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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 the plants' resistance. However, it is time-consuming and costly, and it is sometimes difficult to achieve satisfactory results. Plants induce defense responses to natural elicitors by interpreting multiple genes that encode proteins, including enzymes, secondary metabolites, and pathogenesis-related (PR) proteins. 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 marker 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. Plants can improve their resistance to pathogens through the exogenous application of HSs as this promotes the activity of enzymes related to plant resistance. Finally, we consider the potential use of humic substances as a natural chemical priming agent to boost plant resistance in agriculture

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

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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 reduce 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]. Plants activate a complex signaling network that leads to the expression of defense genes, encoding pathogenesis-related (PR) proteins, and enzymes involved in the synthesis of defensive compounds. PR proteins employ systemic acquired resistance (SAR) response using salicylic acids as a cell signaling molecule, while 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 (HSs) 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 HSs than in the control group. The increase in PAL activities was followed by an enhancement in 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 leading to antimicrobial activity, are 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. 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*, *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].

HSs are the major components 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]. HSs also stimulate 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 HSs 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 [23–25].

This study aimed to assess the effectiveness of HSs as natural elicitors of SAR in plants. Enzymatic activities associated with the SAR response, i.e., PAL, POX, and β-1,3-glucanase, were analyzed. HSs were 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 HSs via foliar spray.

2.1. Humic Substances

Commercial HSs from peat (Iridium®, Ourinhos, São Paulo, Brazil) with 30 g L^{-1} of organic carbon were diluted in water to a concentration of 48 mg of Total Organic Carbon (C)L^{-1} for plant leaf application. HSs used in the study were 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 a 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%, and 160–190 ppm (carboxyl-C): 10%. The CPMAS ^{13}C NMR spectrum of HSs used in the study is shown in Figure 1.

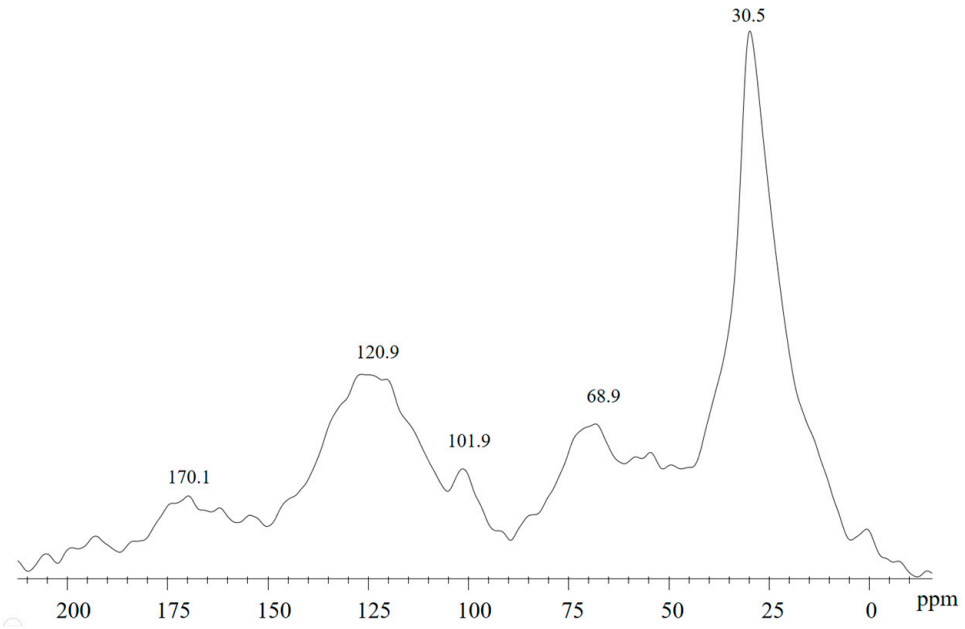

Figure 1. CP/MAS ^{13}C NMR spectrum of HSs used in this study.

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. First, 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 to 5% w/v of polyvinylpyrrolidone (PVP) and 1.2 mL L^{-1} of β-mercaptoethanol), and subsequently centrifuged for 25 min at 10,000× g at 4 °C. After centrifugation and separation of the suspension, the enzymatic reaction medium was composed of 1 mL of crude enzymatic suspension + 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 min, after which the reaction was stopped with 200 µL of 6 M L^{-1} HCl, and the absorbance was measured with a 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) × (V/v × d × Cε290), 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): POX was extracted from frozen powdered leaves. The powdered leaf sample (1 g) 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 min 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). Then, 50 µL of the enzyme extract was added, and the decomposition of hydrogen peroxide was detected at 470 nm. The results were expressed in µmol of H_2O_2 decomposition min^{-1} g^{-1} FW using the following equation EA = [(A × TV)/(Cε470 × t × v)]/FW, where EA = enzyme activity; TV (total reaction volume) = 2.60 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 randomized experimental design with four biological replicates and two treatments: application of the HS 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 h 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 HSs on plant growth and metabolism. After 48 h of treatment, leaves were collected, ground in liquid 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 HSs at 4 mM C L^{-1} via leaf spray. Next, 48 h after the HS application, 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 orange (*Citrus sinensis*) cultivars Baia and 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 the US Soil Survey Classification System. The experiment was entirely randomized, with five plants per treatment in 3.5 m × 5 m plots. The one-year-old Baia cultivar was planted in October 2021, and the one-year-old Seleta cultivar was planted in October 2022. The aqueous suspension of HSs 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 of application and 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 using a tractor sprayer. Commercial HSs were applied at a concentration of 48 mg C/L at an application rate of 2400 L/ha. The orchards were composed of 16-, 7-, and 3-year-old cv. Natal, cv. Pera, and cv. Valencia oranges,

respectively. Leaf samples from both orchards were randomly taken from treated and untreated (control) leaflets near the fruit on ten different trees per cultivar. The leaves were collected 48 h after the treatments and stored in liquid N_2 for transport. After grinding 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 performed daily, with 200 mL of water. At the vegetative stage with three true leaves (V3 stage), an application of 80 mL per pot was employed with HSs at 48 mg C L^{-1} at pH 6.0. The leaves were collected after 48 h of application and 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: Pre-sprouted RB966928, RB 855155, CTC7515 BT, and CTC4 sugarcane plantlets were transferred to a pot (700 mL) filled with commercial growth substrate (Basaplant). After one week of transplanting, 100 mL of aqueous suspension of HSs with 48 mg C L^{-1} at pH 6.0 or with water (control) was applied to the plantlets using a hand sprayer. The leaves were collected 48 h after application and ground in liquid N_2, and after the enzymatic extract had been obtained, 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, 50 mL of HSs at 48 mg C L^{-1} at pH 6.0 or water (control) was added to the pots, simulating drip irrigation, which is typically used in tomato production. There were four replicates in a completely randomized design. The leaves were collected 48 after treatment, ground in liquid N_2, homogenized with extraction buffer, and stored in a freezer (−20 °C) until analysis.

2.4. Data Analysis

To evaluate the effect of HSs across the different experiments conducted on various plant species, statistical analysis was first performed for each plant species. Student's *t*-test was applied to compare enzymatic activity between the control and HS-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 [28] 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 achieved 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: y_i (effect size) and 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. To complement the interpretation of treatment effects in terms of relative likelihood, the risk ratio (RR) was calculated to estimate the random effect model according to algorithm provided by the R metafor package [28]. RR was calculated as $RR = ((a/n_1)/(c/n_2))$, where a is the number of events observed in the treated group, n_1 is the total number of individuals in the treated group, c is the number of events in the control group, and n_2 is the total number of individuals in the control group. For this, enzymatic activity data were dichotomized based on a defined threshold of biological relevance, allowing the estimation of the relative probability of increased enzymatic activity in HS-treated groups compared to controls. This measure expresses the ratio between the probabilities of the event occurring in the treated and control groups, enabling a standardized interpretation of treatment effects across different experimental conditions. For statistical analysis, the logarithmic transformation of the RR was used, as commonly recommended in meta-analyses, to approximate normality and stabilize the variance. A RR of 1 indicates no difference in risk between the two groups. A RR greater than 1 suggests an increased risk in the treated group, while a risk ratio less than 1 suggests a decreased risk. The RR provides a dimensionless measure of effect size that facilitates interpretation across heterogeneous experimental conditions.

3. Results

This study measured the enzymatic activity of three proteins that are known to be involved in 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 HSs when the experiments were analyzed independently (Figures 2 and 3). The PAL activity promotion in the citrus treated with HSs was higher than the overall average (128% vs. 76%) (Figure 2a,d,g,j,m). In Rio de Janeiro, PAL activity increased by 57% and 108% for plants aged one and two years, respectively, compared to the control (Figure 2a,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 (Figure 2g,j,m). The PAL activity increase caused by HSs in coffee was similar to that observed in citrus, resulting in a 122% enhancement compared to the control (Figure 2v). 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 2n). In soybeans (Figure 3a,d,g) and tomatoes (Figure 2s), PAL activity was 48% and 53% higher, respectively, while in sugarcane it was 21% higher than in the control. Although significant differences were observed among varieties, all responded positively compared to untreated plants.

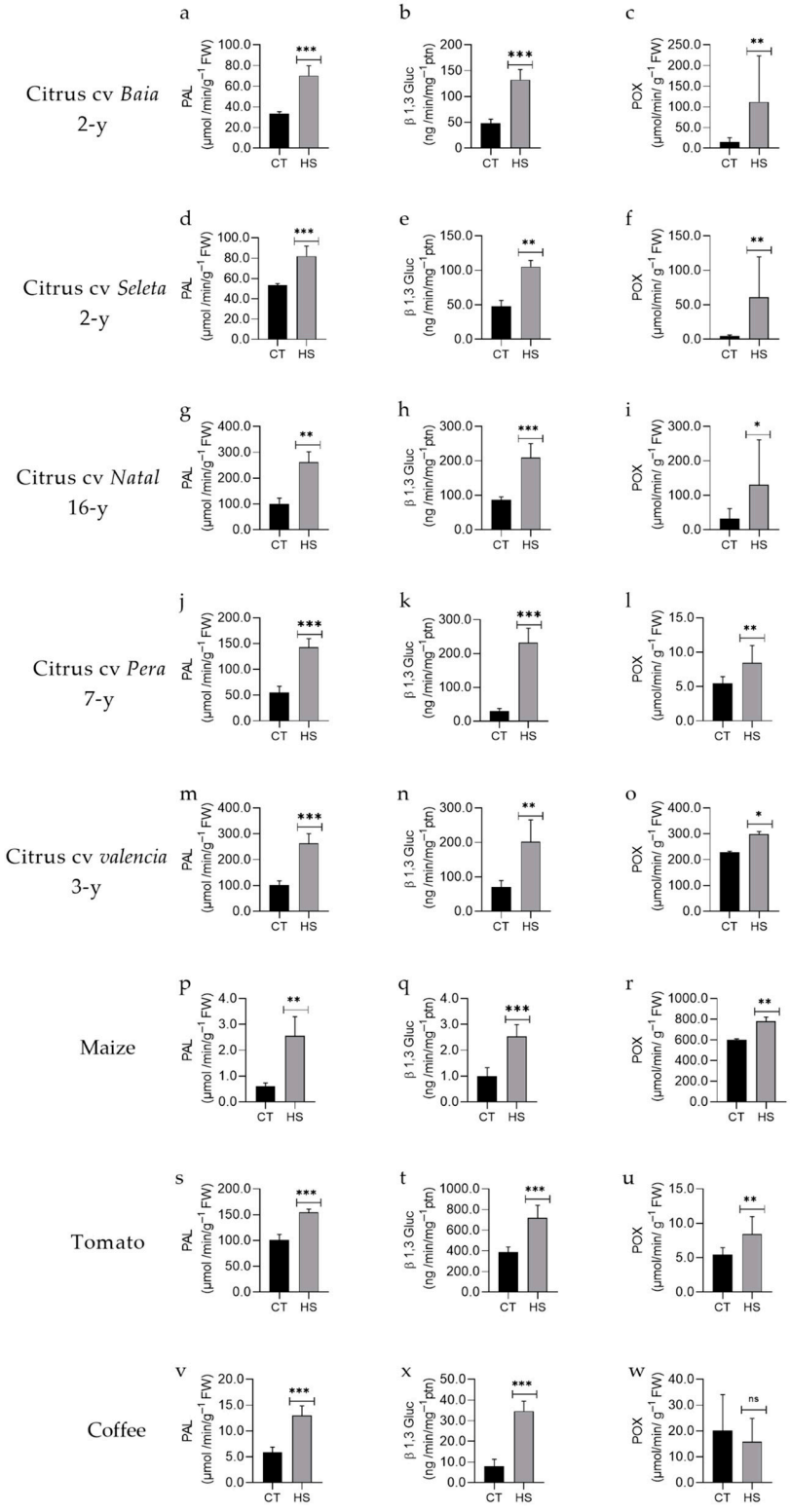

Figure 2. Enzymatic activity of phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (GLUC), and peroxidase (POX) in different crops. Bars represent mean values from independent experiments with citrus cv. Baía (**a**–**c**), cv. Seleta (**d**–**f**), cv. Natal (**g**–**i**), cv. Pera (**j**–**l**), cv. Valencia (**m**–**o**), maize (**p**–**r**), tomato (**s**–**u**), and *Coffea arabica* (**v**–**x**). 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 ($p > 0.05$).

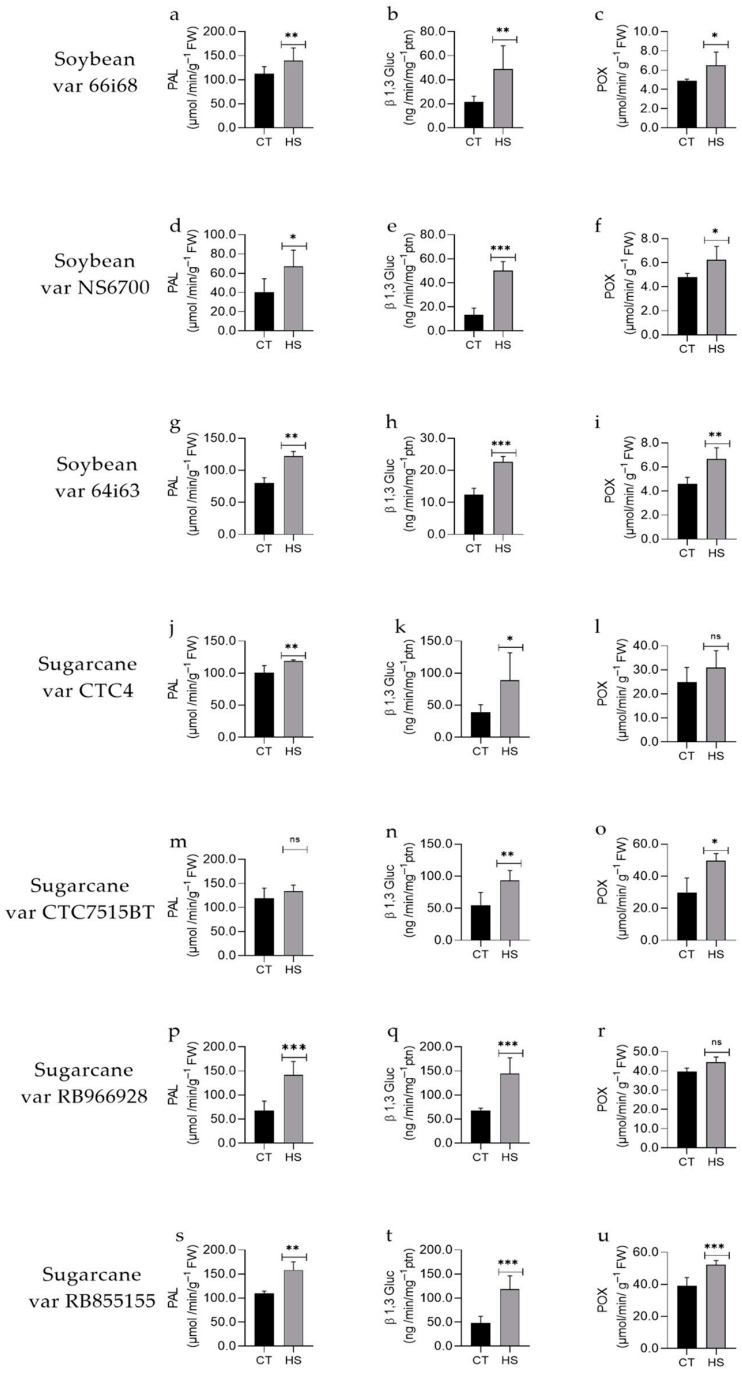

Figure 3. Enzymatic activity of phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (GLUC), and peroxidase (POX) in different crops. Bars represent mean values from independent experiments with soybean var. 66i68 (**a**–**c**), var. NS6700 (**d**–**f**), var. 64i63 (**g**–**i**), Sugarcane var. CTC4 (**j**–**l**), var. CTC 7515BT (**m**–**o**), var. RB 966,928 (**p**–**r**), and var. RB855155 (**s**–**u**). 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 ($p > 0.05$).

The activity of β-1,3-glucanase (GLUC) was also promoted by HSs, 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 (Figure 2e,h,k,n). Coffee plants treated with HSs showed a 273% increase in GLUC activity compared to the control plants (Figure 2x). Similarly, maize seedlings treated with HSs exhibited a

significant increase in GLUC activity, with an average increase of 160% compared to the control (Figure 2q).

HSs significantly affected the POX activity (Figures 2 and 3). 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 2363% observed in citrus var. Seleta at Rio de Janeiro (Figure 2c,f,i,l,o). The activity of POX was 29% higher in maize seedlings treated with HSs compared to control plants. The overall effect of HS application on the activity of PAL, GLUC, and POX enzymes can be observed in Figures 4 and 5. The enzyme activities were promoted by HSs, highlighting their use as a natural elicitor of SAR.

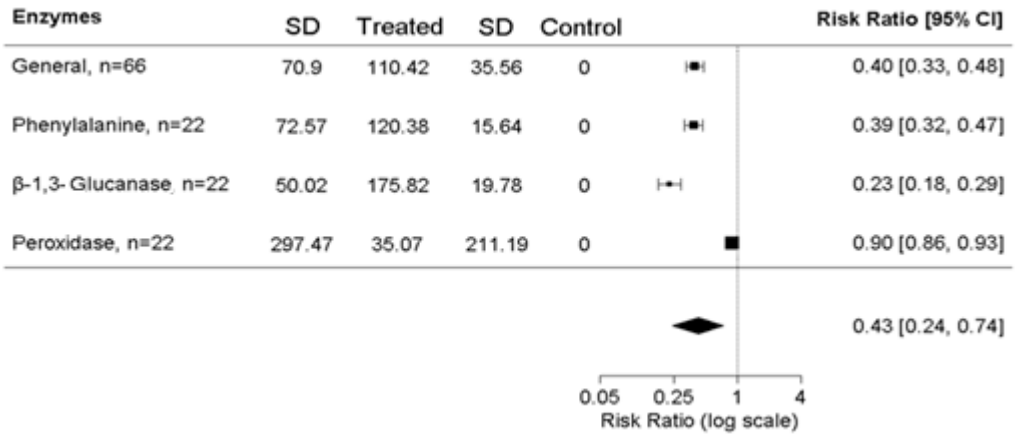

Figure 4. The general effect of HS application on the enzymatic activity of PAL, GLUC, and POX. The square size corresponds to the average effect size, and the error bars are 95% confidence level; the CI (confidence interval) is 95% based on a random effects model. Diamond—the final effect size of the studies included in the meta-analysis; risk ratio—risk rate; Q-Test for heterogeneity; SD: standard deviation.

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 4. 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 HSs. 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 HSs to enhance enzymatic defenses in plants, particularly via PAL and GLUC pathways.

The effects by crop are presented in Figure 5 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 5b) 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 5c) 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.

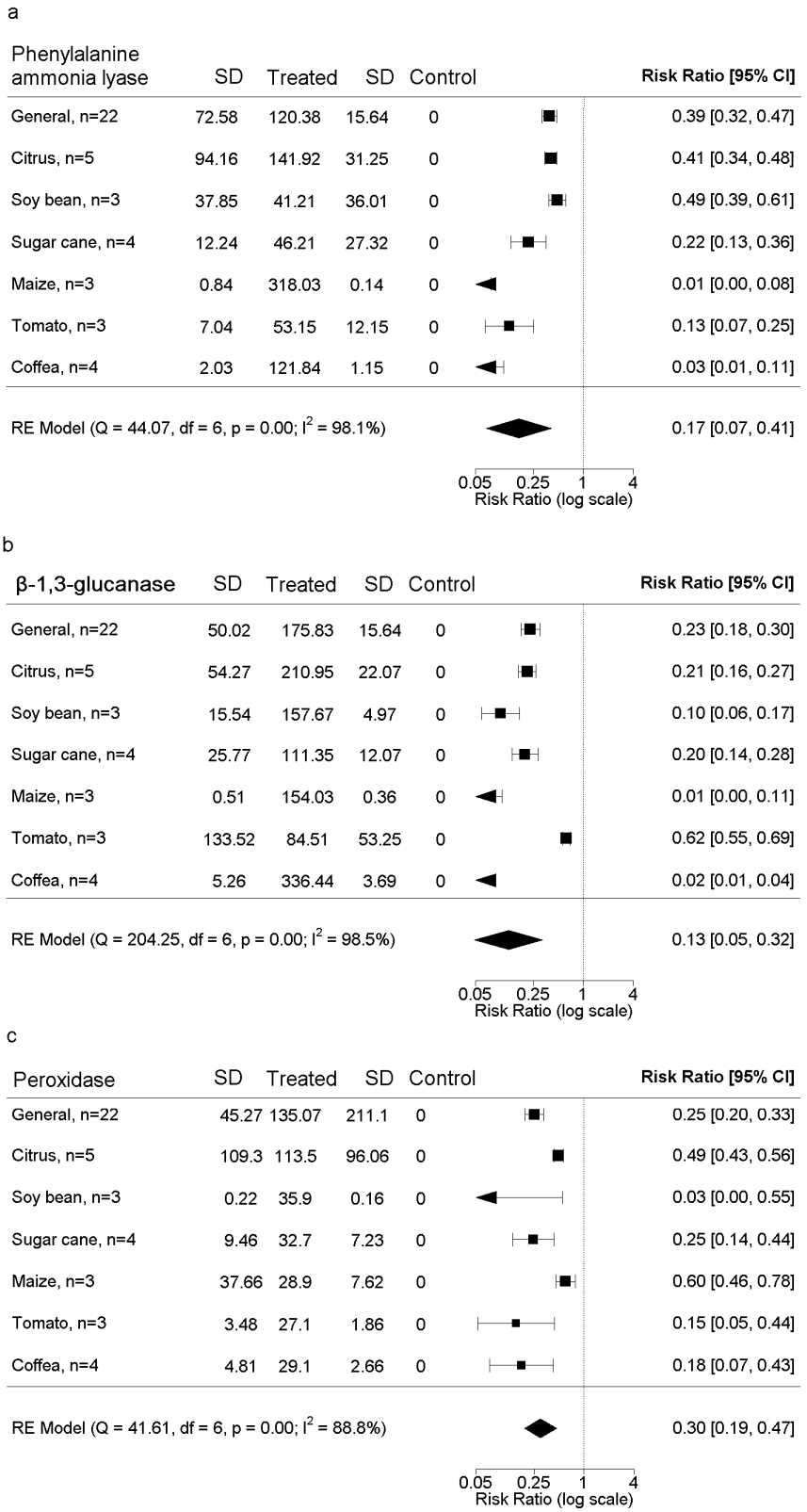

Figure 5. Effect of humic substances (HSs) on the GLUC (**a**), PAL (**b**), and POX (**c**) activities in Citrus, soybean, sugarcane, maize, tomato, and coffee. The size of the square corresponds to the size of the average effect, and the bars represent the error at a 95% confidence level; the CI (confidence interval) is 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 HSs generally enhance 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 HSs to activate defense-related enzymes, particularly GLUC, across diverse cropping systems.

4. Discussion

The study demonstrated that applying HSs directly to leaves in low concentrations can increase the activity of enzymes used as SAR markers. Enzyme activities were recorded two days after applying HSs via foliar spray on various types of plants, using different cultivars for the same type of plant. Figures 2 and 3 showed the observed variation in the 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 produces phenolic polymers [29]. 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 [30].

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

However, HSs are not widely used to induce plant defense against pests and pathogens. Although some experimental results suggest the possibility of using HSs for this purpose, 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 the 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 HSs is the first step towards integrating this biotechnology into 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 [11,40]. This is the well-founded hormonal hypothesis for the physiological effects of HSs [41], which act 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 they can be used to mitigate the effects of various abiotic stresses, including

drought, salinity, extreme temperature, and heavy metal toxicity, which have the induction of cell oxidative stress in common. The potential role of HSs in preventing oxidative stress in plants was observed by Cordeiro et al. [44]. García et al. [45] reported an enhancement in POX activity, decrease in H_2O_2 concentration, and increase in 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]. Plants can develop improved resistance to pathogen attacks through the external application of natural compounds [48]. This chemical priming results in enhanced resistance, involving earlier, faster, and/or stronger responses to pathogen attacks, which are often observed after a stress-free period. In this study, we did not induce any disease in the plants treated with HSs. Instead, we monitored the enzymes associated with SAR to verify that the plants had entered a state of attention. Chemical priming of SAR can be achieved by inducing their biosynthesis pathways through HS treatment. SA is a pivotal phytohormone in the way plants respond to biotic and abiotic stresses. Plants synthesize SA via two independent metabolic pathways: the isochorismate synthase pathway and the PAL pathway. While the former is well understood, the complete biosynthesis of SA from phenylalanine in plants was only recently characterized via the functional analysis of the OSD1 to OSD4 genes in rice (*Oryza sativa*) [49]. Previously, isotope-labeling experiments demonstrated that SA can be synthesized from phenylalanine (Phe) via trans-cinnamic acid (trans-CA) and benzoic acid (BA) [50]. Zhu et al. [49] showed that cinnamoyl-CoA ligase OSD1 catalyzes the conversion of trans-cinnamic acid to cinnamoyl-CoA, which subsequently transforms into benzoyl-CoA via the β-oxidative pathway in peroxisomes. The resulting benzoyl-CoA is then converted into benzyl benzoate by the peroxisomal benzoyltransferase OSD2. Benzyl benzoate is then hydroxylated to form benzyl salicylate by the cytochrome P450 OSD3 enzyme located in the endoplasmic reticulum membrane. The resultant compound is ultimately hydrolyzed to form SA by the cytoplasmic carboxylesterase OSD4 enzyme. According to the authors, activating the PAL pathway in rice significantly enhances SA levels and the plant immunity pathway. It is well known that humic acids increase PAL activity, resulting in higher levels of transcription in treated plants [14]. This leads to an increase in the content of phenolic compounds, including SA.

In addition, the transcriptomic analysis of seedlings primed by HSs showed a significant transcription level of genes encoding stress perception and cell signalization, including kinases, phosphatases proteins, and functional and regulatory (transcription factor) proteins, which are involved in specific gene response against abiotic stress without the presence of a 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 [51]. HSs 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 HSs, 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 HSs 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 HSs 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 HSs 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 (RRs) 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 HSs 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. Plants can improve their resistance to pathogens through the exogenous application of HSs as this promotes the activity of enzymes related to plant resistance. Finally, we consider the potential use of HSs as natural chemical priming agents to boost plant resistance in agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15151688/s1.

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Abbreviations

The following abbreviations are used in this manuscript:

HS	Humic substance
POX	Peroxidase
PAL	Phenylalanine Ammonia-Lyase
GLUC	B 1,3-Glucanase
SAR	Systemic acquired resistance
ISR	Induced systemic resistance
TF	Transcription factor
PR	Pathogenesis-Related
ET	Ethylene
SA	Salicylic acid
JA	Jasmonic acid
PGPB	Plant growth-promoting bacteria

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