated with HS exhibited a significant increase in GLUC activity, with an average increase of 160% compared to the control (Figure 1 q).

HS significantly affected the POX activity (Figures 1 and 2). On average, there was a 1.5-fold increase compared to control plants. However, the range of POX variation was broad, with an increase of 2,363% observed in citrus var Seleta at Rio de Janeiro (Figures 1 c, f, i, l and o). The activity of POX was 29% higher in maize seedlings treated with HS compared to control plants. The overall effect of HS application on the activity of PAL, GLUC, and POX enzymes can be observed in Figures 3 and 4. The enzyme activities were promoted by HS highlighting their use as a natural elicitor of SAR.

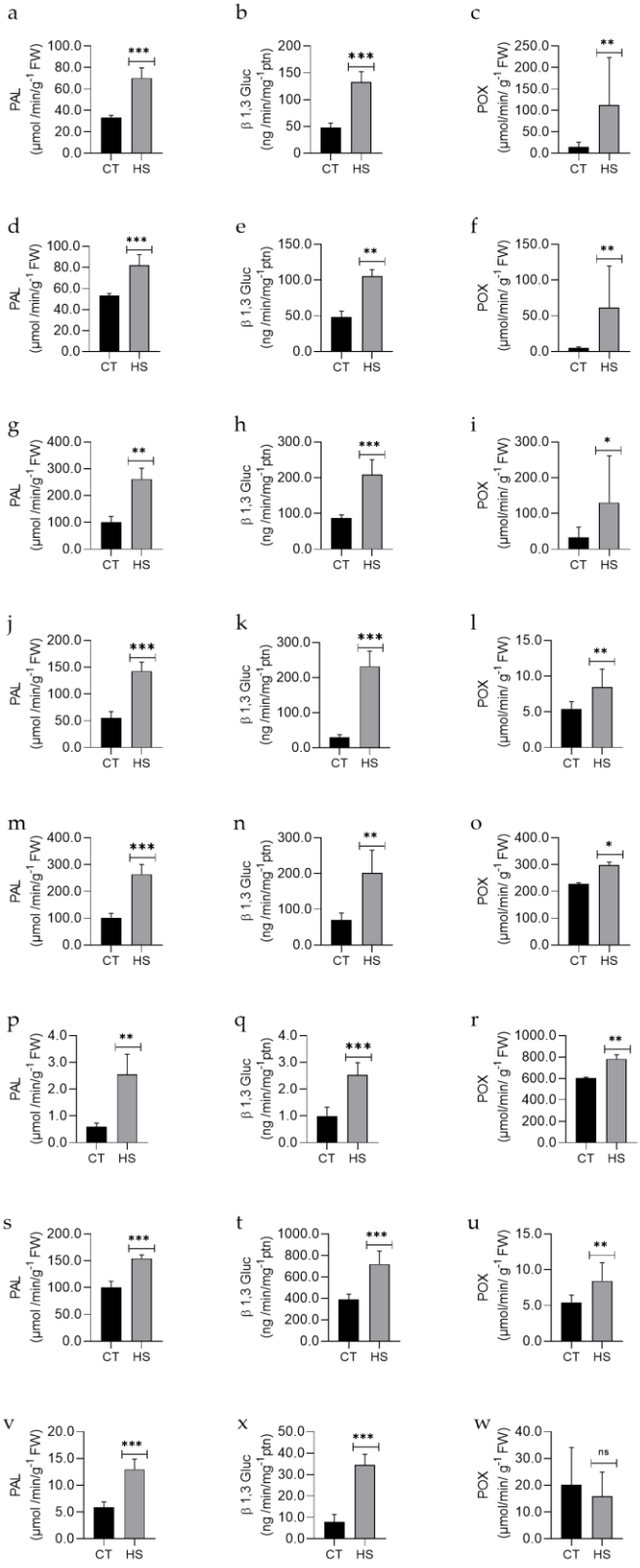


Figure 1. Enzymatic activity of phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (GLUC), and peroxidase (POX) in different crops. Bars represent mean values from independent experiments with citrus cv. Baía (a, b, c), cv. Seleta (d, e, f), cv. Natal (g, h, i), cv. Pera (j, k, l), cv. Valência (m, n, o), maize (p, q, r), tomato (s, t, u), and *Coffea arabica* (v, w, y), respectively. The enzymes assessed in each crop were PAL (first column), GLUC (second column), and POX (third column). Data were analyzed using Student's *t*-test. Asterisks indicate statistically

significant differences compared to the control: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). "ns" indicates no statistically significant difference ($p > 0.05$).

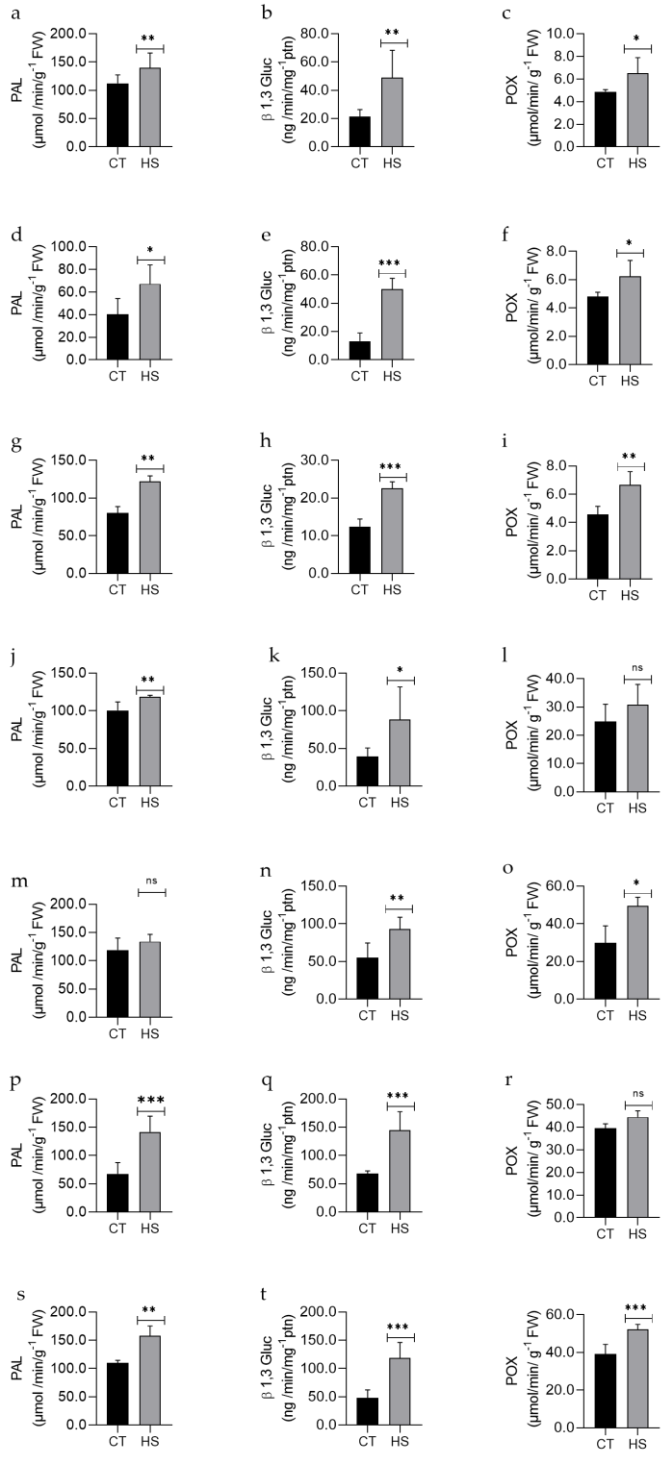


Figure 2. Enzymatic activity of phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (GLUC), and peroxidase (POX) in different crops. Bars represent mean values from independent experiments with soybean var. 66i68 (a, b, c), var. NS6700 (d, e, f), var. 64i63 (g, h, i), Sugarcane var. CTC4 (j, k, l), var. CTC 7515BT (m, n, o), var. RB 966928 (p, q, r), and var. RB855155 (s, t, u), respectively. The enzymes assessed in each crop were PAL (first column), GLUC (second column), and POX (third column). Data were analyzed using Student's *t*-test. Asterisks indicate statistically

significant differences compared to the control: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). "ns" indicates no statistically significant difference ($p > 0.05$).

A meta-analysis was performed using enzymatic activity data to integrate the effects of HS treatment on different plant defense related enzymes, accounting for variability across experiments conducted in distinct plant species and experimental conditions. This statistical approach enables the calculation of a global treatment effect estimate while considering heterogeneity among individual studies, using a random-effects model.

The overall effects of HS treatment on defense-related enzyme activity are presented in Figure 3. A consistent and significant increase was observed for the enzymes PAL (RR = 0.39; 95% CI: 0.32–0.47) and GLUC (RR = 0.23; 95% CI: 0.18–0.29), indicating a strong activation of these SAR-associated responses. In contrast, POX showed a much smaller effect (RR = 0.90; 95% CI: 0.86–0.93), suggesting a limited or variable response to HS. The pooled estimate across all enzyme studies (General, n = 66) confirmed a significant overall effect (RR = 0.40; 95% CI: 0.33–0.48), supporting the potential of HS to enhance enzymatic defenses in plants, particularly via PAL and GLUC pathways.

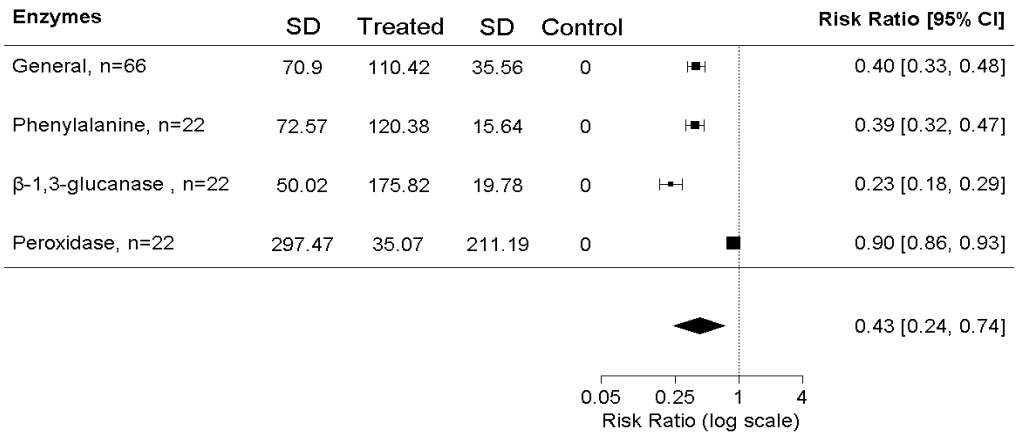


Figure 3. The general effect of HS application on the enzymatic activity of PAL, GLUC, and POX. The square size corresponds to the average effect size, and the error bars are 95% confidence level; the CI – Confidence Interval is 95% based on a random effects model. Diamond – the final effect size of the studies included in the meta-analysis; Risk Ratio – Risk rate; Q - Test for Heterogeneity; I^2 - % of total variability due to heterogeneity; SD: standard deviation.

The effects by crop are presented in Figure 4 for the enzymes PAL (a), GLUC (b), and POX (c). For PAL (Figure 5a), a significant overall effect was observed (RR = 0.17; 95% CI: 0.07–0.41), with strong responses particularly in maize and coffee. GLUC (Figure 4b) also showed a strong overall effect (RR = 0.13; 95% CI: 0.05–0.32), with soybean, coffee, and maize displaying the most pronounced responses. POX (Figure 4c) presented moderate heterogeneity ($I^2 = 88.8\%$), with a consistent overall increase in activity (RR = 0.30; 95% CI: 0.19–0.47). All crops showed a positive response to HS treatment, although the magnitude varied,

suggesting species-specific sensitivity to humic substances.

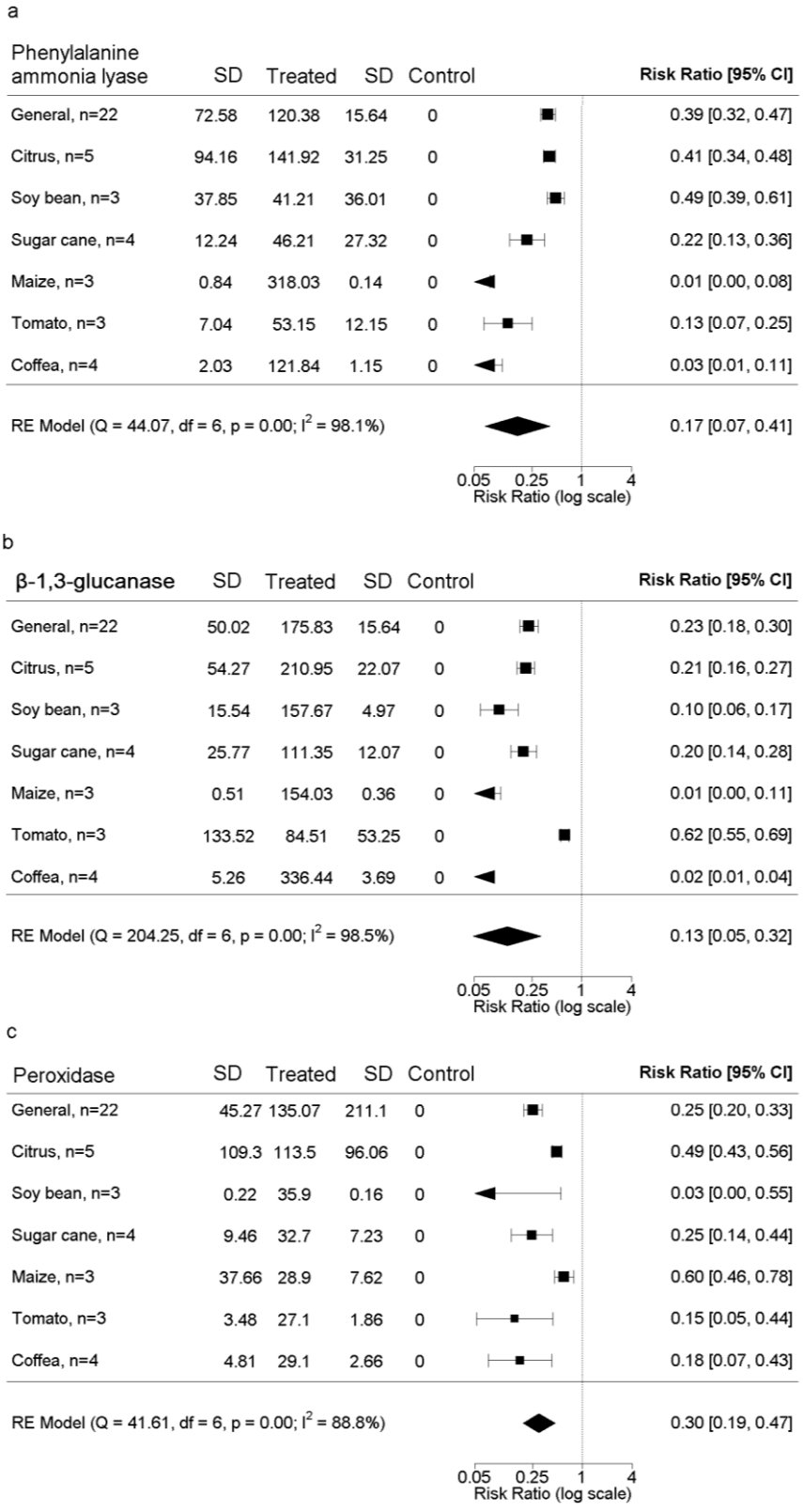


Figure 4. Effect of humic substances (HS) on the GLUC (a), PAL (b) and POX (c) activities in Citrus, soybean, sugarcane, maize, tomato, and coffee. The size of the square corresponds to the size of the average effect, and the bars represent the error at a 95% confidence level; CI – Confidence Interval based on a random effects model; Diamond – the final effect size

of the studies included in the meta-analysis; Risk Ratio – Risk rate. Q - Test for Heterogeneity; I^2 - % of total variability due to heterogeneity. SD: standard deviation

These data confirm that HS generally enhances the activity of SAR associated enzymes, with GLUC standing out, followed by PAL. In contrast, the greater variability observed in POX activity suggests a more complex or context dependent response to HS treatment, potentially influenced by plant species or environmental conditions. Taken together, the results provide strong evidence for the ability of HS to activate defense-related enzymes, particularly GLUC, across diverse cropping systems.

4. Discussion

The study demonstrated that applying HS directly to leaves in low concentrations can increase the activity of enzymes used as SAR markers. Enzyme activities were recorded two days after applying HS via foliar spray on various types of plants, using different cultivars for the same type of plant. Figures 1 and 2 showed the observed variation in results, which depended on the type of plant, cultivar, age, and the enzyme used as an activity marker. POX was the enzyme most subject to variation and the most analytically unstable. POX reduces the H_2O_2 level inside the cell by oxidation of phenolic compounds and ultimately producing phenolic polymers [28]. β-1,3 Glucanase plays a crucial role in indicating the induction of PR proteins and resistance in plants, so much so that it is the most studied PR protein (PR-2), whose activity is increased when plants are treated with a defense response elicitor [24]. PAL is present at high levels in all tests; it indirectly affects the protection system by altering the production of phenolic compounds. These compounds may or may not antagonize the action of pathogens. PAL also plays a vital role in the biosynthesis of SA, the plant hormone that signals SAR, in addition to producing phytoalexins [29].

Organic matter has been used in agriculture for thousands of years and is commonly associated with soil fertility and plant health. Using soluble HS as a plant biostimulant at low concentrations has recently gained popularity among farmers, leading to a million-dollar market for humic substance-based products. Its use as a plant biostimulant is based on its notable effect on nutrient absorption and use efficiency, mitigation of abiotic stress effects and improvements in production quality [21,22] with direct consequences on production cost decrease.

However, HS is not widely used to induce plant defense against pests and pathogens. Although some experimental results suggest this possibility, it is essential to note that these evaluations require wide validation. Silva and Canellas [20] conducted a meta-analysis of research results and found up to a 75% reduction in damage caused by pathogens, after HS use, in plants under controlled conditions. The effectiveness of HS control depends on various factors, including the source of HS, the concentration used, the type of plant, and the type of disease analyzed. Bacterial diseases were more effectively controlled than fungal diseases [20].

This study monitored the activity of three different enzymes related to plant SAR in the main export crops of Brazil. Understanding SAR induction by HS is the first step towards integrating this biotechnology into a more environmentally friendly disease control management.

Further studies, evaluating the effective plant resistance to pathogens and pests, after HS treatment, must be carried out.

Humic substances can induce various physiological responses, such as hormone auxins [31, 32], gibberellins [33, 34], cytokinins [35, 36], alkamides [37], nitric oxide [38, 39], and jasmonic acid [40, 11]. This is the well-founded hormonal hypothesis for the physiological effects of HS [41], which acts as a control hub for plant hormone balance [42]. The central regulators of systemic defense responses are plant hormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) [43]. Furthermore, it is well established in the scientific literature that it can be used to mitigate the effects of various abiotic stresses, including drought, salinity, extreme temperature, and heavy metal toxicity, that have the induction of cell oxidative stress in common. The potential role of HS in preventing oxidative stress in plants was observed by Cordeiro et al. [44]. García et al. [45] reported enhancement of POX activity, decrease of H_2O_2 concentration and increase of cell proline levels, leading to decreased reactive oxygen species (ROS) contents and thereby restoring the cytosolic redox homeostasis [46].

Humic substances have been used as a chemical priming plant defense agent, showing typical hormesis response based on biochemical markers [47]. In addition, the transcriptomic analysis of seedlings primed by HS showed a significant transcription level of genes encoding stress perception and cell signalization, including kinases, phosphatases proteins, and functional and regulatory (transcription factors) proteins, which are involved in specific gene response against abiotic stress without the presence of stress agent [47]. Now, we have been challenged to expand the use of this biotechnology to reduce the damage caused by diseases.

SAR is often associated with various cellular defense responses, such as the synthesis of PR proteins, phytoalexins and accumulation of ROS, rapid alterations in the cell wall, and enhanced activity of various defense-related enzymes, including increased activity of GLUC, POX, and PAL [48]. HS promoted all three enzymes. This presents an opportunity to shift towards a more sustainable agricultural paradigm that prioritizes ecological balance and reduced environmental impact. By applying HS, farmers can potentially decrease their reliance on synthetic pesticides, which often harm biodiversity, soil health, and human health. Instead, farmers can utilize plants' natural defenses, minimizing synthetic inputs while maintaining or even improving crop yields. Promoting SAR mechanisms by HS represents a promising approach to sustainable agricultural disease management.

The meta-analysis conducted in this study further strengthens the evidence for the positive effects of HS on enzymes used as marker of SAR by statistically integrating enzymatic activity data across different crops and experimental conditions. This quantitative synthesis allowed for the detection of consistent patterns and the identification of enzymes with the most consistent responses, despite the inherent variability among studies. By accounting for heterogeneity, the meta-analytic approach provided a more comprehensive and reliable assessment of the true effect size of HS treatments. It not only confirmed the activation of key enzymes such as GLUC, POX, and PAL, but also clarified the relative consistency and impact of each across systems. This reinforces the potential of HS as a viable, biologically based strategy for enhancing plant immunity and

reducing dependence on agrochemicals, supporting the broader shift towards sustainable and resilient agricultural practices.

For the interpretation of this meta-analytic approach, it is important to highlight how forest plots and risk ratios (RR) represent treatment effects. In forest plots, each line corresponds to an individual study or dataset, with the size of the square representing the study's weight in the analysis and the horizontal lines indicating the 95% confidence interval (CI). The vertical line at RR = 1 represents the null effect; thus, values to the left of this line suggest a beneficial effect of the treatment (increased enzyme activity), while values to the right suggest a detrimental or null effect. A narrow CI that does not cross 1 indicates a statistically significant result. In this study, GLUC and PAL generally exhibited RRs below 1 with tight confidence intervals, indicating a consistent and positive response to HS treatment. Conversely, POX showed wider CIs and higher RR values, reflecting greater variability and the potential for treatment response to be influenced by species or environmental context. This detailed visualization enhances understanding of both the magnitude and reliability of treatment effects across diverse experimental conditions.

5. Conclusions

The application of HS increases the activity of PAL, GLUC, and POX associated with the mechanism of SAR in plants such as citrus, coffee, tomatoes, maize, sugarcane, and soybeans. Variations within species, as seen in citrus, soybeans and sugarcane, highlight the importance of considering genetic diversity to assess treatment efficacy.

Although there was wide variability in POX activity, it significantly shaped the overall effect of HS treatment. Notably, coffee did not significantly increase POX activity, indicating potential differences in the prioritization of oxidative enzymes between crops. GLUC was the most responsive to HS treatment as an elicitor in all plants studied.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, L.P.C. and R.M.S.; methodology, formal analysis, investigation, R.M.S.; resources, L.P.C and F.L.O.; writing—original draft preparation, L.P.C. writing—review and editing, R.M.S, FLO. and E.H.N; supervision, L.E.P.P. and E.H.N. All authors have read and agreed to the published version of the manuscript."

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Conflicts of Interest: "The authors declare no conflicts of interest."

Abbreviations

The following abbreviations are used in this manuscript:

HS - Humic Substances

POX - Peroxidase

PAL - Phenyl Alananine Ammonia Lyase

GLUC - B 1,3-Glucanase

SAR - Systemic Acquired Resistance

ISR - Induced Systemic Resistance

TFs - Ttranscription factors

PR - Pathogenesis-Related Proteins

ET - Ethylene

SA - Salicylic acid

JA - Jasmonic acid

PGPB - Plant growth-promoting bacteria

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ARTIGO 2: HUMIC SUBSTANCES FROM DIFFERENT SOURCES MODULATE SALICYLIC ACID MEDIATED DEFENSE IN PLANTS INFECTED BY POWDERY MILDEW

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Article

Humic Substances from Different Sources Modulate Salicylic Acid-Mediated Defense in Plants Infected by Powdery Mildew

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Abstract

Modern agriculture relies heavily on chemical inputs to sustain productivity, yet their intensive use poses environmental and health risks. Sustainable strategies based on biostimulants have emerged as promising alternatives to reduce agrochemical dependence. Among these compounds, humic substances (HS) stand out for their ability to modulate plant growth and activate defense responses. This study aimed to evaluate the effects of HS from different sources—vermicompost (Vc) and peat (Pt)—on the salicylic acid (SA)-mediated defense pathway in tomato plants (*Solanum lycopersicum* cv. Micro-Tom) infected with *Oidium* sp. The HS were characterized by solid-state ^{13}C CPMAS NMR to determine the relative distribution of carbon functional groups and structural domains, including alkyl, O-alkyl, aromatic, and carbonyl carbon fractions, as well as hydrophobicity-related indices. Enzymatic activities of lipoxygenase, peroxidase, phenylalanine ammonia lyase, and beta 1,3-glucanase were determined spectrophotometrically, and RT-qPCR quantified gene transcription levels involved in SA signaling and defense (*MED25, MED16, MED14, NPR1, ICS, PAL, LOX1.1, MYC2, JAZ, jar1, CAT, POX, SOD, APX, ERF, PR-1, PR-2, PR-4 e PR-5*). Both HS significantly reduced disease severity and activated key SA-related defense genes, including the regulatory gene *NPR1* and the effector genes *PR1*, *PR2* and *PR5*, with Pt providing greater protection. Notably, HS amplified defense-related gene expression and enzymatic activities specifically under infection, showing a stronger induction than in non-infected plants. These results demonstrate that structural differences among HS drive distinct and enhanced defense responses under pathogen challenge, highlighting their potential as sustainable tools for improving plant immunity in agricultural systems.

Keywords: induced resistance; hormone; biostimulants

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1. Introduction

Plant health is essential for maintaining global food security. Climate change has intensified abiotic and biotic stresses in agricultural systems, increasing the vulnerability of crops to pathogens and reducing global food security [1,2]. Tomato (*Solanum lycopersicum*) is one of the most economically important horticultural crops worldwide, with high demand for both fresh consumption and processed products [69]. However, tomato production is particularly sensitive to fungal diseases, which are major contributors to yield instability. Among these pathogens, *Oidium* spp., the causal agent of powdery mildew, is responsible for significant economic losses due to reduced photosynthetic capacity, premature leaf senescence, and decreased fruit quality [74]. Tomato has also been widely used as a model system to investigate defense-response mechanisms and hormone-mediated immunity [70], further supporting its relevance for studies on biotic stress responses. In modern agriculture, disease management still primarily relies on direct pathogen control, predominantly via pesticide application. Although effective in the short term, such practices have caused significant environmental impacts and

promoted the emergence of resistant microorganisms, compromising long-term sustainability [3]. Alternatively, plant breeding offers a promising, yet medium- to long-term, solution, whereas the use of elicitors or systemic resistance inducers has emerged as a short-term strategy that harnesses plants' intrinsic adaptive mechanisms to bolster immunity against biotic stresses [4–6].

Plant immunity operates through two primary layers: pattern-triggered immunity (PTI), activated by the recognition of pathogen-associated molecular patterns (PAMPs), and effector-triggered immunity (ETI), resulting from the detection of specific effector proteins secreted by pathogens [5]. Both layers converge to elicit a broad spectrum of defense responses, ranging from physical barriers to biochemical and molecular alterations [7], often orchestrated by complex hormonal signaling networks. Within these networks, salicylic acid (SA) and jasmonic acid (JA) act as central regulators, coordinating distinct but interconnected defense strategies [8,9].

SA plays a central role in defense against biotrophic and hemibiotrophic pathogens, which rely on living host tissues to complete their life cycles [10]. Activation of the SA-dependent signaling pathway induces Systemic Acquired Resistance (SAR), a durable defense state characterized by the accumulation of a subset of pathogenesis-related (PR) proteins, whose induction profiles vary depending on the pathogen, along with enhanced reactive oxygen species (ROS) production, and cell wall reinforcement [9,11]. Key enzymes involved in SAR include β-1,3-glucanase (PR-2), peroxidase (POX), and phenylalanine ammonia-lyase (PAL), which contribute to the synthesis of phenolic compounds and tissue lignification, thereby strengthening structural barriers against pathogen colonization [9,12,13].

In contrast, JA, which may interact synergistically or antagonistically with ethylene (ET) depending on the context, regulates defense against necrotrophic pathogens and herbivorous insects that exploit host cell death [14,15]. The JA/ET-dependent signaling pathway underlies Induced Systemic Resistance (ISR), involving transcriptional activation of defense-related genes, enhanced activity of oxidative enzymes such as lipoxygenase (LOX), and accumulation of secondary metabolites with antimicrobial properties [16,17].

The crosstalk between SA- and JA-mediated pathways is complex and antagonistic, reflecting the need for fine-tuned regulation of immune responses according to pathogen lifestyle and environmental conditions [18,19]. At the molecular level, the integration and fine-tuning of these signaling networks rely on regulatory hubs, including NPR1 (Nonexpressor of Pathogenesis-Related Genes 1), the Mediator complex, and Nudix hydrolases. NPR1 is an important transcriptional co-regulator that integrates SA signaling, turns on PR genes, and ensures that SAR is established.

Its redox-dependent conformational changes allow nuclear translocation and interaction with TGA transcription factors, linking hormonal signaling to gene expression [48,49]. The Mediator complex, a conserved multiprotein assembly, bridges transcription factors and RNA polymerase II, coordinating the transcriptional reprogramming necessary for immune activation and hormonal crosstalk [53,54]. Similarly, Nudix

hydrolases (NUDXs) function as metabolic regulators and signaling modulators, maintaining cellular redox homeostasis and contributing to immune priming by balancing ROS production and detoxification [11,52]. Together, these elements form an essential regulatory network that integrates SA- and JA-dependent responses, ensuring specificity and balance in plant immunity [11,18,49,53].

Within this regulatory framework, natural elicitors have garnered attention for their capacity to modulate defense pathways and enhance plant immunity. Humic substances (HS) are recognized as biostimulants that promote both plant growth and resilience to biotic and abiotic stresses [20–22]. Recent studies demonstrate that HS can activate both the SA-dependent SAR pathway and the JA/ET-mediated ISR pathway [23,24].

HS represents the most stable and bioactive fraction of natural organic matter, arising from the decomposition and transformation of plant and microbial residues [25]. The chemical composition is heterogeneous and strongly dependent on origin, vermicompost (Vc) or peat (Pt), for example, resulting in variable proportions of aromatic, aliphatic, and oxygenated functional groups [26]. This chemical diversity is crucial for determining the intensity and specificity of HS physiological effects [27,28]. Molecular heterogeneity underlies a wide range of metabolic effects, including enhanced membrane permeability, activation of ion transporters, and stimulation of key processes such as photosynthesis, the Krebs cycle, and ATP and amino acid synthesis [29–33].

Furthermore, HS stimulates enzymatic activities associated with plant defense, such as peroxidase (POX), phenylalanine ammonia-lyase (PAL), and pathogenesis-related protein 2 (PR-2) [24,34], while also modulating reactive oxygen species (ROS) metabolism.

ROS act as signaling molecules that regulate metabolic activities through transduction mechanisms [35]. At the molecular level, HS also influences gene expression in hormonal pathways, functioning as signaling molecules that interact with membrane receptors and trigger canonical defense responses [23,24,31,34,36,37].

Despite these advances, significant gaps remain regarding how HS affect specific plant defense pathways. In particular, the activation of the SA pathway, crucial for defense against biotrophic pathogens such as *Oidium sp*. remains poorly understood. Considering that the HS chemical structure can guide biological interactions, elucidating how substances from different sources influence key regulatory components of the SA pathway, such as *NPR1*, PR genes (*PR-1, PR-2, PR-4* and *PR-5*), as well as antioxidant enzymes associated with defense signaling. Such clarification is necessary to understand how HS modulate plant immunity. This study aimed to investigate how HS extracted from vermicompost (Vc) and peat (Pt) regulate the SA pathway and defense responses in plants infected with *Oidium* sp. *(On)*.

2. Results

2.1. Characteristics of the Humic Substances Used

Solid-state ^{13}C NMR analyses revealed marked differences between HS obtained from Vc and Pt (Figure 1). Vc (Figure 1a) showed a larger

area from signals originating from the presence of carbohydrate moieties and peptide linkages centered at 71.7 ppm (O-alkyl C and C–N/O groups). The major signal at 54.3 ppm is often attributed to lignin fragments, as methoxy groups are found in this region. It was possible to observe the presence of unsubstituted aromatic carbons (117.9–126.4 ppm) and carboxylic groups (172.7 ppm). This chemical feature is typical of an organic matter source with a larger proportion of oxygenated compounds, associated with carbohydrate and protein residues. In contrast, the Pt spectrum (Figure 1b) showed a predominance of hydrophobic and aliphatic carbons with an intense signal at 30.5 ppm, along with signals at 120.9 ppm (aromatic carbons) and 170.1 ppm (carboxyl/carbonyl groups). This pattern indicates a more hydrophobic, recalcitrant structure typical of sedimentary organic matter.

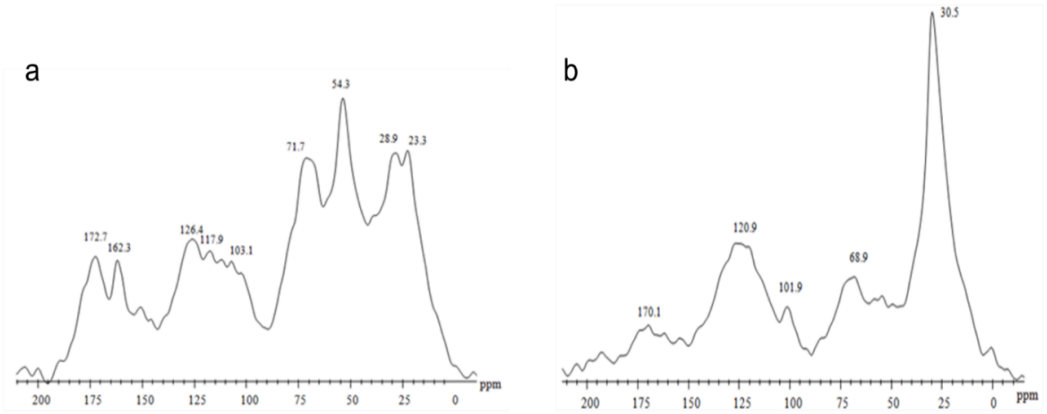

Figure 1. Spectrum of CP/MAS 13C NMR of humic substances (HS) extracted from two sources: (**a**) vermicompost-derived HS obtained from an experimental field area using bovine manure and plant residues, and (**b**) peat-derived HS supplied as a raw commercial humic extract by DNAgro Biotechnology.

The ^{13}C CPMAS NMR spectra of HS isolated from Pt and Vc revealed the usual composition of complex natural organic materials with a carbon distribution extended along all the chemical shift range (Figure 1). Vc was characterized by the predominance of CH_3O/C-N interval and O-alkyl-C components, which represented the 16.7% and 28.1% of the total area, respectively (Table 1), while humic substances isolated from Pt showed the alkyl-C (45-0 ppm) interval corresponding to 50.2% of total spectrum area.

Table 1. Organic carbon distribution (%) in ^{13}C NMR CP/MAS spectra and associated structural indexes [a].

	C=O 190–160	O-aryl-C 160–140	aryl-C 140–110	O-alkyl-C 110–60	CH_3O/C-N 60–45	alkyl-C 45–0	HB	A/OA	Ar	LR
HS-Pt	10.0	3.5	22.7	9.8	3.7	50.2	3.6	5.1	26.3	1.1
HS-Vc	6.5	8.4	20.3	28.1	16.7	19.8	1.3	0.7	28.7	2.0

The hydrophobic index is the ratio of signal intensities in chemical shift intervals for apolar alkyl and aromatic C components over those of hydrophilic C molecules: (1) HB = Σ[(0–45) + (45–60)/2 + (110–160)]/Σ[(45–60)/2 + (60–110) + (160–

190)]. The alkyl ratio determines the relative contribution of apolar versus polar alyl molecules. (2) A/OA = (0–45)/(60–110). The aromaticity index is the total area assigned to aromatic compounds. (3) Ar = Σ[110–160). The lignin ratio relates the area of methoxyl-C + N-alkyl groups to that of O-aryl-C. (4) LR = (45–60)/(145–160).

2.2. Enzymatic Activity

Considering the chemical differences observed between the HS from Vc and Pt, we investigated whether these contrasts would translate into distinct plant responses. The activity of LOX, PAL, POX, and PR-2, key enzymes involved in plant defense pathways, was evaluated. Application of the HS significantly affected the activity of these defense-related enzymes (Figure 2). LOX activity (Figure 2a) increased in both Vc and Pt treatments compared with the control, with no significant difference between them. However, in the presence of powdery mildew, an increase was observed only in Vc + *On*. Infection strongly induced POX activity (Figure 2b) and further enhanced it in the combined treatments, particularly in Pt + *On*, which exhibited the highest values. PAL activity (Figure 2c) was also stimulated by Vc and Pt, with a more pronounced response in the pathogen-inoculated treatments, where Vc + *On* and Pt + *On* showed higher activity than the control. Finally, PR-2 (Figure 2d) was significantly induced by infection, reaching the highest levels in Pt + *On*, followed by Vc + *On*. Overall, the results indicate that HS, particularly when combined with powdery mildew infection, modulates plant enzymatic activity.

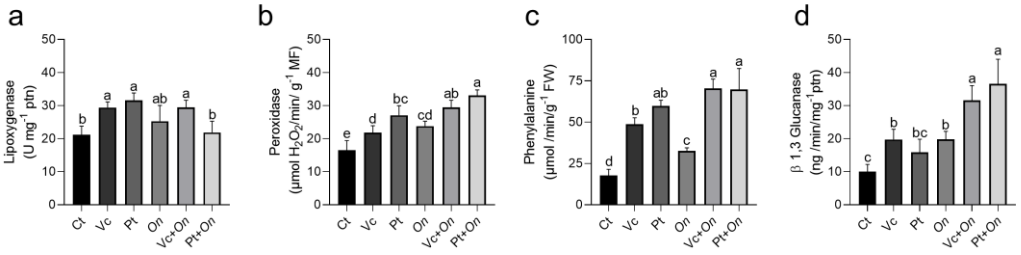

Figure 2. Activity of defense-related enzymes in tomato plants subjected to different treatments. (**a**) LOX, (**b**) POX, (**c**) PAL, and (**d**) PR-2 activities were measured in leaves collected 72 h after treatment and inoculation with *Oidium* sp. Bars represent mean ± standard deviation (SD). Different letters indicate significant differences among treatments by Fisher's LSD test ($p < 0.05$).

2.3. Gene Expression

In general, HS modulates the expression of genes related to systemic defense. In this study, we evaluated 20 genes involved in systemic defense pathways and grouped the results by biological function: co-regulators, hormonal signaling and the phenylpropanoid pathway, antioxidants, and pathogenesis-related (PR) genes.

The treatments differentially modulated the expression of co-regulator genes (Figure 3). *MED25* expression was significantly upregulated in plants treated with HS derived from Pt, whereas pathogen inoculation (*On*) or the combined treatment Pt + *On* led to a pronounced

downregulation compared with the control. In contrast, plants treated with HS from Vc or with the combined treatment Vc + *On* maintained expression levels comparable to the control. For *MED16* (Figure 3b), all treatments showed downregulation compared to the control. In *MED14* (Figure 3c), Pt + *On* showed high expression, followed by Vc + *On* and *On*. Plants exposed only to HS from Vc and HS from Pt showed downregulation compared to the controls. Finally, *NPR1* (Figure 3d), a key regulator of systemic acquired resistance, was upregulated in infected plants, with greater upregulation in Vc + *On* and Pt + *On*. Treatments with HS from Vc or HS from Pt alone did not significantly alter transcript regulation compared to the control.

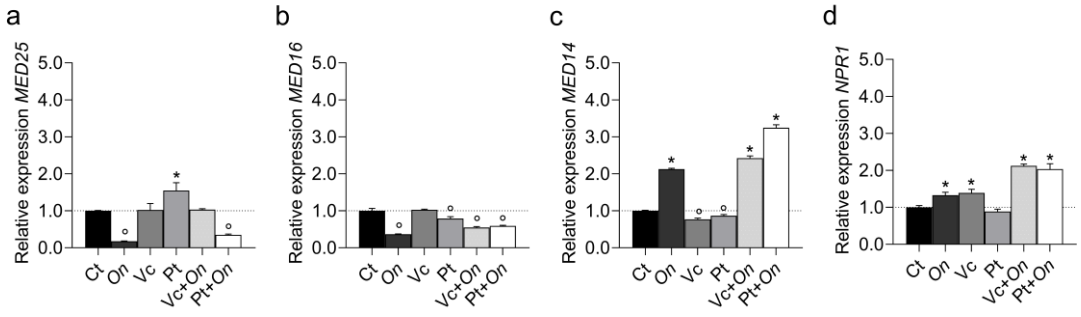

Figure 3. Relative expression of co-regulator genes MED25 (Mediator complex subunit 25) (**a**), MED16 (Mediator complex subunit 16) (**b**), MED14 (Mediator complex subunit 14) (**c**), and NPR1 (Nonexpressor of Pathogenesis-Related genes 1) (**d**) in leaves of tomato cv. Micro-Tom under different treatments: control (Ct), inoculation with Oidium (On), application of humic substances from vermicompost (Vc), humic substances from peat (Pt), vermicompost humic substances and Oidium sp. (Vc + On), and peat humic substances and Oidium sp. (Pt + On). Bars represent mean ± standard deviation (n = 3 biological replicates). Statistical differences in relation to the control were assessed using Dunnett's multiple comparisons test ($p < 0.05$).

The expression of genes involved in hormonal signaling pathways showed distinct modulation across treatments (Figure 4). In the JA pathway, *LOX1.1* (Figure 4a) and *MYC2* (Figure 4b) were strongly upregulated in plants treated with Vc, with relative expression levels approximately six and fivefold higher than the control, respectively. Both genes also showed moderate induction in Pt + *On* and Vc + *On*, while *On* and Pt alone resulted in lower expression levels. The gene *jar1* (Figure 5c) exhibited significant induction only in Vc, with reduced expression in the remaining treatments. In contrast, *JAZ* (Figure 4d) was highly induced in On, reaching the highest relative expression among treatments, followed by Pt + *On*. Regarding the ET pathway, *ERF* (Figure 4e) showed increased expression in Vc and Pt + *On*, whereas *On* caused a marked reduction compared to the control.

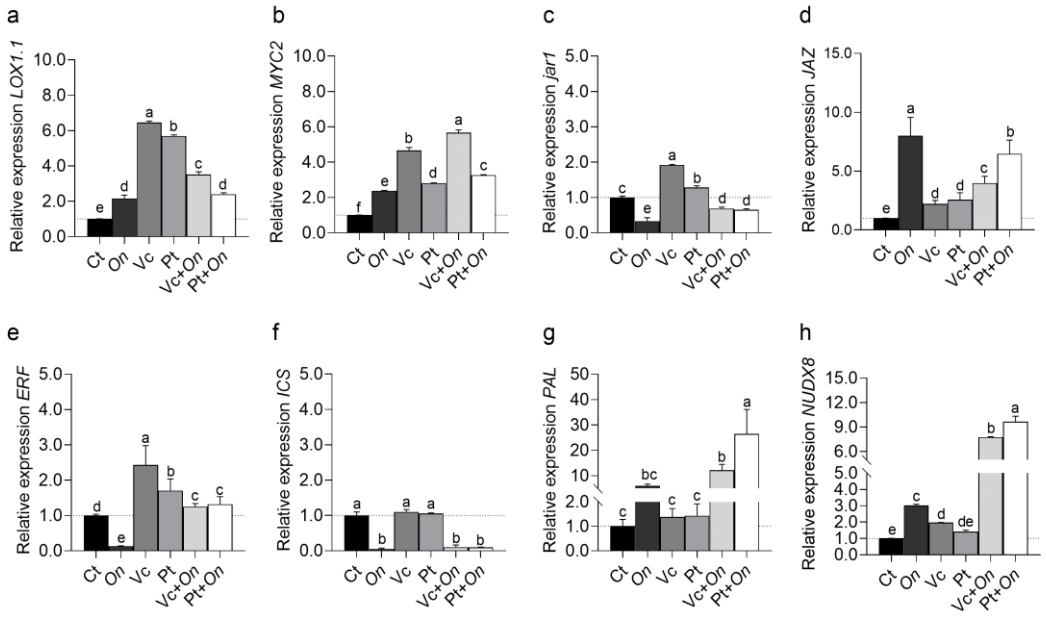

Figure 4. Relative expression of genes involved in JA and SA signaling pathways, ethylene response, and nucleotide metabolism in tomato plants (cv. Micro-Tom) under different treatments. (**a**) *LOX1.1*, (**b**) *MYC2*, (**c**) *JAR1*, and (**d**) *JAZ* are key components of the JA signaling pathway; (**e**) *ERF* is associated with ethylene response; (**f**) *ICS* and (**g**) *PAL* are related to SA biosynthesis; and (**h**) *NUDX8* is involved in NADPH metabolism and stress response. Treatments: control (Ct), inoculation with *Oidium* sp. (On), application of humic substances from vermicompost (Vc) or peat (Pt), and combined treatments (Vc + On, Pt + On). Bars represent mean ± standard deviation (n = 3 biological replicates). Statistical differences in relation to the control were assessed using Dunnett's multiple comparisons test ($p < 0.05$).

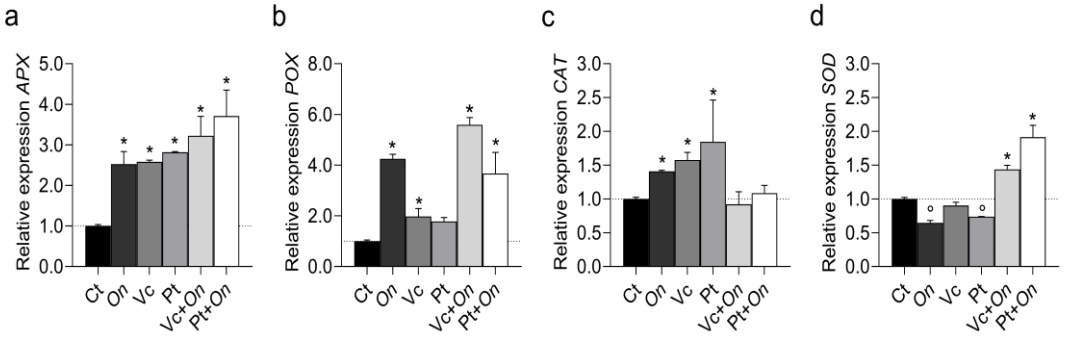

Figure 5. Relative expression of antioxidant-related genes in tomato plants (cv. Micro-Tom) under different treatments. (**a**) *APX* (ascorbate peroxidase), (**b**) *POX* (peroxidase), (**c**) *CAT* (catalase), and (**d**) *SOD* (superoxide dismutase). Treatments: control (Ct), inoculation with *Oidium* sp. (On), application of humic substances from vermicompost (Vc) or peat (Pt), and combined treatments (Vc + *On*, Pt + *On*). Bars represent mean ± standard deviation (n = 3 biological replicates). Statistical differences in relation to the control were assessed using Dunnett's multiple comparisons test ($p < 0.05$).

In the SA pathway, the *ICS* and *PAL* genes (Figure 4f,g), both involved in SA biosynthesis and, for PAL, also participating in the

phenylpropanoid pathway, displayed contrasting expression profiles. Expression of *ICS* was strongly repressed in On, Vc + *On*, and Pt + *On* treatments compared with the control. In contrast, *PAL* expressions were significantly induced, particularly in the Pt + *On* and Vc + *On* treatments. The *NUDX8* gene (Figure 4h), associated with nucleotide metabolism and SAR, also exhibited a marked upregulation in Pt + *On* and Vc + *On*.

The expression of antioxidant metabolism genes varied among treatments (Figure 5). The APX gene (Figure 5a) showed increased expression in all treatments compared to the control, with the highest transcription levels observed in Pt + *On*, followed by Vc + *On*, Pt, Vc, and *On*. In POX (Figure 5b), expression in Vc + *On* was approximately sevenfold higher than the control. On also promoted upregulation. Meanwhile, Vc and Pt alone showed lower expression levels, similar to the control. The CAT gene (Figure 5c) showed moderate variation between treatments, with a slight induction in Pt and Vc, while *On*, Vc + *On*, and Pt + *On* did not differ significantly from the control. Furthermore, SOD (Figure 5d) was significantly upregulated in Pt + *On*, followed by Vc + *On*, while treatments with *On*, Vc, and Pt resulted in low or similar levels to the control.

The expression of *pathogenesis-related (PR)* genes varied markedly among treatments (Figure 6). The *PR-1* gene (a) showed slight induction in Vc, whereas all other treatments resulted in reduced expression compared with the control, with the lowest transcript levels observed in On and Pt + *On*. The *PR-2* gene (b) was strongly upregulated in Pt + *On* and Vc + *On*, reaching approximately 8- and 6-fold higher levels than in the control, respectively. In contrast, *On*, Vc, and Pt alone exhibited lower expression levels. Similarly, *PR-4* (c) displayed a pronounced induction of Vc + On, followed by Pt + On, whereas the *On*, Vc, and Pt treatments showed only modest increases compared with the control. For *PR-5* (d), expression was highest in Vc, whereas On, Pt, Vc + *On*, and Pt + *On* resulted in intense repression relative to the control.

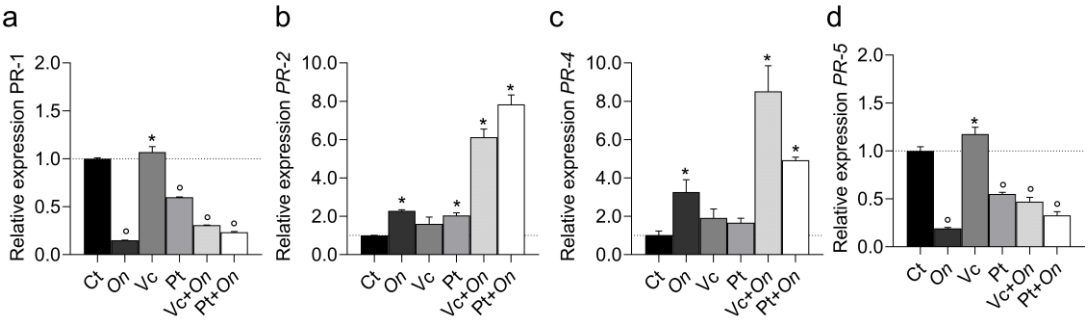

Figure 6. Relative expression of *Pathogenesis-Related* (PR) genes *PR1* (**a**), *PR2* (**b**), *PR4* (**c**), and *PR5* (**d**) in leaves of tomato cv. Micro-Tom under different treatments: control (Ct), inoculation with *Oidium* (*On*), application of humic substances from vermicompost (Vc), humic substances from peat (Pt), vermicompost humic substances and *Oidium* sp. (Vc + *On*), and peat humic substances and Oidium sp. (Pt + *On*). Bars represent mean ± standard deviation (n = 3 biological replicates). Statistical differences in relation to the control were assessed using Dunnett's multiple comparisons test ($p < 0.05$).

2.4. Severity of the Disease

Phenotypic evaluation confirmed that the structural and functional differences in HS were directly reflected in the plant–pathogen interaction (Figure 7). Seven days after inoculation with *Oidium* sp., control plants showed visible symptoms and a severity index of approximately 12%, while the HS treatments from Vc and Pt showed severity index of 2.5% and 0.8%, respectively. These results complement the results obtained in the enzymatic and molecular analyses.

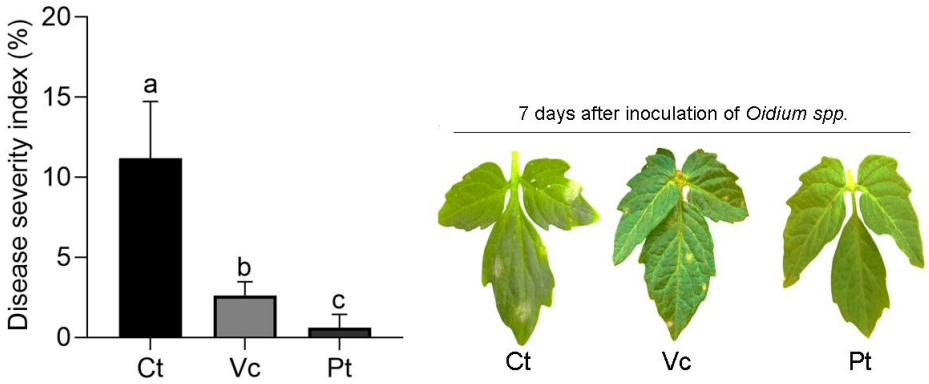

Figure 7. Disease severity index (%) in tomato plants subjected to different treatments (Ct, Vc, and Pt), evaluated 7 days after inoculation with *Oidium* sp. Bars represent mean ± standard error (SE). Different letters above the bars indicate significant differences among treatments according to the Kruskal–Wallis test followed by Dunn's multiple comparison test with Bonferroni adjustment ($p < 0.05$). Representative leaves from each treatment are shown on the right.

3. Discussion

The results obtained in this study indicate that HS extracted from Vc and Pt, despite exhibiting marked chemical differences, act as multifunctional elicitors of defense responses in tomato (*Solanum lycopersicum* cv. Micro-Tom) infected with *Oidium* sp., a biotrophic pathogen whose interaction with the host depends on the balance between activation and suppression of defense pathways. Both HS stimulated resistance mechanisms; however, the HS from Pt was more effective, reducing disease severity to 0.8%, compared to 2.5% for Vc and 12% in the control (Figure 7).

The tomato cultivar Micro-Tom was selected as a model system due to its high genetic uniformity, short life cycle, compact growth habit, and well-characterized hormonal and defense signaling pathways, which make it widely used in studies of plant immunity and hormone-regulated responses. Importantly, Micro-Tom is considered moderately susceptible to powdery mildew, allowing reliable discrimination of induced resistance responses without masking effects of strong genetic resistance [71–73]. This intermediate sensitivity makes it an appropriate biological model to assess the capacity of humic substances to activate defense mechanisms against biotrophic pathogens.

From a chemical standpoint, the greater efficacy of the Pt-derived HS may be associated with its higher hydrophobic character, as indicated by

the ~30 ppm peak in the ^{13}C NMR spectrum, which is typical of long-chain aliphatic carbons (Figure 1). Hydrophobic domains promote interactions with membranes and may enhance the transport and delivery of signaling molecules to responsive sites, thereby increasing hormonal and enzymatic activation. In contrast, the Vc-derived HS exhibits a higher content of oxygenated polar groups, greater solubility, and a lower degree of hydrophobicity, which may explain its comparatively weaker effects. These findings are consistent with previous studies that have correlated humic hydrophobicity with plant bioactivity [27,28,38]. Spectroscopic and multivariate statistical analyses have demonstrated that fractions with a higher proportion of aliphatic and aromatic hydrophobic structures show stronger correlations with the stimulation of physiological and enzymatic processes in plants [39,40].

The structural basis for the differential bioactivity between peat- and vermicompost-derived humic substances was further supported by solid-state ^{13}C CPMAS NMR analyses. The spectra revealed a typical heterogeneous distribution of carbon functional groups, but with marked contrasts between the two materials. Humic substances isolated from peat showed a strong predominance of alkyl-C structures (0–45 ppm), representing 50.2% of the total spectral area, whereas vermicompost-derived HS were enriched in O-alkyl-C (28.1%) and CH_3O/C–N regions (16.7%) (Table 1). These data indicate that peat HS are enriched in long-chain aliphatic domains, while vermicompost HS contain a higher proportion of carbohydrate-like and oxygenated structures [75–77].

The dominant alkyl-C region in peat HS, with intense resonances around 30–33 ppm, is typically associated with long-chain methylene groups derived from waxes, cutin, fatty acids, and other lipid-like structures. These hydrophobic domains are known to enhance molecular interactions with biological membranes and to facilitate the delivery of signaling molecules, thereby increasing the efficiency of defense activation. In contrast, vermicompost HS exhibited stronger signals in the 60–110 ppm region, characteristic of O-alkyl-C groups derived mainly from polysaccharides, hemicellulose, and low-molecular-weight carbohydrates, which confer a more polar and hydrophilic character [77].

These spectral differences were corroborated by structural indices calculated from NMR data, including higher hydrophobicity (HB) and alkyl/O-alkyl (A/OA) ratios in peat-derived HS, reflecting the predominance of apolar aliphatic structures. These chemical features are consistent with the greater biological activity observed for peat HS in this study, as hydrophobic fractions of humic substances have been repeatedly associated with stronger stimulation of enzymatic, hormonal, and defense-related plant responses. Conversely, the lower hydrophobicity and higher carbohydrate-like content of vermicompost-derived HS likely contribute to their reduced, although still significant, capacity to induce resistance responses. Together, the NMR-derived structural differences provide a mechanistic explanation for the distinct biological performance of the two HS sources, linking molecular composition, hydrophobicity, and defense-eliciting capacity in tomato plants.

SAR is a key pathway in defense against biotrophic pathogens [10], mediated by SA. In plants, SA is synthesized via two main pathways: the ICS and PAL pathways. Generally, under pathogen attack, the PAL

pathway is preferentially activated, being associated with phenylpropanoid metabolism and the production of phenolics and lignin precursors [41–43]. Schiavon and colleagues [44] were the first to show that HS promotes phenylpropanoid metabolism by inducing PAL enzyme activity and expression, thereby increasing total phenolic content in plants. An upregulation of *PAL* gene expression was also observed in citrus plants two days after HS application under non-infectious conditions [24]. In the present study, *ICS* expression remained similar to the control in HS-treated plants and was repressed in the presence of the pathogen. In contrast, *PAL* was strongly induced, with approximately 25× increases in Pt + *On* and 13× in Vc + *On* compared to the control, indicating a metabolic shift favoring the PAL–SA route and enhancing both structural and chemical defenses against the biotrophic fungus.

Activation of the SA pathway was reflected in the induction of classical defense effector genes, particularly those encoding pathogenesis-related (PR) proteins. The expression of *PR-2* and *PR-4* showed marked upregulation, with approximately 6- and 8-fold increases above the control in the combined treatments Pt + *On* and Vc + *On*, respectively, whereas PR-4 expression reached 8- and 5-fold increases in the same treatments. PR-2 (β-1,3-glucanase) and PR-4 (chitinase) enzymes, act synergistically to degrade fungal cell walls and inhibit hyphal growth, mechanisms that are critical for resistance against *Oidium* sp. [45,46]. Conversely, no significant change was observed in *PR-5* expression, a thaumatin-like protein, suggesting that the HS-induced induction was selective for PRs with direct antifungal enzymatic activity rather than those involved in osmotic adjustment or membrane permeability modulation, as typically associated with PR5, such as xylanase inhibition [47]. This expression pattern indicates that HS, particularly those derived from Pt, direct the defense response toward pathways with greater antifungal efficiency, reinforcing their role as selective elicitors of SAR. Supporting this interpretation, previous studies have shown that other SAR-related PRs, including *PR-3, PR-7*, and *PR-11*, are also upregulated by HS in citrus plants [24], suggesting that HS-induced transcriptional modulation follows a conserved pattern across plant species and may involve the activation of NPR1-dependent regulatory cofactors, with NPR1 acting as a key positive regulator required for SAR activation. NPR1 is a positive regulator required for the activation of SAR against a wide range of pathogens [48]. NPR1 responds to the redox state and activates SA-dependent genes [49,50].

Nudix proteins also act as regulators in plant immunity. They perform several functions to detect and modulate the levels of their substrates, such as nucleotide sugars, deoxyribonucleoside triphosphates (dNTPs), and mRNAs, in order to maintain proper cellular processes and physiological homeostasis. *NUDX8* acts as another positive regulator of SAR, is associated with SA signaling, and influences redox homeostasis [51,52]. Both *NPR1* and *NUDX8* were positively regulated in plants treated with HS from Pt and Vc, and under the combined treatments from Pt + *On* and Vc + *On*, their expression was amplified. Together, these results support the hypothesis that HS establishes an oxidative priming state that increases the sensitivity and speed of the defense response.

Fine regulation between hormonal pathways was evidenced by the modulation of Mediator complex subunits: *MED14*, *MED16*, and *MED25* exhibited distinct expression patterns. *MED14* (a co-regulator of the SA pathway) was more highly expressed in Pt + *On*, whereas *MED25* (a co-regulator of JA/ET-mediated immunity) was induced by Vc-derived HS but repressed in the combined pathogen treatment. These profiles indicate that Pt-derived HS redirects signaling toward the SA pathway (resistance to biotrophic pathogens), while Vc-derived HS allows a partial balance between SA and JA, a modulation that may explain the intermediate effectiveness observed for Vc. This multiprotein Mediator complex has been recognized as a key regulator of transcriptional reprogramming, functioning as an adapter/co-regulator between sequence-specific transcription factors and RNA polymerase II (RNAPII). The plant Mediator complex is a macromolecular unit composed of 34 subunits, among which MED14, MED16, and MED25 play critical roles in defense pathways against biotic stresses [53,54].

It is also important to highlight the regulation of the JA pathway in this study, which was also investigated due to its antagonistic relationship with the SA pathway and its typical association with defense against necrotrophic pathogens [55,56]. The genes *LOX1*, *MYC2*, and *jar1* (components of JA biosynthesis and signal transduction) were mainly expressed in plants treated with HS from Pt and Vc. The effect of HS on the activation of the JA biosynthetic pathway has already been reported [23,57]. However, in this study, it was demonstrated that under *Oidium* sp. infection, the pathway is repressed. *JAZ*, a repressor protein of JA signaling, showed a substantial increase (~7× in Vc + *On* and ~4× in Pt + *On*). This pattern indicates that, in the presence of a biotrophic pathogen, HS favors the prioritization of the SA pathway through repression of JA effector signaling (via increased *JAZ* expression and decreased *LOX1*/*MYC2*/*jar1*), which is consistent with the classical SA–JA antagonism [58,59] and with the need for plants to suppress responses typically directed against necrotrophs when facing a biotrophic infection. The differentiation between Pt and Vc also suggests that the structural composition of the HS influences the intensity of this prioritization, as Pt induced a stronger suppression of JA signaling than Vc.

Transcriptional activation was accompanied by promising enzymatic and redox responses: significant increases in the activities of PAL, POX, LOX, and PR2 were observed, along with elevations in the expression and activities of the antioxidant system (*APX, POX, CAT, SOD*), particularly in the Pt + *On* and Vc + *On* treatments. The controlled production of reactive oxygen species (ROS) functions as a secondary messenger in defense signaling [60] and contributes to lignin deposition and cell wall crosslinking [61]. Simultaneously, the activation of APX, CAT, and POX is essential to limit oxidative damage and finely modulate the signal. HS from Vc and Pt increased the expression of *APX* and *POX* genes, with even higher expression observed when plants were infected (Pt + *On* and Vc + *On*). Thus, HS influences an integrated response: enhancement of phenolic metabolism (PAL → SA), activation of enzymatic effectors (PRs), and adjustment of the antioxidant system, resulting in both physical and biochemical reinforcement against infection.

Phenotypically, this molecular and biochemical coordination translated into a marked reduction in disease severity. Considering the biotrophic nature of *Oidium* sp., which relies on haustorium formation and maintenance of living tissues, the early activation of oxidative mechanisms, phenol/lignin deposition, and PR protein expression prevents mycelial establishment and the formation of feeding structures, explaining the observed protective effect. Beyond expanding mechanistic understanding, this study represents, to our knowledge, the first report of using HS to control foliar fungal diseases, complementing preexisting evidence of pathogen suppression by HS in soil and field assays [21].

In summary, the HS investigated act as natural inducers of systemic resistance by integrating chemical signals (composition and hydrophobicity), modulating SA biosynthetic pathways (with preference for the PAL pathway), recruiting transcriptional and redox regulators (*NPR1, NUDX8, MEDs*), and adjusting the balance between SA and JA (reducing *LOX1*/*MYC2*/*jar1* and increasing *JAZ*), together with the activation of the antioxidant system (*APX, POX, CAT, SOD*). Pt-derived HS, due to its higher hydrophobicity, enhances this network of responses, whereas Vc-derived HS induces a more balanced modulation between pathways. These findings position HS as a promising and sustainable tool for integrated management of fungal diseases, with practical implications for the development of bioelicitor formulations and crop protection strategies.

4. Materials and Methods

4.1. Extraction and Characterization of Humic Substances

Humic substances (HS) were obtained from two sources: (i) vermicompost (Vc), produced from cattle manure using the earthworm *Eisenia andrei*, and (ii) a commercial peat-based product (Pt) supplied by DNAgro Biotechnology. Extraction of HS from Vc was performed with a 5% KOH solution (Sigma-Aldrich, St. Louis, MO, USA) (1:20, *v*/*v*) under agitation for 4 h, followed by centrifugation (15 min, 5000× g). The supernatant was neutralized to pH 7.0. Aliquots of each product (Vc and Pt) were frozen and lyophilized for subsequent chemical characterization.

Total organic carbon (TOC) content: performed by dry combustion using a TOC analyzer from Shimadzu (Tokyo, Japan), based on the Dumas combustion method. In this system, each liquid sample is injected into a high-temperature combustion furnace (~680 °C) under an oxygen-rich atmosphere, promoting complete oxidation of all carbon-containing compounds to carbon dioxide (CO_2). The resulting gases pass through purification traps for moisture and nitrogen oxides, and purified CO_2 is subsequently quantified by a non-dispersive infrared detector (NDIR). Carbon concentrations were calculated from calibration curves prepared with potassium hydrogen phthalate standards. For TOC measurements, crude humic substance (HS) extracts from vermicompost and peat were diluted 1:1000 using ultrapure water. A final volume of 15 mL was used per reading, with six analytical replicates for each HS source. Ultrapure water, the same used for sample dilution, was included as the analytical blank. Carbon concentration is expressed as mg C L^{-1}. The HS extracted

from vermicompost contained 1388 ± 12.5 mg C L^{-1} and the peat-derived HS 11,681 ± 151.5 mg C L^{-1}, expressed as mean ± standard deviation.

Solid-state ^{13}C NMR spectroscopy: spectra were obtained by CP/MAS on a Bruker-400 spectrometer operating at 75.4 MHz for ^{13}C. Acquisition parameters included: spectral width of 50 kHz, acquisition time of 50 ms, pulse width of 5 μs (90°), contact time of 1 ms, 4 s recycle delay, ^1H decoupling in gated mode, and 10,000 transients. Hexamethylbenzene (δ = 17.3 ppm) was used as a secondary reference. Signal integrals were calculated in the following chemical shift regions: aliphatic C (0–50 ppm), O-alkyl/peptide C (50–110 ppm), aromatic/olefinic C (110–150 ppm), heteroatom-substituted aromatic C (150–165 ppm), and carbonyl/amidic/ester C (165–200 ppm) [62].

4.2. Plant Material and Growth Conditions

Seeds of tomato (*Solanum lycopersicum* cv. Micro-Tom) were obtained from the Laboratory of Hormonal Control and Development, Luiz de Queiroz College of Agriculture, University of São Paulo (ESALQ-USP, Piracicaba, São Paulo, Brazil). The cultivar Micro-Tom is widely recognized as a model system for the study of hormonal signaling pathways in plants. Seeds were surface-sterilized in a 5% NaClO solution containing 2 drops of detergent for 10 min, then rinsed thoroughly with sterile distilled water. The seeds were sown in 300 mL pots containing a 1:1 (*v*/*v*) mixture of commercial substrate and vermiculite, supplemented with 8 g of NPK (10–10–10) per liter of substrate. Plants were grown in a greenhouse under an average temperature of 25 °C and a relative humidity of approximately 60%. The experimental design was completely randomized, with 12 replicates per treatment. Six treatments were established: (i) Control (no HS application and no *Oidium* sp inoculation); (ii) Pt (peat-derived HS, non-inoculated); (iii) Vc (vermicompost-derived HS, non-inoculated); (iv) Control + *Oidium* sp inoculation; (v) Pt + *Oidium* sp inoculation; and (vi) Vc + *Oidium* sp inoculation.

All HS suspensions were applied at a concentration of 8 mM C L^{-1}. The first application was carried out via substrate drenching at the two-true-leaf stage. At 21 days after germination, a second application was performed by foliar spraying. Inoculation with *Oidium* sp. was conducted 48 h after the foliar application of HS. The conidial suspension was obtained by gently scraping infected tomato leaves, and the concentration was adjusted to 1.8×10^6 conidia mL^{-1} using a Neubauer chamber. Plants were uniformly sprayed with the conidial suspension and maintained in the greenhouse under the same environmental conditions described above.

4.3. Enzyme Assays

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5): PAL activity was determined as described by Pascholati et al. [63] with minor modifications. Leaf tissue (100 mg) was ground in liquid nitrogen and homogenized in 10 mL of 0.1 M sodium borate buffer (pH 8.8), supplemented with 5% (*w*/*v*) polyvinylpyrrolidone (PVP) and 1.2 mL L^{-1} β-mercaptoethanol. After centrifugation (10,000× g, 25 min, 4 °C), the supernatant was used for the enzymatic reaction, consisting of 1 mL of

enzyme extract, 1 mL of 0.1 M phenylalanine, and 1 mL of 0.2 M borate buffer (pH 8.8). Samples were incubated at 36 °C for 60 min in the dark, and the reaction was stopped with 200 µL of 6 M HCl. Absorbance was measured at 290 nm, and activity was expressed as µmol of trans-cinnamic acid min^{-1} g^{-1} fresh weight, using a molar extinction coefficient of $\varepsilon_{290} = 10^4$.

Peroxidase (POX, EC 1.11.1.7): POX was extracted from leaf tissue ground in liquid nitrogen. Approximately 1 g of tissue was homogenized in 5 mL of 0.1 M sodium acetate buffer (pH 5.0), containing 1% (*v*/*v*) PVP and 1 mM EDTA. The homogenate was centrifuged (10,000× g, 10 min, 4 °C), and the supernatant was used for activity determination. The assay was carried out at 30 °C in a reaction mixture containing 2.0 mL of 0.2 M phosphate buffer (pH 5.8), 0.5 mL of 0.38 M H_2O_2, 50 µL of 0.02 M guaiacol, and 50 µL of enzyme extract. The increase in absorbance was monitored at 470 nm for 1 min, and activity was expressed as µmol of H_2O_2 decomposed min^{-1} g^{-1} fresh weight, using $\varepsilon_{470} = 26.6$ mM^{-1} cm^{-1} [64].

Lipoxygenase (LOX, EC 1.13.11.12): LOX activity was determined according to Axelrod et al. (1981) [68]. Fresh leaves were ground in liquid nitrogen to a fine powder, and the crude extract was used for the assays. The 10 mM sodium linoleate stock solution was prepared from linoleic acid, Tween 20, and 0.5 N NaOH, and stored at −20 °C in light-protected tubes. The reaction mixture contained 1.0 mL of enzyme extract, 4.0 mL of sodium linoleate solution, and 1.0 mL of 50 mM phosphate buffer (pH 6.0). Product formation was monitored spectrophotometrically at 234 nm at 30 s intervals for 120 s. All analyses were performed in triplicate, and enzyme activity was expressed as the change in absorbance min^{-1} g^{-1} fresh weight [66].

β-1,3-glucanase (GLU, EC 3.2.1.29): activity was determined according to Lever (1972) [65] by measuring glucose released from laminarin. Reaction mixtures contained 150 µL of enzyme extract and 150 µL of 2 mg mL^{-1} laminarin solution, incubated at 40 °C for 60 min. Then, 50 µL of the mixture was added to 1.5 mL of p-hydroxybenzoic acid hydrazide (PAHBAH) reagent and heated at 100 °C for 10 min. After cooling to 30 °C in an ice bath, absorbance was measured at 410 nm. Values were corrected against non-incubated controls and quantified using a glucose standard curve. Enzymatic activity was expressed as ng of glucose released min^{-1} mg^{-1} protein [65].

4.4. Gene Expression Analysis by RT-qPCR

Total RNA was extracted from leaves of three independent biological replicates using the TRIzol reagent (Invitrogen). RNA quality and quantity were assessed using a NanoDrop 1000 spectrophotometer and by electrophoresis on a 1.5% agarose gel. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the GoScript™ Reverse Transcriptase Kit (Promega). Quantitative real-time PCR (RT-qPCR) reactions were performed on a StepOne Fast Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix. The cycling conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s and 58 °C for 30 s. Three biological and three technical replicates were analyzed for each gene. Gene expression levels were normalized

using actin (Slactin-4) as the reference gene, and relative transcript abundance was calculated using the $2^{-\Delta\Delta CT}$ method [67]. Primer sequences used for all target genes are provided in Supplementary Table S3.

4.5. Disease Severity Assessment

Powdery mildew severity in tomato plants was evaluated using the standard area diagram scale proposed by Lage et al. [68], developed for whole tomato leaves exhibiting symptoms of *Oidium* sp. The scale includes six severity levels (1, 5, 10, 20, 40, and 60% of infected leaf area). For each plant, the third and fourth fully expanded leaves below the apex were evaluated, and the percentage of diseased area was visually estimated by direct comparison with the diagrammatic scale. The mean severity per plant was used as the individual severity index (S_p), and the disease index (DI) for each treatment was calculated as the average of all S_p values within that treatment group.

4.6. Statistical Analysis

Data on enzymatic activity were subjected to analysis of variance (ANOVA), and when treatment effects were significant ($p \leq 0.05$), means were compared using the LSD test. Enzymatic analysis statistics were performed in R (R Core Team, version 4.3) using the ExpDes.pt package. Disease severity data, expressed as the percentage of infected leaf area, were analyzed using GraphPad Prism (version 8.1). Although homoscedasticity was not violated (Brown–Forsythe test, $p = 0.0676$), normality assumptions were not met (D'Agostino–Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov tests; $p < 0.05$). Therefore, disease severity was analyzed using the nonparametric Kruskal–Wallis test ($p < 0.05$), followed by Dunn's multiple-comparison test with Bonferroni adjusted p values. Gene expression data were analyzed in GraphPad Prism using one-way ANOVA, and significant differences relative to the control ($p < 0.05$) were indicated as follows: * for induction (higher expression) and ° for repression (lower expression). All ANOVA summary tables are provided in the Supplementary Material (Tables S1, S2 and S4). Graphs were generated using GraphPad Prism.

5. Conclusions

In this study, HS demonstrated a multifunctional role in inducing plant defense in tomato plants infected by *Oidium* sp., acting as modulators of hormonal signaling, redox balance, and the expression of defense-related genes. Despite the structural differences observed between HS isolated from Vc and Pt, both sources were equally effective in activating pathways associated with SAR, enhancing the expression of antioxidant genes and PR proteins, and significantly reducing the severity of infection caused by *Oidium* sp., a biotrophic pathogen whose containment strongly depends on mechanisms mediated by SA and SAR. These results reinforce the role of HS as sustainable, integrative biostimulants that coordinate multiple defense pathways and strengthen the plant immune system. Therefore, the use of HS represents a promising strategy to contain the development of Oidium sp. Infection in tomato

crops, supporting more sustainable and resilient disease management practices in commercial production systems.

Supplementary Materials: The following supporting information can be found at: https://www.mdpi.com/article/doi/s1, Table S1. Summary of statistical results for relative gene expression (Dunnett's test). Table S2. Statistical analysis of disease severity (*Oidium* sp.). Table S3: Oligonucleotide sequence; Table S4: Enzyme anova.

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Data Availability Statement:

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

HS	Humic substances
JA	Jasmonic acid
SA	Salicylic acid
ET	Ethylene
On	*Oidium* sp
SAR	Systemic acquired resistance
ISR	Induced systemic resistance
PR	Pathogenesis-Related
Vc	Vermicompost
Pt	Peat
DI	Disease severity index
PAL	Phenylalanine Ammonia Lyase
POX	Peroxidase
LOX	Lipoxygenase
PR2	B 1,3-Glucanase
ROS	Reactive oxygen species
APX	Ascorbate peroxidase
SOD	Superoxide dismutase

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ARTIGO 3: MOLECULAR REWIRING OF TOMATO DEFENSE BY HUMIC SUBSTANCES

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MOLECULAR REWIRING OF TOMATO DEFENSE BY HUMIC SUBSTANCES

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Abstract: Humic substances (HS) have been associated with the activation of plant immunity, although the underlying molecular mechanisms are not fully understood. This study evaluated the effects of two structurally distinct HS one derived from vermicompost (HS-Vc) and the other from peat (HS-Pt) on the physiological, biochemical, transcriptional, and proteomic responses of tomato plants (*Solanum lycopersicum* cv. Micro-Tom) infected with *Xanthomonas euvesicatoria*. Solid-state ^{13}C NMR analysis revealed significant structural differences between the two HS: HS-Vc was enriched in O-alkyl carbons associated with carbohydrates and peptides, whereas HS-Pt was characterized by more recalcitrant hydrophobic and aromatic structures. Proteomic analysis identified 995 proteins and demonstrated extensive metabolic reprogramming triggered by bacterial infection. HS-Vc maintained a proteomic profile similar to the control, suggesting a modulatory effect and preservation of homeostasis. In contrast HS-Pt induced significant alterations in pathways related to photosynthesis, oxidative phosphorylation, and redox regulation. Gene expression analyses revealed significant induction of key genes involved in induced systemic resistance, including regulators and effectors associated with the jasmonic acid pathway and PR genes, as well as modulation of central antioxidant genes (SOD, CAT, and APX). Complementary enzymatic assays of phenylalanine ammonia-lyase (PAL), peroxidase (POX), lipoxygenase (LOX), and β-1,3-glucanase (GLU) confirmed the coordinated activation of defense mechanisms. In infected plants, both HS mitigated the impact of the pathogen through specific adjustments in proteins, defense-related genes, and antioxidant components, resulting in enhanced physiological stability. Taken together, our results show that different types of HS trigger distinct regulatory responses in tomato defense of tomato plants. HS-Vc acts primarily as a homeostatic modulator, while HS-Pt promotes a more dynamic and responsive metabolic state. These findings highlight the potential of HS as promising biological inputs for sustainable management of plant diseases.

Keywords: Proteomics, biostimulants, redox metabolism.

1. Introduction

Plant immunity is sustained by a multilayered defense system orchestrated by highly complex and context-dependent hormonal signaling networks (Ali et al., 2025; Li et al., 2025). In general, jasmonic acid (JA) and ethylene (ET) pathways are associated with resistance against necrotrophic pathogens and activation of induced systemic resistance (ISR), whereas salicylic acid (SA) plays a central role in defense against biotrophic pathogens and systemic acquired resistance (SAR) (Glazebrook, 2005; Pieterse et al., 2012; Haghpanah et al., 2025). These hormonal circuits do not operate in isolation: they are strongly integrated with reactive oxygen species (ROS)-mediated signaling networks, which function as secondary messengers in redox adjustment and defense–hormonal communication, influencing both activation and antagonism among SA–JA/ET pathways (Rojas et al., 2014; Schieber & Chandel, 2014; Miller et al, 2017).

In addition to endogenous signaling, plant immunity can be modulated by the perception of exogenous elicitors capable of adjusting the host defensive state, frequently linked to immune priming, a process in which sub-lethal stimuli prepare the plant for faster, stronger, and metabolically more efficient responses to future stresses (Meena et al., 2022; Malik et al., 2020). Within this framework, humic substances (HS) have been proposed as natural elicitors due to their ability to interact with biological interfaces, modulate hormonal pathways, and regulate stress-responsive enzymes that integrate nutrition, physiology, and defense (Canellas & Olivares, 2014; Nardi et al., 2021; Silva & Canellas, 2022; Souza et al., 2022).

HS are highly heterogeneous supramolecular mixtures composed of dynamic associations of organic molecules enriched in oxygenated functional groups such as carboxyl (–COOH), phenolic (Ar–OH), and O-alkyl-C domains alongside aromatic and aliphatic regions whose structural organization directly reflects their geological or biogenic origin (Piccolo, 2002; Piccolo et al., 2018; Maffia et al., 2025; Piccolo & Drosos, 2025). This molecular diversity determines key physicochemical properties, including redox reactivity, hydrophobicity, chelating capacity, and affinity for biological interfaces, enabling interactions with lipid bilayers, membrane proteins, and apoplastic components, as well as modulation of defense-related hormonal and redox-responsive enzymes (Trevisan et al., 2010; Canellas & Olivares, 2014; Nardi et al., 2016; Zandonadi et al., 2025). These include peroxidases of class III (POD), lipoxygenases (LOX), superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), β-1,3-glucanases (GLU), glutathione transferases (GST), and phenylpropanoid-related enzymes, which collectively contribute to ROS homeostasis, redox signaling, and immune–hormonal communication (Piccolo, 2002; Nardi et al., 2021; Canellas et al., 2014; Roomi et al., 2018; Zandonadi et al., 2025).

Although recent evidence indicates that HS can activate both SA-dependent SAR and JA/ET-mediated ISR, while modulating antioxidant and phenylpropanoid pathways (Schiavon et al., 2010; García et al., 2016; Faccin & Piero, 2022; Silva et al., 2023a; 2023b; Zandonadi et al., 2025), mechanistic understanding remains limited, largely due to their intrinsic chemical heterogeneity. Moreover, integrative studies assessing how chemically distinct HS shape the plant proteome and hormonal balance under hemibiotrophic bacterial infection a condition that demands economical metabolic adjustments and a fine-tuned equilibrium between frequently antagonistic routes such as SA and JA/ET are still scarce.

Xanthomonas euvesicatori (*Xe*) exhibits a hemibiotrophic lifestyle, characterized by an initial asymptomatic colonization phase followed by a transition to a necrotrophic stage, in which tissue collapse, and typical disease symptoms develop (Chowdhury et al., 2017). This alternation between biotrophic and necrotrophic strategies within a single pathosystem requires continuous reprogramming of host defense responses. Because it integrates both lifestyles, the tomato–Xe interaction constitutes a particularly suitable biological model for investigating phytohormone-mediated systemic resistance mechanisms, enabling an integrated analysis of the roles of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) in the coordination of plant defense and in maintaining hormonal crosstalk under biotic stress (Glazebrook, 2005; Glazebrook & Roby, 2018).

Given this knowledge gap, this work proposed to investigate how HS from contrasting sources reprogram the tomato proteome, hormonal gene transcription, and enzyme activity, influencing redox-integrative hubs and sustaining a primed state at low physiological cost. Consistent with the mechanistic discussion developed throughout this study, we hypothesized that structural differences between HS-Vc and HS-Pt translate into distinct regulatory impacts on foliar proteome dynamics, redox signaling nodes, and the functional coordination of SA–JA/ET defense pathways.

Accordingly, the present study aimed to investigate the effects of humic substances of contrasting origins on hormonal rebalancing and molecular reprogramming in tomato plants (*S. lycopersicum* cv. Micro-Tom) infected with *Xe*. By integrating chemical, proteomic, transcriptional, and biochemical analyses, we sought to elucidate how structural contrasts between HS-Vc and HS-Pt translate into differential regulatory effects on leaf proteome profiles, redox networks, defense-related enzymes, and hormonal pathway coordination under bacterial infection.

2. Material and methods

2.1. Obtaining and chemical characterization of HS

Humic substances (HS) were obtained from two distinct sources. The first consisted of HS extracted from vermicompost (Vc) produced from cattle manure using the earthworm

Eisenia andrei. The second source was a commercial peat-derived product (Pt) provided by DNAgro Biotechnology (Ourinhos, Brazil). HS-Vc were extracted using a 5% (w/v) potassium hydroxide (KOH) solution (Sigma-Aldrich, St. Louis, MO, USA) at a 1:20 (v/v) ratio, under continuous agitation for 4 h. The suspension was subsequently centrifuged at 5000× *g* for 15 min, and the supernatant was adjusted to pH 7.0 with HCl (6 M) Aliquots of both HS preparations (Vc and Pt) were frozen and freeze-dried prior to chemical characterization.

Total organic carbon (TOC) content was determined by dry combustion using a Shimadzu TOC analyzer (Tokyo, Japan). For said analysis, crude HS extracts obtained from Vc and Pt were diluted 1:1000 in ultrapure water. A final volume of 15 mL was analyzed per measurement, with six analytical replicates conducted for each HS source. Ultrapure water used for sample dilution was included as the analytical blank. Total organic carbon concentrations were expressed as mg C L^{-1}. The vermicompost-derived HS contained 1,388 ± 12.5 mg C L^{-1}, whereas peat-derived HS presented 11,681 ± 151.5 mg C L^{-1}, reported as mean ± standard deviation (Silva et al., 2025).

The Solid-state ^{13}C NMR spectra were obtained using cross-polarization and magic angle spinning (CP/MAS) on a Bruker-400 spectrometer equipped with a solid-state probe. Experimental conditions were as follows: 75.4 MHz observation frequency for ^{13}C, 50 kHz spectral width, 50 ms acquisition time, 5 μs (90°) pulse, 4 s pulse delay, 1 ms contact time, and ^1H decoupling in gated mode with 10,000 transients accumulated. Hexamethylbenzene (δ = 17.3 ppm) was used as the secondary reference. Integrals were obtained for the following chemical shift regions: aliphatic C (0–50 ppm), O-alkyl or peptide C (50–110 ppm), aromatic and olefinic C (110–150 ppm), heteroatom-substituted aromatic C (150–165 ppm), and carbonyl, amide, or ester C (165–200 ppm) (Aquino et al., 2019).

2.2 Plant material, growth conditions, and bacterial inoculation

Tomato seeds (*Solanum lycopersicum* cv. Micro-Tom) were surface-sterilized in 5% sodium hypochlorite (NaClO) containing two drops of neutral detergent for 10 min, followed by repeated rinsing in distilled water to remove residues. Seeds were sown in 300 mL pots filled with a 1:1 (v/v) mixture of commercial substrate and expanded vermiculite, supplemented with 8 g L^{-1} of NPK (10-10-10). The experiment was conducted in a growth chamber at 28/24 °C (day/night), 80% relative humidity, and an 8 h light / 16 h dark photoperiod, in a completely randomized design with six treatments and 12 replicates per treatment.

Treatments consisted of: Control (no HS, no Xe), HS-Vc, HS-Pt, HS-Vc + Xe, HS-Pt + Xe, and Xe (no HS, infection only). HS suspensions were prepared at 8 mM C L^{-1} and applied to the substrate at the second pair of true leaves, followed by a foliar spray 21 days after germination.

The *Xe* inoculum was prepared from a pure colony grown in liquid medium at 28 °C

(24–48 h, shaking) and resuspended in sterile solution. The culture showed an initial OD_{600} = 0.7, and the blank solution used for optical reference presented OD_{600} = 0.59. For plant inoculation, the bacterial suspension was adjusted to OD_{600} = 0.3.

Tomato plants were inoculated by foliar spray until runoff, using the OD_{600} = 0.3 Xe suspension, identical to the procedure applied to mock controls (carrier solution only + surfactant). After spraying, plants were maintained at high humidity (> 90%) for 24 h to support infection establishment.

Forty-eight hours after the foliar HS spray, plants assigned to infected treatments were inoculated with Xe. Seventy-two hours post-inoculation, leaf tissues from four biological replicates per treatment were harvested for biochemical and molecular analyses.

2.3. Proteomic analyses

Approximately 200 mg of leaf tissue were ground in liquid nitrogen and homogenized in 800 µL of extraction buffer A (0.5 M Tris, 0.7 M sucrose, 0.1 M KCl, 1% DTT, 1 mM PMSF, and 1× protease inhibitor). Samples were incubated under agitation for 30 min at 4 °C and centrifuged to collect the supernatant. The remaining pellet was resuspended in 1 mL of extraction buffer B (50 mM Hepes, pH 8.0; 1% Triton X-100; 1 M NaCl; 1× protease inhibitor), incubated for 1 h at 4 °C with agitation, and centrifuged, with the supernatant collected. Proteins were precipitated from the supernatants using different strategies: TCA precipitation for supernatant A and phenol precipitation for supernatant B. Following the addition of the respective precipitation solutions, samples were vortexed, incubated overnight at –20 °C, and centrifuged (15,000 rpm, 10 min, 4 °C). The resulting pellets were washed successively with 100% acetone containing DTT, combined in a 1.5 mL tube, and resuspended in 8 M urea/100 mM Tris-HCl (pH 8.5). Protein concentration was determined using the Bradford assay (Sigma Aldrich, cat. 12161503). Protein integrity was verified by agarose gel electrophoresis prior to digestion. For enzymatic digestion, proteins were processed in Vivacon 10 kDa filters (Sartorius) with trypsin (12.5 ng/µL) and dried under vacuum using a SpeedVac concentrator (Thermo Fisher Scientific). The resulting peptides were resuspended in 60 µL of 3% acetonitrile/0.1% formic acid (theoretical concentration: 0.5 µg/µL; initial injection volume: 4 µL = 2 µg). Samples were analyzed in technical triplicates by LC-MS/MS (LTQ Orbitrap XL, Thermo Fisher Scientific). Data were processed using MaxQuant v2.5.2.0.

2.4. Differential transcription level of genes by RT-qPCR

Total RNA was extracted from three independent biological replicates using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA) as described by Silva et al., 2023. RNA quantity and quality were assessed with a NanoDrop1000 spectrophotometer (Thermo Scientific, USA) and verified on 1.5% agarose gel. The cDNA was synthesized from 1 µg of

total RNA using the GoScript™ Reverse Transcriptase kit (Promega, A5001), following the manufacturer's instructions (Silva et al., 2023). RT-qPCR was performed on a StepOne Fast Real-Time PCR System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems). Cycling conditions were: 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s and 58 °C for 30 s. Three biological replicates and three technical replicates were analyzed for each gene. Relative transcript levels were normalized to *Actin* and calculated by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Primers were designed using Primer3Plus and validated by NCBI BLAST and Primer Premier software.2.10.

2.4. Enzyme Assays

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5): PAL activity was determined as described by Pascholati et al. (1986) with minor modifications. Leaf tissue (100 mg) was ground in liquid nitrogen and homogenized in 10 mL of 0.1 M sodium borate buffer (pH 8.8), supplemented with 5% (w/v) polyvinylpyrrolidone (PVP) and 1.2 mL L^{-1} β-mercaptoethanol. After centrifugation (10,000 g, 25 min, 4 °C), the supernatant was used for the enzymatic reaction, consisting of 1 mL of enzyme extract, 1 mL of 0.1 M phenylala-nine, and 1 mL of 0.2 M borate buffer (pH 8.8). Samples were incubated at 36 °C for 60 min in the dark, and the reaction was stopped with 200 µL of 6 M HCl. Absorbance was meas-ured at 290 nm, and activity was expressed as µmol of trans-cinnamic acid min^{-1} g^{-1} fresh weight, using a molar extinction coefficient of ε_{290} = 10^4.

Peroxidase (POX, EC 1.11.1.7): POX was extracted from leaf tissue ground in liquid nitro-gen. Approximately 1 g of tissue was homogenized in 5 mL of 0.1 M sodium acetate buffer (pH 5.0), containing 1% (v/v) PVP and 1 mM EDTA. The homogenate was centrifuged (10,000 g, 10 min, 4 °C), and the supernatant was used for activity determination. The as-say was carried out at 30 °C in a reaction mixture containing 2.0 mL of 0.2 M phosphate buffer (pH 5.8), 0.5 mL of 0.38 M H_2O_2, 50 µL of 0.02 M guaiacol, and 50 µL of enzyme extract. The increase in absorbance was monitored at 470 nm for 1 min, and activity was ex-pressed as µmol of H_2O_2 decomposed min^{-1} g^{-1} fresh weight, using ε_{470} = 26.6 mM^{-1} cm^{-1} (Hammerschmidt ,1982).

Lipoxygenase (LOX, EC 1.13.11.12): LOX activity was determined according to Axelrod et al. (1981). Fresh leaves were ground in liquid nitrogen to a fine powder, and the crude ex-tract was used for the assays. The 10 mM sodium linoleate stock solution was prepared from linoleic acid, Tween 20, and 0.5 N NaOH, and stored at −20 °C in light-protected tubes. The reaction mixture contained 1.0 mL of enzyme extract, 4.0 mL of sodium linole-ate solution, and 1.0 mL of 50 mM phosphate buffer (pH 6.0). Product formation was monitored spectrophotometrically at 234 nm at 30 s intervals for 120 s. All analyses were performed in triplicate, and enzyme activity was expressed as the change in absorbance min^{-1} g^{-1} fresh weight.

β-1,3-glucanase (GLU, EC 3.2.1.29): activity was determined according to Lever (1972) by measuring glucose released from laminarin. Reaction mixtures contained 150 µL of en-zyme extract and 150 µL of 2 mg mL^{-1} laminarin solution, incubated at 40 °C for 60 min. Then, 50 µL of the mixture was added to 1.5 mL of p-hydroxybenzoic acid hydrazide (PAHBAH) reagent and heated at 100 °C for 10 min. After cooling to 30 °C in an ice bath, absorbance was measured at 410 nm. Values were corrected against non-incubated controls and quantified using a glucose standard curve. Enzymatic activity was expressed as ng of glucose released min^{-1} mg^{-1} protein.

2.5. Statistical Analysis

Data on enzymatic activity were analyzed using analysis of variance (ANOVA), and when treatment effects were significant ($p \leq 0.05$), means were compared using the Least Significant Difference (LSD) test. Enzymatic statistical analyses were performed in R (R Core Team, version 4.3) using the ExpDes.pt package. Gene expression data were analyzed in GraphPad Prism using one-way ANOVA, and treatments showing significant differences relative to the control ($p < 0.05$) were considered statistically distinct. Graphs for gene expression and enzymatic activity results were generated using GraphPad Prism.

For proteomic data, protein identification was carried out using MaxQuant, and differential protein accumulation was assessed through the NormalyzerDE web pipeline, applying the empirical Bayes Limma approach (moderated t-test). Resulting P-values were corrected for multiple testing using the Benjamini–Hochberg procedure (FDR), with a significance cutoff set at adjusted p-value ≤ 0.05. Differentially accumulated proteins were defined using a fold change > 1.3 or < −1.3. Principal Component Analysis (PCA) was performed in R (R Core Team, v4.3) using the normalized intensity matrix to verify the separation of experimental groups. Functional annotation and enrichment analyses were conducted based on the statistical output generated by NormalyzerDE.

3. Results

3.1. Chemical characterization of HS

The solid-state ^{13}C NMR spectra revealed clear structural distinctions between HS-Vc and HS-Pt (Figure 1). The Vc spectrum (Figure 1a) exhibited broader resonance signals associated with carbohydrate- and peptide-like structures, particularly a strong peak centered at 71.7 ppm, corresponding to O-alkyl carbons and C–N/O functional groups. A prominent signal at 54.3 ppm was attributed to lignin-derived methoxy groups. In addition, resonances in the range of 117.9–126.4 ppm indicated the presence of unsubstituted aromatic carbons, whereas a signal at 172.7 ppm was assigned to carboxylic groups. These spectral features suggest that HS-Vc contained a higher proportion of oxygenated compounds related to carbohydrate and protein

residues. In contrast, the Pt spectrum (Figure 1b) was dominated by hydrophobic and aliphatic carbons, characterized by an intense peak at 30.5 ppm, along with signals at 120.9 ppm (aromatic carbons) and 170.1 ppm (carboxyl/carbonyl groups), indicating a more hydrophobic and recalcitrant organic structure typical of sedimentary materials.

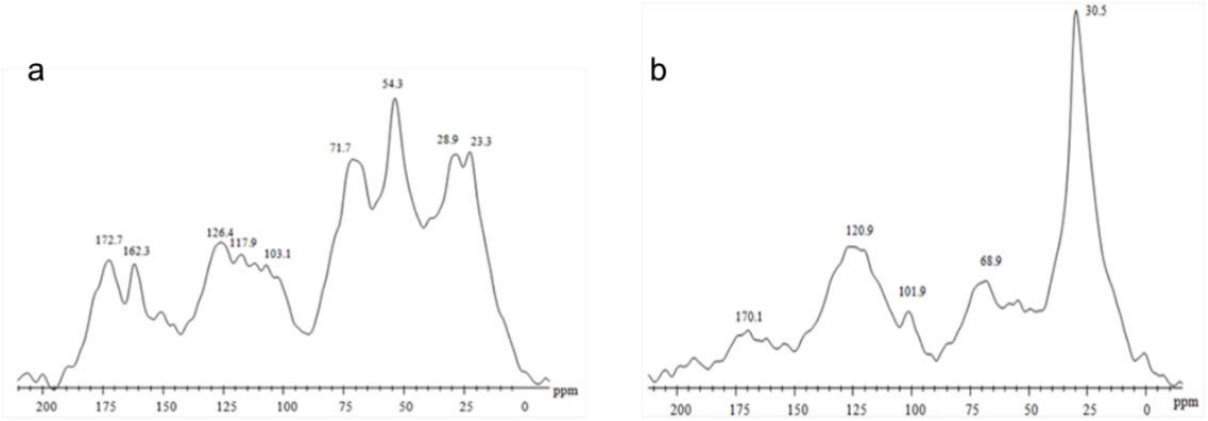

Figure 1. Solid-state ^{13}C CP/MAS NMR spectra of humic substances (HS) isolated from vermicompost (a) and peat (b).

The 13C CPMAS NMR spectra of HS isolated from Pt and Vc revealed the usual composition of complex natural organic materials with a carbon distribution extended along all the chemical shift range (Figure 1). Vc was characterized by the predominance of CH3O/C-N interval and O-alkyl-C components, which represented the 16.7% and 28.1% of the total area, respectively (Table 1), while humic substances isolated from Pt showed the alkyl-C (45-0 ppm) interval corresponding to 50.2% of total spectrum area.

	C=O 190–160	O-aryl-C 160–140	aryl-C 140–110	O-alkyl-C 110–60	CH_3O/C-N 60–45	alkyl-C 45–0	HB	A/OA	Ar	LR
HS-Pt	10.0	3.5	22.7	9.8	3.7	50.2	3.6	5.1	26.3	1.1
HS-Vc	6.5	8.4	20.3	28.1	16.7	19.8	1.3	0.7	28.7	2.0

The hydrophobic index is the ratio of signal intensities in chemical shift intervals for apolar alkyl and aromatic C components over those of hydrophilic C molecules: (1) HB = Σ[(0–45) + (45–60)/2 + (110–160)]/Σ[(45–60)/2 + (60–110) + (160–190)]. The alkyl ratio determines the relative contribution of apolar versus polar alyl molecules. (2) A/OA = (0–45)/(60–110). The aromaticity index is the total area assigned to aromatic compounds. (3) Ar = Σ[110–160). The lignin ratio relates the area of methoxyl-C + N-alkyl groups to that of O-aryl-C. (4) LR = (45–60)/(145–160).

3.2. Changes in the leaf proteome in response to HS treatments and bacterial infection

Overall, HS promoted significant alterations in the leaf proteome of tomato plants, regardless of Xe infection. Proteomic analysis identified a total of 995 proteins across the analyzed samples (Supplementary Table S1). To investigate global differences among treatments, a Principal Component Analysis (PCA) was performed considering only differentially abundant proteins (Figure 2). The first two principal components explained 85.1% of the total variance, with 74.6% attributed to PC1 and 10.5% to PC2. The control (Ct), Xe, and

HS-Vc treatments clustered closely, indicating similar proteomic profiles and suggesting only minor proteomic changes in the HS-Vc+Xe treatment induced by bacterial infection. In contrast, the HS-Pt and, more markedly, the HS-Pt+Xe treatments displayed a clear separation along PC1, reflecting a more pronounced proteomic reprogramming

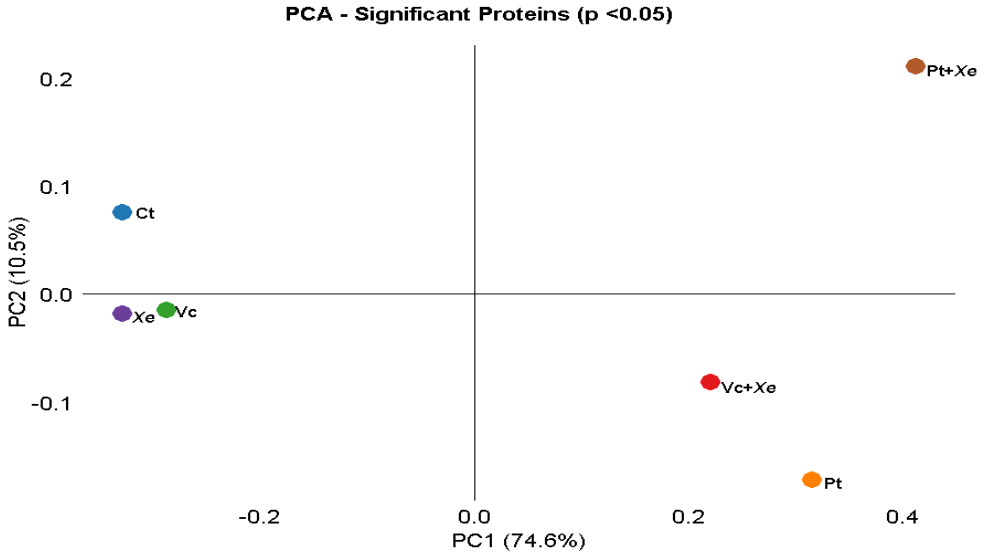

Figure 2. PCA of significantly altered proteins across treatments. Proteins showing significant differences between treatments were identified using ANOVA with FDR-adjusted $p < 0.05$. Each point represents the mean log2-transformed intensity of a significant protein for each treatment. Colors indicate experimental treatments: Ct (control), Xe (*X. euviscatoria*), Vc (Vermicompost), Pt (Peat), Vc + Xe, and Pt + Xe. PC1 and PC2 axes display the percentage of variance explained, highlighting treatment-specific proteome changes.

In a comparative analysis relative to the Ct, 5, 47, and 148 differentially expressed proteins (padj < 0.05) were identified in the HS-Vc, SH-Pt, and Xe treatments, respectively. In the HS-Vc treatment, all differentially abundant proteins showed increased abundance compared with the Ct (Table 1), including enzymes associated with amino acid metabolism, such as chloroplastic leucine aminopeptidase 1 and acetylornithine deacetylase, as well as a protease inhibitor related to plant defense. These results indicate that HS-Vc induces a modest and targeted proteomic response under basal conditions.

Table 1. Differentially expressed proteins in tomato leaves treated with HS from Vc compared with Ct, under non-infected conditions.

Protein ID	Protein name	Functional annotation	Localization	Fold change	p_{adj}
Q10712	Leucine aminopeptidase 1	Proteolysis and amino acid turnover	Chloroplast	1.7	5,59E-04
A0A3Q7HRC7	Acetylornithine deacetylase	Amino acid metabolism	Cytosol	1.7	4,15E-02

Q43710	Proteinase inhibitor type-2 TR8	Defense-related protease inhibitor	Extracellular	1.8	7,24E-03
A0A3Q7FP33	Uncharacterized protein	Putative metabolic protein	Unknown	2.1	7,92E-03
P25306	Threonine dehydratase 2 biosynthetic	Branched-chain amino acid biosynthesis	Chloroplast	1.5	4,79E-02

Note: Fold change values represent relative protein abundance (Vc/Ct). Adjusted *p*-values (*padj*) were calculated using the Benjamini–Hochberg false discovery rate (FDR) correction. Only proteins with *padj* < 0.05 were considered significantly differentially expressed.

In contrast, HS-Pt application under non-infected conditions promoted extensive proteomic reprogramming, involving both positively and negatively regulated proteins across multiple functional categories (Table 2). Proteins associated with photosynthesis and the photosynthetic apparatus constituted the most affected group and were predominantly downregulated, including several chlorophyll *a–b* binding proteins and components of photosystems I and II, indicating a coordinated suppression of light harvesting and electron transport. Conversely, proteins involved in the Calvin cycle and carbon fixation, such as RuBisCO activase and sedoheptulose-1,7-bisphosphatase, showed increased abundance, suggesting adjustments in carbon assimilation.

Pronounced changes were also observed in proteins related to energy and respiratory metabolism, with a predominance of downregulation among mitochondrial components of the tricarboxylic acid cycle, the electron transport chain, and oxidative phosphorylation. In parallel, increased abundance of cytosolic enzymes from glycolysis and the pentose phosphate pathway indicates a reorganization of cellular energy metabolism. HS-Pt treatment also modulated proteins involved in amino acid metabolism and protein quality control, including the induction of aminopeptidases, branched-chain amino acid biosynthetic enzymes, mitochondrial chaperonins, and the peroxisomal Lon protease. Additionally, changes were detected in proteins associated with oxidative stress responses, redox regulation, intracellular transport, signaling, nuclear structure, and translation, collectively reflecting a broad physiological adjustment.

Table 2. Differentially expressed proteins in tomato leaves treated with HS-Pt compared with Ct plants, under non-infected conditions agrupadas por categoria funcional.

Protein ID	**Protein name**	**Functional annotation**	**Localization**	**Fold change**	**p_{adj}**
Photosynthesis and the photosynthetic apparatus					
P10707	Chlorophyll a–b binding protein 1D	Light harvesting and photosynthesis	Chloroplast	-1.7	1.72E-02
A0A3Q7G195	Chlorophyll a–b binding protein	Light harvesting and	Chloroplast	-1.3	1.88E-02

		photosynthesis			
Q2MIA0	Photosystem I P700 chlorophyll a	Photosystem I core protein	Chloroplast	-1.4	1.88E-02

Table 2. Continuation.

Q672Q6	Photosystem II oxygen-evolving	Photosystem II oxygen evolution	Chloroplast	-2.3	2.55E-02
P10708	Chlorophyll a–b binding protein 7	Light harvesting and photosynthesis	Chloroplast	-1.5	3.70E-02
A0A3Q7FU35	Chlorophyll a–b binding protein	Light harvesting and photosynthesis	Chloroplast	-1.4	3.70E-02
Q7M1K8	Chlorophyll a–b binding protein	Light harvesting and photosynthesis	Chloroplast	-2.1	4.25E-02
A0A3Q7HDJ6	Chlorophyll a–b binding protein	Light harvesting and photosynthesis	Chloroplast	-1.4	4.42E-02
A0A3Q7GQW4	Chlorophyll a–b binding protein	Light harvesting and photosynthesis	Chloroplast	-1.6	4.48E-02
P27525	Chlorophyll a–b binding protein CP24	Light harvesting and photosynthesis	Chloroplast	-1.4	4.48E-02
A0A3Q7HIP8	Photosystem I reaction center V	Photosystem I stabilization and electron transport	Chloroplast	-1.5	4.65E-02
A0A3Q7I009	RuBisCO activase	Regulation of RuBisCO activity and carbon fixation	Chloroplast	1.5	1.88E-02
A0A3Q7J0I3	RuBisCO large subunit-binding protein subunit alpha	RuBisCO assembly and stabilization	Chloroplast	1.3	4.53E-02
A0A3Q7GLK1	Sedoheptulose-1,7-bisphosphatas	Calvin cycle	Chloroplast	1.3	4.86E-02
Energy and respiratory metabolism					
A0A3Q7FLI5	Mitochondrial outer membrane protein porin of 34 kDa	Mitochondrial metabolite transport and energy metabolism	Mitochondrion	-1.7	1.88E-02
Q8GTQ9	Succinate–CoA ligase (ADP-forming) subunit alpha-1	Tricarboxylic acid cycle	Mitochondrion	-2.7	3.70E-02
A0A3Q7EIY6	ATP synthase subunit O	Oxidative phosphorylation	Mitochondrion	-2.1	3.70E-02

A0A3Q7F8L3	NADH dehydrogenase flavoprotein 1	Mitochondrial electron transport (Complex I)	Mitochondrion	-2.2	4.25E-02
A0A3Q7JX44	ATP synthase delta chain	Oxidative phosphorylation	Mitochondrion	-1.8	4.86E-02

Table 2. Continuation.

A0A3Q7HUQ3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis and energy metabolism	Cytosol	1.3	3.70E-02
A0A3Q7GF14	6-phosphogluconate dehydrogenase	Pentose phosphate pathway	Cytosol	1.6	4.53E-02
Amino acid and protein metabolism and quality control					
Q8GZD8	Neutral leucine aminopeptidase preprotein	Proteolysis and amino acid turnover	Chloroplast	1.5	1.72E-02
A0A3Q7HRC7	Peptidase M20 dimerisation domain-containing protein	Protein processing and amino acid metabolism	Cytosol	1.4	2.66E-02
P25306	Threonine dehydratase 2 biosynthetic	Branched-chain amino acid biosynthesis	Chloroplast	1.5	2.66E-02
B6ECN9	Aminoaldehyde dehydrogenase 2	Amino acid catabolism	Cytosol	1.4	3.70E-02

Table 3. Continuation.

A0A3Q7I956	Chaperonin CPN60-2	Protein folding and quality control	Mitochondrion	5.6	3.70E-02
A0A3Q7HU70	Lon protease homolog 2	Protein quality control and degradation	Peroxisome	22.1	3.70E-02
A0A3Q7IP94	Elongation factor G	Chloroplast protein translation	Chloroplast	1.4	3.70E-02
Defense, oxidative stress, and redox					
Q43710	Proteinase inhibitor type-2	Defense-related protease inhibitor	Extracellular	1.7	1.72E-02
A0A3Q7GBP8	Thioredoxin domain-containing protein	Redox regulation	Cytosol	1.3	3.70E-02
A0A3Q7HGQ5	Glutathione transferase	Detoxification and oxidative stress response	Cytosol	-2.1	4.53E-02
A0A3Q7J9P7	Cytochrome c domain-containing protein	Electron transport and redox processes	Mitochondrion	1.4	2.41E-02
Transportation, signage and regulation					

A0A3Q7ETC3	Ras-related protein Rab7	Vesicle trafficking and endosomal transport	Cytosol	-2.5	3.77E-02
D1MAF2	Exportin-1	Nuclear–cytoplasmic transport	Nucleus / Cytosol	1.8	4.65E-02

Table 2. Continuation.

A0A3Q7HG48	RING-type domain-containing protein	Protein ubiquitination and regulation	Cytosol / Nucleus	-1.4	4.86E-02
A0A3Q7IJ97	Tr-type G domain-containing protein	GTP-binding protein involved in signaling	Cytosol	1.3	3.70E-02
Nuclear structure and translation					
P35057	Histone H4	Chromatin organization and transcriptional regulation	Nucleus	-4.1	3.70E-02
A0A3Q7H1U4	Small ribosomal subunit protein uS2	Protein synthesis (chloroplast ribosome)	Chloroplast	-1.5	2.66E-02
Uncharacterized proteins					
A0A3Q7FP33	Uncharacterized protein	Unknown	Unknown	1.8	2.66E-02
A0A3Q7JDN9	Uncharacterized protein	Unknown	Unknown	2.9	2.66E-02
A0A3Q7EUR0	Uncharacterized protein	Unknown	Unknown	-1.9	3.70E-02
A0A3Q7GQM4	Uncharacterized protein	Unknown	Unknown	1.4	3.70E-02

Nota: Fold change values correspond to $\log_2$-transformed abundance ratios relative to Xe. Positive and negative values indicate increased and decreased protein abundance, respectively. Only proteins with padj < 0.05 are shown. Functional annotation and subcellular localization were inferred from UniProt.

Infection with *Xe* alone (Xe vs. Ct) triggered extensive proteomic alterations, characterized by the induction of proteins related to plant defense and redox control, concomitant with a coordinated repression of proteins associated with photosynthesis, primary metabolism, and translation, reflecting the high physiological cost of the plant immune response (Supplementary Table S2).

To assess the effect of HS on the modulation of the infection response, plants treated with HS-Vc+*Xe* and HS-Pt+*Xe* were compared with infected-only plants (*Xe*). In the HS-Vc+*Xe* versus *Xe* comparison, only a limited number of proteins exhibited significant changes in abundance (Table 3), indicating a selective modulation of the infection-induced proteome. Regulated proteins included components associated with amino acid metabolism and chloroplastic nitrogen assimilation, which were predominantly downregulated, as well as proteins related to redox metabolism and defense, such as a 2Fe–2S-type ferredoxin and an

extracellular peroxidase, which showed increased abundance, suggesting a potential reinforcement of antioxidant mechanisms.

Table 3. Differentially expressed proteins in the comparison between HS-Vc–treated plants infected with *Xanthomonas euvesicatoria* (HS-Vc + *Xe*) and plants infected with *X. euvesicatoria* only (*Xe*).

Protein ID	Protein name	Functional annotation	Localization	Fold change	p_{adj}
Q40129	Uncharacterized protein	Putative metabolic protein	Unknown	−1.6	4.18E−04
P25306	Threonine dehydratase 2 biosynthetic	Branched-chain amino acid biosynthesis	Chloroplast	−1.8	1.56E−03
P05119	Wound-induced proteinase inhibitor 2	Defense-related protease inhibitor	Extracellular	−2.2	4.66E−03
A0A3Q7HXA1	2Fe–2S ferredoxin-type domain-containing protein	Electron transfer and redox metabolism	Chloroplast	+2.5	1.77E−02
Q10712	Leucine aminopeptidase 1	Proteolysis and amino acid turnover	Chloroplast	−1.5	1.77E−02
A0A3Q7G5A7	PCI domain-containing protein	Protein complex assembly and regulation	Cytosol / Nucleus	−10.5	3.01E−02
A0A3Q7ESW4	Ferredoxin–nitrite reductase	Nitrogen assimilation and redox metabolism	Chloroplast	−1.7	3.29E−02

Table 3. Continuation.

A0A3Q7E7T2	Peroxidase	Oxidative stress response and defense	Apoplast / Extracellular	+1.8	4.47E−02
Q40131	Steroid 16α-hydroxylase	Secondary metabolism (oxidative reactions)	Endoplasmic reticulum	+1.6	4.47E−02
A0A3Q7I9H4	Bet v I / Major latex protein domain-containing protein	Stress- and defense-related protein	Cytosol	−1.7	4.47E−02

Note: Fold change values correspond to $\log_2$-transformed abundance ratios relative to Xe. Positive and negative values indicate increased and decreased protein abundance, respectively. Only proteins with padj < 0.05 are shown. Functional annotation and subcellular localization were inferred from UniProt.

In contrast, HS-Pt treatment in infected plants (HS-Pt + *Xe* vs. *Xe*) resulted in a more extensive proteomic modulation (Table 4). An increase in the abundance of proteins associated with redox control and stress responses was observed, including peroxidases and ferredoxin-type proteins, whereas proteins involved in central metabolic processes, such as

purine biosynthesis, chlorophyll biosynthesis, and photosystem II stabilization, showed a significant decrease in abundance. In addition, proteins related to the assembly and regulation of protein complexes, including those containing PCI domains, were also strongly repressed. Taken together, these results indicate that HS-Pt exerts a more pronounced modulatory effect on the proteomic response to bacterial infection compared with HS-Vc, reinforcing stress- and redox metabolism associated pathways while concomitantly repressing central metabolic and photosynthetic processes.

Table 4. Differentially expressed proteins in the comparison between HS-Pt–treated plants infected with *Xanthomonas euvesicatoria* (HS-Pt + Xe) and plants infected with *X. euvesicatoria* only (*Xe*).

Protein ID	Protein name	Functional annotation	Localization	Fold change	p_{adj}
Q40129	Uncharacterized protein	Putative metabolic protein	Unknown	−1.6	5.49E−04
A0A3Q7E7T2	Peroxidase	Oxidative stress response and defense	Apoplast / Extracellular	+2.1	1.77E−02
A0A3Q7HXA1	2Fe–2S ferredoxin-type domain-containing protein	Electron transfer and redox metabolism	Chloroplast	+2.5	2.21E−02
A0A3Q7HTP1	NAD(P)-binding domain-containing protein	Redox-related metabolic process	Cytosol	+1.6	2.21E−02
A0A3Q7HJ62	Water stress and hypersensitive response domain-containing protein	Stress and defense response	Cytosol	+1.6	2.21E−02
A0A3Q7FPU6	Phosphoribosylformyl glycinamidine synthase	Purine biosynthesis	Chloroplast	−12.3	2.86E−02
Q672Q6	Photosystem II oxygen-evolving complex protein 3 (PsbQ)	Photosynthesis (PSII stabilization)	Chloroplast / Thylakoid lumen	−2.3	3.48E−02
A0A3Q7HCH3	NADP-dependent oxidoreductase domain-containing protein	Oxidoreductase activity	Cytosol	+1.3	4.21E−02
Q09IV6	Solanesyl diphosphate synthase	Isoprenoid / plastoquinone biosynthesis	Chloroplast	+1.6	4.35E−02
A0A3Q7G5A7	PCI domain-containing protein	Protein complex assembly and regulation	Cytosol / Nucleus	−5.1	4.35E−02
A0A3Q7HE62	NAD(P)-binding domain-containing protein	Redox and metabolic regulation	Cytosol	−5.3	4.35E−02
A0A3Q7IBI0	NADPH-protochlorophyllide oxidoreductase	Chlorophyll biosynthesis	Chloroplast	−2.1	4.79E−02

A0A3Q7HA43	Histidine kinase/HSP90-like ATPase domain-containing protein	Signal transduction / stress response	Cytosol	−2.4	4.79E−02

Note: Fold change values correspond to $\log_2$-transformed abundance ratios relative to *Xe*. Positive and negative values indicate increased and decreased protein abundance, respectively. Only proteins with $p_{adj} < 0.05$ are shown. Functional annotation and subcellular localization were inferred from UniProt.

3.3. Gene expression associated with hormonal pathways, defense, and oxidative stress

The expression of genes associated with JA/ET and SA biosynthesis and signaling pathways, as well as genes related to defense and oxidative stress, was evaluated by RT-qPCR (Figure 3). Overall, *Xe* infection induced significant transcriptional changes, indicating the coordinated activation of multiple pathways involved in the biotic stress response. The genes *lox1.1*, *jar1*, and *MYC2*, involved in JA biosynthesis and signaling (Figure 3a–d), were significantly upregulated in infected plants compared to the control, confirming the activation of the jasmonate pathway in response to infection. HS-Vc treatment resulted in subtle changes in these genes, maintaining expression levels similar to those of plants infected with *Xe* alone. In contrast, HS-Pt treated plants, particularly under infection, showed a stronger induction of *lox1.1*, *JAR1*, and *MYC2*. The expression of *JAZ1,* a negative regulator of the pathway, did not differ from the control in infected plants. In contrast, *JAZ1* expression was significantly upregulated in plants treated with both HS sources. This pattern suggests that HS may attenuate the JA signaling, potentially favoring the necrotrophic phase of Xe while enhancing protection against its biotrophic phase. However, because JAZ1 regulation occurs primarily at the post-transcriptional level through proteasomal degradation rather than transcriptional control, changes in transcript abundance should be interpreted with caution, as they may not direct reflect JAZ1 status.

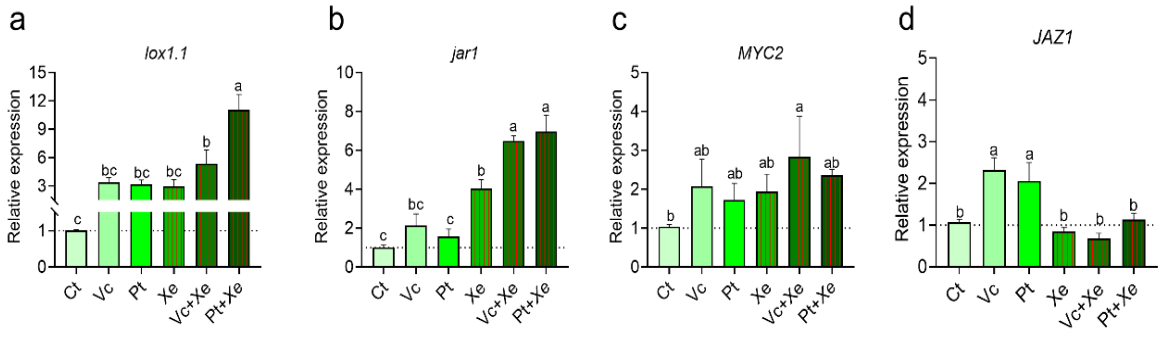

Figure 3. Relative expression of genes associated with the jasmonate signaling pathway in tomato plants cv. Micro-Tom subjected to different treatments. (a) *lox1.1*, (b) *jar1*, (c) *MYC2*, and (d) *JAZ1*. Treatments: control (Ct), inoculation with *X. euvesicatori* (*Xe*), application of HS derived from vermicompost (Vc) or peat (Pt), and combined treatments (Vc + *Xe*, Pt + *Xe*). Gene expression data were normalized using *actin* as the reference gene. Bars represent the mean ± standard deviation (n = 3 biological replicates). Different lowercase letters indicate statistically significant differences in relation to the control group, as

determined by Dunnett's multiple comparisons test ($p < 0.05$).

The *ERF*1 gene, associated with ET signaling and the integration of ET- and JA-mediated responses, was induced by bacterial infection, with additional upregulation in HS-Pt + *Xe* plants compared to *Xe* alone, whereas HS-Vc had a less pronounced effect (Figure 5a). The *PAL5* gene, involved in secondary metabolism and SA biosynthesis, was upregulated by *Xe* infection. HS treatments modulated its expression, with HS-Pt showing the strongest effect (Figure 5b), suggesting adjustments in the SA pathway, which is known to antagonize JA signaling. Complementarily, the expression of *NPR1* (Figure 5c), a central regulator of the SA pathway and repressor of JA signaling, was unaltered by infection and upregulated by SH treatments, indicating a rebalancing of these hormonal pathways. *ICS1*, a key gene involved in salicylic acid biosynthesis, was significantly induced by Pt treatment, whereas its expression tended to decrease under combined HS + Xe treatments, suggesting a fine regulation of SA production during the defense response (Figure 5d).

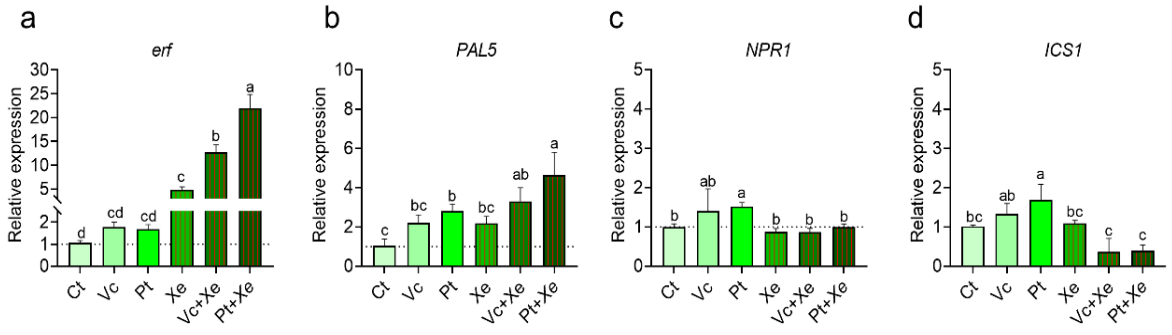

Figure 5. Relative expression of genes associated with salicylic acid (SA) and ethylene (ET) signaling pathways in tomato plants cv. Micro-Tom subjected to different treatments. (a) *erf*, (b) *PAL5*, (c) *NPR1*, and (d) *ICS1*. Treatments: control (Ct), inoculation with *X. euvesicatori* (*Xe*), application of humic substances (HS) derived from vermicompost (Vc) or peat (Pt), and combined treatments (Vc + *Xe*, Pt + *Xe*). Gene expression data were normalized using *actin* as the reference gene. Bars represent the mean ± standard deviation (n = 3 biological replicates). Different lowercase letters indicate statistically significant differences in relation to the control group, as determined by Dunnett's multiple comparisons test ($p < 0.05$).

Pathogenesis-related genes, in special *PR-1* and *PR-5*, were strongly induced in infected plants relative to controls, confirming activation of the defense response (Figure 6). In general, HS-Vc + *Xe* maintained expression levels similar to those of *Xe*-infected plants, whereas HS-Pt + *Xe* induced a stronger response, evidencing a distinct modulation of immunity.

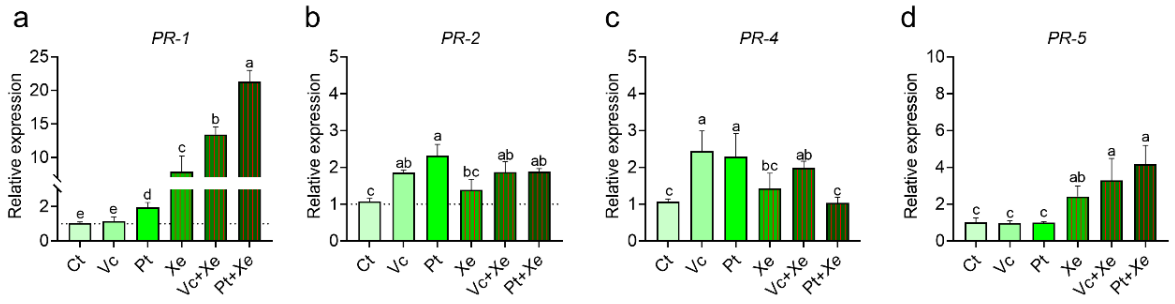

Figure 6. Relative expression of pathogenesis-related (PR) genes in tomato plants cv. Micro-Tom subjected to different treatments. (a) *PR-1*, (b) *PR-2*, (c) *PR-4*, and (d) *PR-5*. Treatments: control (Ct), inoculation with *X. euvesicatori* (*Xe*), application of humic substances derived from vermicompost (Vc) or peat (Pt), and combined treatments (Vc + *Xe*, Pt + *Xe*). Gene expression data were normalized using *actin* as the reference gene. Bars represent the mean ± standard deviation (n = 3 biological replicates). Different lowercase letters indicate statistically significant differences in relation to the control group, as determined by Dunnett's multiple comparisons test ($p < 0.05$).

Oxidative stress-related genes (*cat1*, *apx1*, *sod*, and *pox*) were upregulated by bacterial infection, reflecting increased oxidative pressure during the infection process. HS-Pt treatment, especially under infection, enhanced the expression of these genes compared to *Xe* alone, whereas HS-Vc had a milder effect (Figure 7).

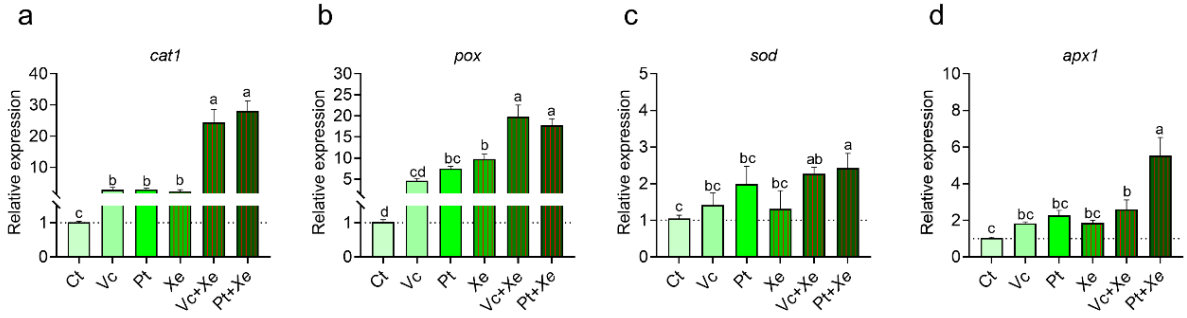

Figure 7. Relative expression of oxidative stress–related genes in tomato plants cv. Micro-Tom subjected to different treatments. (a) *cat1*, (b) *apx1*, (c) *sod*, and (d) *pox*. Treatments: control (Ct), inoculation with *X. euvesicatori* (Xe), application of humic substances derived from vermicompost (Vc) or peat (Pt), and combined treatments (Vc + Xe, Pt + Xe). Gene expression data were normalized using *actin* as the reference gene. Bars represent the mean ± standard deviation (n = 3 biological replicates). Different lowercase letters indicate statistically significant differences in relation to the control group, as determined by Dunnett's multiple comparisons test ($p < 0.05$).

The enzymatic activity analyses corroborated the proteomic and molecular results, highlighting the modulatory effect of HS on key defense enzymes. Lipoxygenase (LOX), associated with the jasmonate (JA) pathway, showed a marked increase in the HS-Vc treatment, indicating that Vc strongly induced basal activation of this pathway, while Pt also enhanced LOX activity, albeit to a lesser extent. Peroxidase (POX) exhibited a progressive increase from Ct to Xe, HS-Vc, and HS-Pt, with HS-Pt, Vc + Xe, and Pt + Xe showing the highest values, indicating reinforcement of oxidative barrier formation. Phenylalanine

ammonia-lyase (PAL), a key enzyme of the phenylpropanoid metabolism, was strongly stimulated in the Vc + Xe and Pt + Xe treatments, suggesting that HS enhanced the production of phenolic compounds during infection. Finally, PR-2, a classical marker of pathogenesis-related proteins, increased in all HS treatments, with Pt and Pt + Xe being the most responsive. Altogether, these enzymatic responses demonstrate that Vc and Pt activate defense biochemical mechanisms in distinct but complementary ways, reinforcing the pattern observed in the gene expression data.

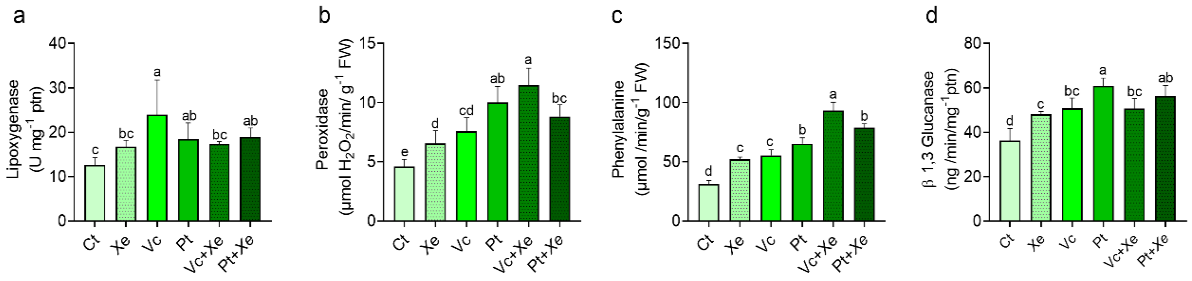

Figure 8. Activity of defense-related enzymes in tomato plants subjected to different treatments. (a) LOX, (b) POX, (c) PAL, and (d) PR-2 activities were measured in leaves collected 72 h after treatment and inoculation with *Xanthomonas euvesicatoria*. Bars represent mean ± standard deviation (SD). Different letters indicate significant differences among treatments by Fisher's LSD test ($p < 0.05$).

4. Discussion

Humic substances (HS) are highly heterogeneous supramolecular assemblies formed by dynamic associations of organic molecules enriched in oxygen-containing functional groups, such as carboxyls (–COOH), phenolic moieties (Ar–OH), and O-alkyl-C domains, as well as aromatic and aliphatic regions whose structural organization reflects their geological or biogenic origin (Piccolo et al., 2018; Maffia et al., 2025). This chemical diversity underpins key physicochemical properties, including redox reactivity, hydrophobicity, chelating capacity, and affinity for biological interfaces, enabling direct interactions with lipid bilayers, membrane proteins, and apoplastic components. Through these interactions, HS modulate stress-responsive enzymes and hormone-mediated pathways that coordinate plant defense and ROS homeostasis (Piccolo, 2002; Canellas et al., 2011; Nardi et al., 2021; Souza et al., 2022; Faccin & Piero, 2022; Zandonadi et al., 2025).

Here, we used two HS with contrasting origins: HS extracted from vermicompost (HS-Vc) and HS extracted from peat (HS-Pt). Solid-state ^13C CPMAS NMR revealed marked chemical differences between them. HS-Pt exhibited a strong predominance of Alkyl-C regions (0–45 ppm; 50.2%), including intense resonances at ~30–33 ppm attributed to methylene (–CH_2–) groups from long-chain structures derived from waxes, cutin, and fatty acids, as well as hydrophobic aromatic contributions associated with lignin. These nonpolar domains favor direct interactions with biological membranes, increasing functional permeability and facilitating the transport and delivery of signaling molecules to responsive sites, thereby

enhancing the activation of hormone-mediated and antioxidant defense pathways that regulate ROS containment and cellular redox status (Piccolo, 2002; Canellas et al., 2011, 2012; Roomi et al., 2018; Nardi et al., 2018, 2021). In contrast, HS-Vc was enriched in O-Alkyl-C (60–110 ppm; 28.1%) and CH_3O/C–N (16.7%) domains, typical of carbohydrate-like and oxygenated nitrogenous structures derived from polysaccharides, hemicellulose, and microbial biomolecules. This chemical composition likely reflects the combined contribution of earthworm mucus and microbial biomass turnover, both of which are known to enrich vermicompost-derived humic substances in carbohydrate-like and oxygenated nitrogenous domains. The HS-Vc profile confers higher aqueous solubility and lower hydrophobicity, as reflected by its reduced Alkyl/O-Alkyl (A/OA) ratio (Martinez-Balmori et al., 2014; Spaccini et al., 2019). More hydrophobic humic fractions tend to induce stronger physiological and enzymatic responses in plants, whereas more hydrophilic materials, although bioactive, display lower efficiency in triggering resistance mechanisms (Silva et al., 2025).

The chemical differences were directly reflected in proteome conditioning and subsequent hormonal modulation. Under non-infected conditions, HS-Vc caused only subtle proteomic changes, notably inducing enzymes associated with nitrogen metabolism and amino acid biosynthesis, such as leucine aminopeptidase 1, acetylornithine deacetylase, and threonine dehydratase 2. This profile suggests that vermicompost-derived HS primarily supports the supply and recycling of nitrogenous precursors and carbon skeletons, potentially improving the availability of assimilable N and key metabolic intermediates, but without triggering broad proteomic reprogramming or major physiological adjustments. A similar effect of vermicompost humic acids on amino acid accumulation has been reported in maize plants (Canellas et al., 2022).

The application of HS-Pt alone triggered a substantially broader reconfiguration of the tomato proteome, with 47 proteins showing differential abundance. The dominant trend was the coordinated repression of photosynthetic and energy metabolism-related proteins, indicating a strategic reduction of growth-associated energy allocation a classical metabolic defense response in which plants prioritize immune signaling and defense-integrated hormonal pathways (Rojas et al., 2014; Guo et al., 2018). Importantly, this repression did not reflect energetic collapse. Instead, central integrative nodes of carbon metabolism were preserved or induced, including RuBisCO activase, the α-subunit of the RuBisCO large subunit-binding protein, sedoheptulose-1,7-bisphosphatase, GAPDH, and 6-phosphogluconate dehydrogenase. This protein set supports a model of selective carbon-flux modulation, maintaining metabolic hubs that provide precursors and reducing power (NADPH/NADH), both essential for functional hormonal crosstalk between SA, JA, and ET and for downstream defense signaling. Consistently, increased RuBisCO activity has also been observed in HA-treated plants (Esringü et al., 2016; Amerian et al., 2024). The promotion of RuBisCO activity

by HA was previously reported by Ertani et al. (2011), who also observed increases in chlorophyll, glucose, and fructose contents, suggesting a positive influence of humic fractions on photosynthetic processes even during immune-driven metabolic adjustments.

In parallel, the strong accumulation of mitochondrial chaperones and proteases, such as CPN60-2, Lon protease 2, and EF-G, with high fold-change values, indicates that HS-Pt increased cellular oxidative pressure in a controlled manner, supporting the use of ROS as secondary messengers without causing irreversible oxidative damage, a condition that requires reinforcement of the proteostasis machinery. The induction of Lon protease 2 is especially relevant, as Lon regulates mitochondrial oxidative stress and retrograde signaling that directly influence SA–JA balance, mechanistically connecting redox status, energy metabolism, and immunity (Rigas et al., 2014; Lingard & Bartel, 2009; Figaj et al., 2020). The marked repression of histone H4 (FC −4.1) further suggests an impact on chromatin-level regulation, consistent with fine-scale epigenetic adjustments in hormonal and immune gene expression during defense activation. Histones, which enable nuclear DNA packaging into chromatin (Kornberg, 1974; Socolova et al., 2022), play a major role in regulating chromatin-level processes such as transcription and replication, and consequently exert broad effects on organismal growth, development, and fitness (Kouzarides, 2007).

Xanthomonas euvesicatoria (Xe) infection, in the absence of HS application, resulted in the most extensive proteomic reconfiguration in tomato plants (148 differentially abundant proteins), characterized by strong induction of defense-related and redox regulatory proteins alongside marked repression of proteins associated with photosynthesis, primary metabolism, and basal translation. This response confirms the high physiological cost of immune activation and the classic resource reallocation from growth to immunity, a hallmark of hemibiotrophic interactions in which plants dynamically adjust metabolism to counter pathogens that shift from an early biotrophic to a later necrotrophic phase (Lorang, 2019; McCombe et al., 2022; Zhou et al., 2023).

Conversely, tomato plants pretreated with HS prior to Xe infection exhibited a dramatic reduction in the magnitude of stress-driven proteomic remodeling (10 and 14 differential proteins in HS-Vc + Xe and HS-Pt + Xe, respectively). This finding indicates that HS functioned not as stress aggravators, but as metabolic conditioners capable of establishing an efficient and physiologically balanced immune priming state. This behavior aligns with recent evidence showing that primed plants often display a restricted scale of proteomic shifts while maintaining heightened responsiveness and faster activation of immune pathways with reduced energetic penalties (Tanou et al., 2012; Chen et al., 2025). In the HS-Vc + Xe system, core nodes of electron transfer and oxidative regulation remained active (peroxidases and 2Fe-2S ferredoxins), along with mild modulation of steroid metabolism, suggesting moderate JA–ET hormonal integration. Similar low-cost priming adjustments have been reported for other natural and synthetic elicitors that enhance stress tolerance and immune readiness without

causing severe disruption to primary metabolism (Sanchez-Lucas et al., 2025; Chen et al., 2025).

In contrast, HS-Pt + Xe triggered coordinated repression of nitrogen-intensive and energetically costly pathways (purine metabolism and M20 peptidases) while preserving or inducing redox-integrative proteins, particularly thioredoxins. This pattern suggests that peat-derived HS (HS-Pt) redirect cellular redox control toward alternative networks less dependent on glutathione-mediated regulation, favoring a responsive, intermediate redox state that is metabolically economical and functionally suited for ROS-mediated immune signaling. This is consistent with the emerging concept of balanced redox priming, in which controlled ROS accumulation acts as a central secondary messenger that integrates defense, hormone signaling, and protein quality control without causing irreversible oxidative damage (Miller et al., 2023; Haghpanah, et al., 2025).

The redox reconfiguration promoted by HS-Pt was further supported by transcriptional data. HS-Pt enhanced the expression of key jasmonate pathway genes (*lox1.1*, *jar1*, *MYC2*) without driving excessive constitutive activation, as indicated by the concurrent modulation of *JAZ1*, a negative regulator of JA signaling. Induction of *ERF* transcription factors reinforced JA–ET functional coordination, while changes in *PAL5* and *NPR1* expression indicated modulation of the SA–JA balance, maintaining antagonistic pathways active without complete suppression an essential requirement for flexible and effective hormonal crosstalk during hemibiotrophic defense responses. In parallel, the modulation of redox-associated genes (*cat1*, *apx1*, *sod*, *pox*) and *nudix8* confirms that HS contribute to ROS homeostasis and redox buffering, processes now recognized as central to both SAR establishment and antibacterial defense signaling (Liu et al., 2021; Meena et al., 2024; Miller et al., 2023). Zandonadi et al. (2025) also reported that humic acid application led to increased activity of the antioxidant enzymes superoxide dismutase and catalase, as well as upregulation in the expression of these genes.

Biochemical and enzymatic assays consolidated these molecular insights: HS-Vc strongly increased lipoxygenase activity, indicating basal JA pathway priming, whereas HS-Pt significantly intensified peroxidase and PAL activities under infection, supporting reinforcement of oxidative barriers and stimulation of phenolic biosynthesis defense mechanisms widely reported in elicited and primed defense responses (Silva et al., 2025). The consistent rise in β-1,3-glucanase activity across all treatments confirms the activation of PR-associated defense layers, a response frequently potentiated in primed plants, especially when induced by organic biostimulants such as HS, chitosan, laminarin, and microbial-derived elicitors (Castro & Bach, 2004; Nowak et al., 2022).

Santos-Jiménez et al. (2022) observed that passion fruit plants (*Passiflora edulis*) treated with a combination of humic acids (HA) + peptidoglycated galactomannan + a bacterial consortium (*Serratia marcescens*, *Bacillus* sp., and *Herbaspirillum seropedicae*) showed

induced transcription of PR-3 and PAL, 24 hours after treatment. Notably, PR-3 and PAL genes are key molecular markers of SAR. HS can also stimulate phenylpropanoid metabolism, promoting the accumulation of several phenolic compounds in plants (Schiavon et al., 2010).

Collectively, these results demonstrate that HS origin dictates the mode of immune conditioning: vermicompost-derived HS (HS-Vc) support primarily nutritional and basal priming adjustments with minimal physiological disruption, while peat-derived HS (HS-Pt) induce selective redox-centered reprogramming that amplifies defense outputs while preserving the hormonal communication among SA–JA–ET without abolishing antagonistic interactions required for immune flexibility. The integration of chemical, proteomic, transcriptional, and biochemical evidence supports that peat-derived HS act as more efficient systemic priming agents by reconfiguring proteostasis and cellular redox control as functional hubs of hormonal crosstalk. This reinforces their potential as a sustainable, low-environmental-impact strategy for bacterial spot management in tomato crops and for reducing dependency on chemical pesticides (Silva et al., 2023a; 2023b; Meena et al., 2024; Miller et al., 2023; Zhao et al., 2025).

5. Conclusion

HS modulated the tomato proteome and gene expression in a source-dependent manner. HS-Vc caused only subtle proteomic shifts, largely linked to amino acid metabolism, suggesting limited physiological reprogramming. In contrast, HS-Pt triggered a selective metabolic adjustment characterized by coordinated repression of photosynthesis and basal translation, while strongly activating defense- and redox-related genes and enzymes, including key signaling nodes such as *lox1.1, myc2, jar1, erf, PAL, POX*, and integrative stress-response proteins such as thioredoxins and Lon protease 2, which are central to ROS homeostasis and protein quality control. Notably, even under Xe, both HS sources markedly restricted the overall magnitude of stress-driven proteomic remodeling, supporting a balanced and physiologically sustainable priming state. This primed condition did not suppress hormonal crosstalk, but rather enhanced its functional coordination. Among the sources tested, HS-Pt exhibited greater efficiency in establishing and sustaining the defensive response, mechanistically linking its chemical composition, redox buffering, ROS signaling control, and the orchestrated activation of immunity. Collectively, these results indicate that HS, particularly those derived from peat, hold strong potential as a sustainable strategy to enhance tomato resilience against bacterial diseases. Therefore, peat-derived HS emerge as promising tools for integrated, low-environmental-impact disease management aimed at increasing crop durability and reducing dependency on chemical pesticides.

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CONCLUSÕES

Esta tese demonstrou que as SH atuam como moduladoras potentes e integrativas da imunidade vegetal, sendo capazes de induzir, coordenar e sustentar respostas de resistência sistêmica em culturas agrícolas de elevada importância econômica. No primeiro capítulo, foi evidenciado que a aplicação de SH promove a ativação de enzimas clássicas associadas à SAR: fenilalanina amônia-liase (PAL), peroxidase (POX) e β-1,3-glucanase (GLUC), em espécies como citros, café, tomate, milho, cana-de-açúcar, soja e milho. A elevada responsividade da GLUC em todas as culturas destaca essa enzima como um marcador bioquímico robusto da ativação imune mediada por SH. Em contrapartida, a variabilidade observada na atividade de POX, bem como sua não indução significativa em café, indicam que enzimas oxidativas podem ser diferencialmente priorizadas de acordo com a fisiologia e o repertório genético de cada cultura. Além disso, as diferenças intraespecíficas observadas em citros, soja e cana reforçam que a eficácia das SH deve ser interpretada considerando a diversidade genética do hospedeiro, um aspecto essencial para a construção de recomendações agronômicas tecnicamente consistentes e aplicáveis em larga escala.

O segundo capítulo confirmou o papel multifuncional das SH na modulação da defesa do tomateiro contra *Oidium* sp., um patógeno biotrófico cuja contenção depende fortemente de respostas SAR mediadas por SA. Mesmo apresentando diferenças químicas significativas entre as SH derivadas de vermicomposto (SH-Vc) e de turfa (SH-Pt), ambas as fontes ativaram a via SAR, estimularam a expressão de genes antioxidantes e o acúmulo de proteínas PR, e reduziram significativamente a severidade da doença. Esses achados consolidaram as SH como bioestimulantes sustentáveis capazes de modular simultaneamente sinalização hormonal, equilíbrio redox e efetores diretos da imunidade vegetal, contribuindo para o fortalecimento do sistema imune do tomateiro em sistemas de produção agrícola.

O terceiro capítulo aprofundou a compreensão mecanística ao demonstrar que a percepção das SH e seus efeitos sobre a imunidade são dependentes da origem das SH. Enquanto SH-Vc induziu mudanças discretas no proteoma, majoritariamente associadas ao metabolismo de aminoácidos, SH-Pt desencadeou uma reprogramação metabólica seletiva e mais dinâmica, caracterizada pela repressão transitória de processos de alto custo energético, como fotossíntese e tradução basal,

concomitantemente à ativação expressiva de genes e enzimas relacionados à defesa e ao metabolismo redox. No modelo de infecção por *Xanthomonas euvesicatoria* (Xe), cujo ciclo hemibiotrófico possibilita o recrutamento sequencial e integrado das vias dependentes de SA (SAR) e JA/ET (ISR), ambas as fontes de SH limitaram a magnitude do remodelamento proteômico induzido pelo estresse, sustentando um estado de *priming* equilibrado e fisiologicamente sustentável. Esse estado não suprimiu o crosstalk hormonal, mas, ao contrário, favoreceu sua coordenação funcional, otimizando a sinalização imune. Entre as fontes avaliadas, SH-Pt mostrou maior eficiência em sustentar a resposta defensiva, associando mecanisticamente sua composição química à capacidade de tamponamento redox, ao controle fino da sinalização por ROS e à ativação orquestrada de componentes centrais da imunidade e da homeostase proteica.

De forma integrada, esta tese estabeleceu que as SH, especialmente aquelas derivadas de turfa, promovem um *priming* imune biologicamente eficiente, metabolicamente coordenado e ambientalmente sustentável no tomateiro, fortalecendo a resiliência da planta contra a infecção bacteriana por *Xe*. Esses resultados reforçam as SH como insumos biológicos estratégicos para o manejo integrado e sustentável de doenças, contribuindo para a redução da dependência de agroquímicos e apoiando a transição para uma agricultura mais resiliente, durável e ambientalmente responsável.

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