Chemical and Biological Technologies in Agriculture

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Humic substances trigger plant immune responses

Rakiely Martins da Silva[1], Natália Aguiar Canellas[1], Fábio Lopes Olivares[1], Alessando Piccolo[1] and Luciano Pasqualoto Canellas[1*]

Abstract

Background Huanglongbing (HLB) is a devastating citrus disease. Before callose deposition, the bacterial infection causes oxidative stress, starting cell damage. Humic substances are among the most efficient antioxidants found in nature. Furthermore, it is also previously reported that humic substances can induce a phenylpropanoid metabolism contributing to the production of antimicrobial compounds. It has been noted by technicians and growers in the Brazil's main citrus belt that orchards continuously treated with humic substances can live well with the symptoms of HLB.

Methods We treated two young citrus orchards (Citrus sinensis cultivars Baía and Pera) with soluble humic substances (HS) isolated from peat either combined or not with a plant growth-promoting bacteria consortium. The activity of key enzymes related to the response against biotic stresses, such as peroxidase (POX), β-1,3-glucanase (PR) and phenylalanine ammonia-lyase (PAL), as well as the differential transcription level of target genes linked to plant stress response by qRT-PCR was monitored for six months.

Results No differences were obtained between sole humic substances and their application combined with plant growth-promoting bacteria, indicating that the microbial consortium had no modulatory effect on HS-treated plants. The treatments promoted the activities of POX, PAL and β-1,3-glucanase, which remained significantly higher concerning the control throughout the evaluation period. In addition, treatments positively regulated the transcription levels of CsPR-7, CsPR-3 and CsPR-11 genes. The experimental data were qualitatively similar to those found in commercial orange orchards treated continuously for 12 and 6 years with humic substances, where the activity and transcription levels were also more significant than for untreated plants.

Conclusion Our results indicate that humic substances can trigger immune-mediated responses in plants and they can be used as a natural chemical priming agent to mitigate disease symptoms and contribute to more resilient citrus cultivation.

Keywords Stress alleviation, Biostimulant, Sustainable agriculture

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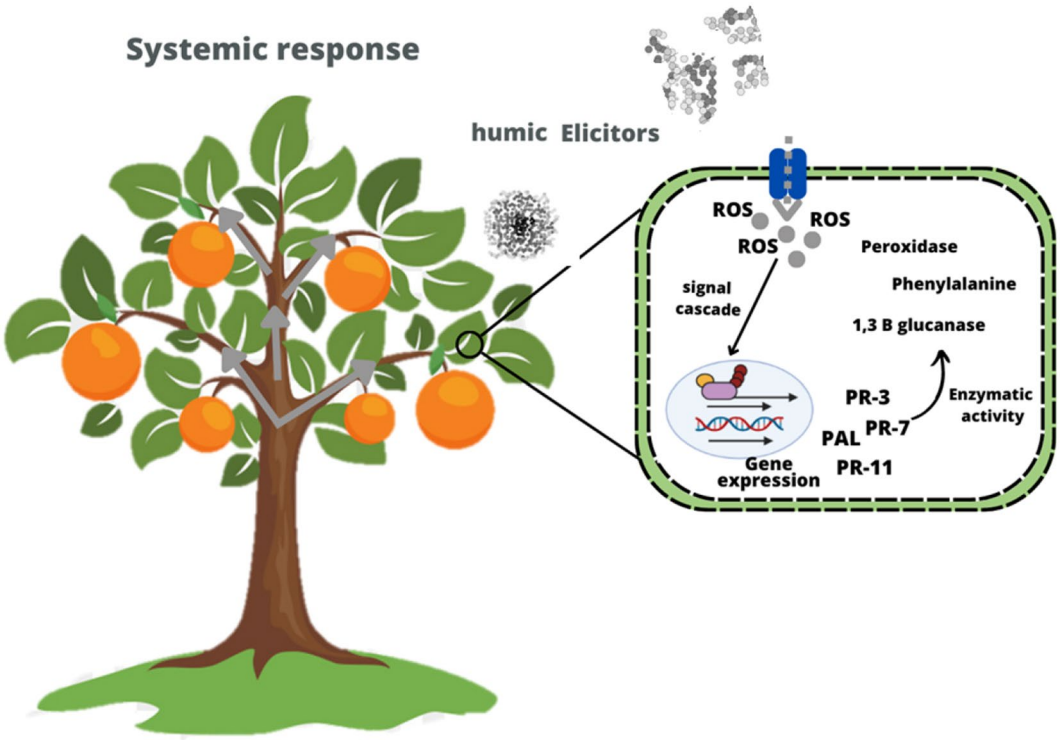
Graphical Abstract



Introduction

Huanglongbing (HLB) is the greatest threat to the global citrus industry. It is a devastating disease caused by the phloem-colonising bacterium *Candidatus Liberibacter asiaticus*, transmitted by the psyllid *Diaphorina citri*. There is no cure for diseased plants. Controlling HLB requires planting healthy seedlings, eliminating diseased plants and controlling psyllids. In São Paulo, the largest producing state in Brazil, almost 50 million orange trees have been eradicated, corresponding to 100,000 ha or 1/4 of the orchards since 2004 [1]. There are no resistant varieties or efficient chemical control available.

The plant innate immune system consists of pattern-triggered immunity (PTI), which is triggered by pathogen-associated molecular patterns (PAMPs) via cell surface-localised pattern recognition receptors, and effector-triggered immunity (ETI), which is instigated by pathogen effector proteins via intracellular receptors called nucleotide-binding, leucine-rich repeat receptors (NLRs) [2–4]. Induced systemic resistance (ISR) responses will increase healthy plant defence by activating PTI and ETI [5]. In conjunction with ISR, there is another systemic resistance in plants that enhances defence against different pathogens, known as systemic acquired resistance (SAR) [6]. The SAR pathway is activated after the formation of a necrotic lesion, either as a part of the hypersensitive response or as a symptom of disease [7].

After cell perception of pathogenic agent reactive oxygen species (ROS) triggers signalisation, a diverse array of plant protectants and activation of defence genes is started, of which products include peroxidases, hydrolytic enzymes (e.g. chitinases and β-1,3-glucanases), pathogenesis-related (PR) proteins and phytoalexin biosynthetic enzymes, like phenylalanine ammonia-lyase (PAL) [8].

Ma and colleagues [9] showed evidence that citrus HLB is an immune-mediated disease, stimulating systemic and chronic immune responses in phloem tissue with the production of ROS and induction of immunity-related genes. A foliar spray of HLB-affected citrus with antioxidants (uric acid and rutin) reduces H_2O_2 concentrations and cell death in phloem tissues, diminishing HLB symptoms.

Humic substances (HS) are dominant components of soil organic matter and one of the most efficient antioxidants found in nature. Typically HS bear functional groups that contain oxygen (O), primarily in carboxyl (C(=O)OH) and carbonyl (−C=O), attached to an R group, and hydroxyl (-OH) groups in alcohols and phenols, related to their reducing capacities or electron-donating capacities [10], probably resulting in the HS ability to regulate ROS accumulation and metabolism [11–14].

The effect of HS in mitigating different plant stresses is well known and generally described as a result of enzymatic and nonenzymatic antioxidant defence rise and increase in compatible solutes production [15]. The potential role of humic acids (HA) in preventing oxidative stress in plants was previously described, including enhancement of peroxidase activity, reduction of H_2O_2 concentration and increase of cell proline levels, leading to decreased ROS contents and thereby restoring the cytosolic redox homeostasis [13, 14]. Antioxidant activities of superoxide dismutase (SOD), peroxidases (POX) and catalase (CAT), which are omnipresent in all aerobic organisms, were promoted by HS, diminishing the injurious effects of ROS [16–18]. Furthermore, the biochemical action against stress operates by producing antimicrobial and toxic substances such as the production of hydrolytic enzymes like β-1,3-glucanase (pathogenesis-related proteins or PR proteins) enabled by SAR [5, 19, 20] providing some degree of resistance and protection against future attacks from microorganisms. HS's induction of PAL activities was previously reported in different plant species [21–23]. The induction of PR by HA was also observed [24]. Moreover, HS showed the most significant effectiveness (75%) in plant pest and disease control, according to data meta-analysis [25], in controlled conditions suggesting their potential role as biotic elicitors in stimulating defence pathways.

The aim of this study was to evaluate whether the application of humic substances together or not with plant growth-promoting bacteria can trigger the defence system of orange trees. Two newly established young orchards were treated with HS and the activity of antioxidant enzymes and differential transcription of pathogen response genes were followed for six months. The results were qualitatively compared with the response of orchards treated for a long time (six and twelve years) with the same humic substances isolated from peat.

Materials and methods

Two *Citrus sinensis* orchards 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 on a Oxisol, according US Soil Survey, otherwise classified as typical "*Latossolo Amarelo distrocoeso*" according to the Brazilian Soil Classification System. The soil had the following characteristics: pH = 4.6; C = 10.4 g/kg; N = 1.1 g/kg; C: N = 9.54; organic matter (OM) = 20.10 g dm^{-3}; P = 4.45 mg dm^{-3}; Al^{+3} = 0.10 $cmol_c$ dm^{-3}; H + Al = 3.2 $cmol_c$ dm^{-3}; Ca = 0.80 $cmol_c$ dm^{-3}; Mg = 1.20 $cmol_c$ dm^{-3}; Sum of bases (SB) = 2.11 $cmol_c$ dm^{-3}; Base saturation (V) = 40 (%); Saturation by Al^{3+} (m) = 4.5% and Cation Exchange Capacity (CEC) = 5.3 $cmol_c$ dm^{-3}. The soil surface layer (0–0.2 m) was collected at three distinct points of the same farm plot. The experiment was entirely randomised, with five plants per treatment spaced in 3.5 m × 5 m. The orange cv *Baia* was planted in October 2021, while the *Seleta* was in October 2022. No incidence of HLBL was reported in the north of Rio de Janeiro state. The seedlings were purchased from a supplier obliged by law to carry out phytosanitary testing. The pot (40 × 50 cm) was fertilised with 20 L of compost of cattle manure per pit, 200 g of lime and 200 g of rock phosphate and 500 g of vermicompost of cattle manure. The treatments were (i) controlled with water application, (ii) one application of HS suspension at 4 mmol C L^{-1} using 2 L per plant and (iii) the HS suspension biofortified with a consortium of plant growth-promoting bacteria. The bacterial strains were obtained from the Microbial Culture Collection of Laboratório de Biologia Celular e Tecidual (LBCT-UENF). The bacteria consortium was formulated using *Herbaspirillum seropedicae* strain HRC54, *Burkholderia silvatlantica* strain UENF 117111 and *Bacillus safensis* strain UENF J1.1. The preinoculum was grown in 5 mL of Digy's liquid medium in a rotatory shaker at 30 °C and 150 rpm for 48 h. Subsequently, a 50 µL suspension of the activated cultures was transferred to a 1 L flask containing 500 mL of the same liquid medium and growth conditions. The individual bacterium density was adjusted to 10^9 cells mL^{-1}. The final dose application per plant results from 200 mL of the combined bacteria strains suspension diluted in 1.8 L of HS. The fourth leaflet from the top of the branch was collected from different branches two days after the treatments were applied for six months. The HS were kindly provided by DNAgro Biotechnology (São Paulo, Brazil). The HS were extracted from peat using KOH 5% ratio 1:20 (v:v). The Total Carbon Concentration (TOC) of HS was measured using a Shimadzu TOC analyser (Tokyo, Japan) (**di cosa?**). Immediately after the field leaflet collection, the samples were transported in an ice container to the laboratory and stocked at -80 C until analysis.

Enzymes activity

Phenylalanine ammonia-lyase assay PAL: (EC 4.3.1.5)

PAL was extracted by homogenising 1 g leaf tissue in 5 ml ice-cold 100 mM potassium phosphate buffer (pH 8.0)

containing 1.4 mM 2-mercaptoethanol and 0.10 g polyvinylpyrrolidone. After centrifuging at 4 °C for 15 min at 15,000xg, the supernatant was chromatographed on Sephadex G-25 equilibrated with the same buffer. Total protein was measured using the Bradford method. A mixture of 0.4 ml of 100 mM Tris–HCl buffer (pH 8.8), 0.2 ml of 40 mM phenylalanine and 0.2 ml of enzyme extract was incubated for 30 min at 37 °C and stopped with 0.2 ml 25% TCA. Phenylalanine was added to the control after incubation and the addition of the acid. After centrifuging for 15 min at 4 °C at 10,000xg, the absorbance of the supernatant was measured at 280 nm relative to the control. PAL activity was expressed as nmol product/mg protein/min.

Peroxidase (POX, E.C.1.11.1.7)

Leaves extract: 1 g of fine powder using liquid N2 was transferred to a 15 mL falcon tube with 1% (v/v) polyvinylpyrrolidone (PVP) and 5 mL of sodium acetate buffer (0.1 M, pH 5) and 1 mL of EDTA (1 mM). The extracts were centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was transferred to Eppendorf tubes and stored at -20°C. The supernatants will evaluate β-1,3-glucanase, POX and soluble protein content. The POX activity was determined at 30° C according to the method described by Hammerschmidt et al. (1982). Reaction medium with 50 µL of guaiacol (0.02 M), 0.5 mL of hydrogen peroxide (0.38 M) and 2.0 mL of phosphate buffer (0.2 M/ pH 5.8). 50 µL of the enzymatic extract will be added, gently shaken and read at a wavelength of 470 nm, for 1 min, with intervals of 10 s. The results will be expressed in Δ absorbance/min/mg protein.

β-1,3-glucanase (E.C.3.2.1.29)

The β-1,3-glucanase activity in the samples was determined by the colourimetric quantification of glucose released from laminarin using p-hydroxybenzoic acid hydrazide (HAPHB) (Lever, 1972). The reaction will consist of 250 µL of enzymatic extract and 250 µL of laminarin (4.0 mg/mL) incubated at 40 °C for 90 min. After this time, 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) will be added and heated at 100 °C for 5 min. Afterwards, the reaction was cooled to 30 °C on ice, and the absorbance was determined at 410 nm against the blank (500 uL extraction buffer + p-hydroxybenzoic acid hydrazide heated at 100 °C for 5 min). Finally, each sample must be subtracted from the control value (corresponding to a mixture identical to that of the sample but without prior incubation). Absorbance readings will be plotted on a standard curve for glucose, and the results will be expressed in ug glucose/min/mg protein.

Differential transcription level of genes with RT-qPCR

Extraction of total RNA

A sample of 100 mg of fresh leaves *tissues* was homogenised with a mortar and pestle in liquid N_2. The homogenate was transferred to new RNAse-free microcentrifuge tubes (1.5 mL), and the RNA was extracted using the mini-plant RNeasy Qiagen ® kit (Germantown, USA). Reverse transcription (RT) followed by polymerase chain reaction (PCR) 1 µg of total RNA was used to produce cDNAs. The synthesis was performed using the high-capacity cDNA reverse transcription kit Applied Biosystems, USA). A PCR with a gradient temperature (59, 60 and 61 °C) was performed to confirm the specificity of the primers and the actual melting temperature. Electrophoresis in 2.0% agarose gel with TAE buffer was also performed to confirm PCR products with the specific primers.

Primers for the genes CsPR1, CsPR27 and CsPR11 were designed with the Primer3 program and their characteristics were evaluated in the Oligo Tech program, and after a rigorous analysis, they were synthesised by IDT technology. Confirmation of primers specificity was obtained in a high-resolution gel, which gave single PCR products at the different temperatures tested and with the expected size. The melting curve performed in StepOne ™ System (Thermo Fisher Scientific, Waltham, MA, USA) also confirmed specificity. The Real-time PCR (RT-qPCR): for statistical validation, two independent tests in the thermal cycler StepOne™ System, with mRNA extracted from the independent experiments, were performed. cDNAs of each experiment were used in quadruplicate for each condition evaluated. The medium for the PCR was prepared as follows (final concentrations): 5 ρM of the forward primer, 5 ρM of the reverse primer (1 µL), 7.5 µL of SYBR Green I component (Applied Biosystems®) and 0.5 µL of ultrapure water. 10 µL of the medium was added to an ELISA plate, and 5 µL of cDNA was added. For the cDNA dilution curve, the following concentrations were used: 0.2, 2, 20 and 200 ng of cDNA template at the control condition. The whole procedure was performed in a laminar flow using sterile materials that were RNAse free. After adding the reagents, the plate was sealed with adhesive and centrifuged gently. The protocol used for the experiment consisted of four steps: (i) denaturation programme (10 min at 95 °C); (ii) amplification programme and quantification repeated 45 times [10 s at 95 °C; 5 s at 61 °C—for both genes (reference and target), 5 s at 72 °C with a single fluorescence acquisition mode]; (iii) melting curve programme (65–95 °C with a heating rate of 0.1 °C/s with continuum fluorescence acquisition) and (iv) cooling programme to lower the temperature to 40 °C. Crossing points (CPs) were obtained and used in the subsequent calculations. CPs are the points at which

the fluorescence achieves significantly higher levels than nonspecific fluorescence. The relative mRNA expression of the genes of interest and the endogenous control [ubiquitin (UBI)] was compared using a nonparametric pairwise fixed reallocation randomisation test as previously described.

Orchards in the citrus belt in São Paulo, Brazil

We visited two areas with orange orchards with continuous use of the same HS for twelve and six years in São Carlos and Brotas, respectively. In São Carlos, the orchards are composed of orange cv *Valencia* and *Pera Natal* in orchards located at Brotas. Both cvs are susceptible to HBL. Once a year, two litres of HS is placed on each orange tree in the soil, and once a month, foliar spraying is carried out using 60 L of commercial humic liquid products per ha containing 30 g TOC L^{-1}, 10 g N L^{-1}, the density of 1.01 g mL^{-1} and pH 8.0. Conventional chemical fertilisation and pest and disease control by traditional methods are used. We performed soil sampling at a depth of 0 to 0.2 and 0.2 to 0.4 cm in the crown projection and between the lines defined as a control in Table 1, showing the main chemical characteristics. Plants in both orchards showed visual symptoms of greening and maintained productivity within the average observed for the state of São Paulo, with 60 kg of oranges per tree. Plant samples were also collected in adjacent areas where the humic product was not used. Leaf samples from both orchards were randomly taken from leaflets from the fruit on ten different trees.

Statistical analysis

The data collected during the experiment were submitted to analysis of variance (ANOVA) using statistical R software version 4.1.2. The LSD test ($p<0.05$) was used to compare differences between means of gene expression and enzyme activity. All data were the average of three replicates.

Results

Enzyme activities

Two days after the application of HS, sampling of the leaves began and was followed monthly for six months. The results of the PAL, POX and β-1,3-glucanase are shown in Fig. 1. All enzymatic activities were more significant than control plants at all times observed. However, the stimulation promoted by HS was different for each enzyme and orange type studied (Fig. 1). No linear model was found to describe the data behaviour, and the typical quadratic model was also insignificant. The maximum PAL stimulation was observed 45 days after application in both *C. sinensis* varieties. After this time, the activity dropped to 90-d until it stabilised at around 1.5-fold higher than the control. Data fluctuation was completely different for β-1,3-glucanase activity at the time and dependent on the orange type. Despite the β-1,3-glucanase, activity remained significantly high concerning control in cv "Seleta". This increase was virtually stable in the first observations, leaping at 60 days and returning to similar values to those observed at 2 and 30 days after application. However, the quadratic model was not significant in describing the data. For cv,

Table 1 Some chemical characteristics of soil samples are collected in two places (São Carlos and Brotas, São Paulo, Brazil) at the crow projection (considered treated = HS) and between the lines defined as control

	São Carlos-12y				Brotas-6y			
	Control		HS		control		HS	
	0–20 cm	20–40 cm	0–20 cm	20–40 cm	0–20 cm	20–40 cm	0–20 cm	20–40 cm
pH (H_2O)	6.90	6.10	6.6	6.7	6.4	6.5	**4.8**	5.1
OM (g dm^{-3})	8.0	9.4	**18.8**	9.4	10.5	9.4	**12.7**	6.7
P (mg dm^{-3})	7.9	2.3	**48.3**	11.4	6.7	4.4	**39.8**	**10.6**
K ($Cmol_c$ dm^{-3})	0.18	0.08	0.23	0.09	0.05	0.04	**0.46**	**0.39**
Ca ($Cmol_c$ dm^{-3})	2.3	0.4	2.4	1.3	1.1	1.4	0.70	0.60
Mg ($Cmol_c$ dm^{-3})	0.7	0.5	0.5	0.5	0.5	0.5	0.5	0.4
H + Al ($Cmol_c$ dm^{-3})	1.5	1.7	1.62	1.5	1.6	1.6	1.9	2.3
Fe (mg dm^{-3})	49.3	91.4	41.5	72.1	14.8	48.7	34.2	34.0
Cu (mg dm^{-3})	6.2	nd	**19.5**	5.5	12.1	5.8	**19.2**	**18.2**
Zn (mg dm^{-3})	11.0	0.9	**36.0**	8.5	1.75	0.46	**4.7**	**5.0**
Mn (mg dm^{-3})	44.6	24.9	53.6	31.1	**55.3**	**46.5**	39.8	39.3

The values correspond to the average of three compost samples (n = 3). Each sample was composed of 10 subsamples. The values in bold correspond to significant differences at the same soil layer by LSD test $p < 0.05$

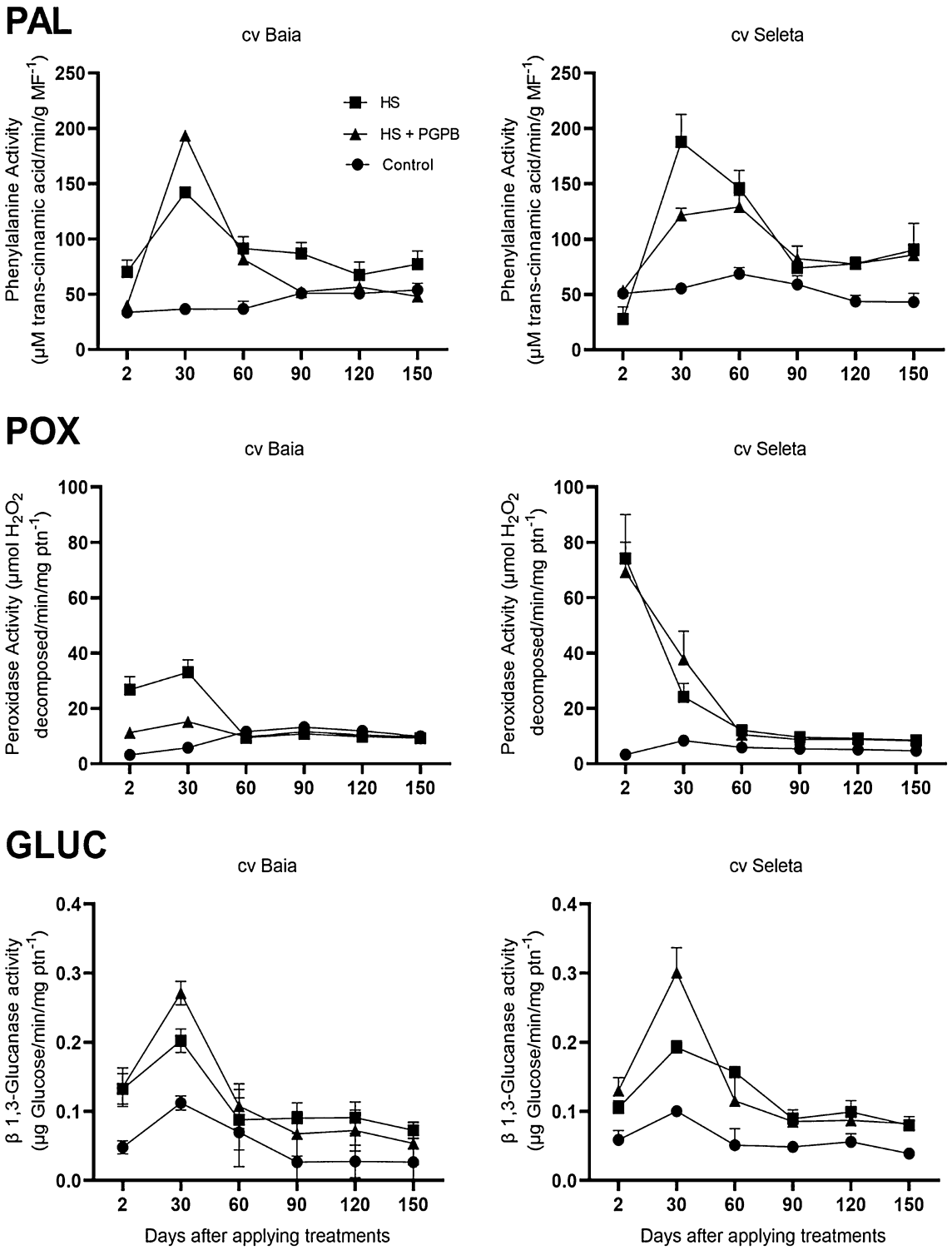


Fig. 1 Phenylalanine ammonia-lyase (PAL), Peroxidase (POX) and β-1,3glucanase activities in orange leaves from untreated (control) and treated plants with humic substances (HS) and HS + plant growth-promoting bacteria. A: cv Baia with two years old and B: cv Seleta with one year old. Leaflets were harvested 48 h after treatments and then monthly for six months

Baía, a significant negative quadratic model described the first part of the experimental time (Fig. 1b). Considering all data, the activity of β-1,3-glucanase was stabilised at 90 days with 2.5-fold larger than control plants. Finally, the POX activity was 22- and 16-fold larger at the initial time (2-d) for Seleta and Baia cultivars, respectively. After this time, a sharp drop was observed with stabilisation at 90-d, where the POX activity was 1.25-fold higher in treated plants. Summarising, the treatment significantly enhanced the enzyme activities used as biochemical markers of plant response, until the end of data collection, within 180 days from HS application. The stabilisation considered as the average activity of both cultivars at the time was 90-d.

Transcription level of target genes (PR3, PR7 and PR11)

The PAL and three PRs genes were used to monitor the molecular gene response of HS and HS + PGPB, and their relative expression is shown in Fig. 2. There was an increased accumulation of CsPAL1 transcripts in HS-treated plants compared to control during all experimental time. The increase in the transcription level of PR3 was significantly more significant than the control for both cultivars only after 48 h of treatments. However, the transcription level of PR7 and PR11 remains significantly higher than the control until 120 days after treatments.

Field campaign

The activities of PAL, POX and β-1,3-glucanase in leaves of oranges treated for 12 and 6 years with HS are shown in Figure 4. The data were expressed concerning untreated plants. Although the commercial orchards were not implemented for experimentation purposes, it is possible to make a qualitative assessment since all other cultural practices were the same. The drop in production and the greening symptoms are visually evident compared to orchards treated with HS.

Discussion

We presented evidence that HS enriched (or not) with plant growth-promoting bacteria can be used as a prime agent to induce citrus defence. HS can attenuate oxidative stress due to their antioxidant properties, hormonal regulation and enzymatic and not enzymatic antioxidant response elicitor [14]. Antioxidative systems, both enzymatic and nonenzymatic, play an essential role in balancing and preventing oxidative damage [26]. This study focussed on PAL, POX and β-1,3-glucanase activities as biological markers of HS action in plant defence. The stress tolerance mechanisms were triggered by applying HS directly on plants at low concentrations. The first stage of this response is the perception and transmission of the signals from HS (Fig. 3).

Activating signalling factors starts with the perception of environmental cues represented by the exogenous application of HS and/or microorganisms in plants, which require active signalling pathways, utilising a complex network of interactions to orchestrate biochemical and physiological responses. We used plant growth-promoting bacteria in combination with HS to reinforce the first signalling since plants need time to differentiate dangerous signals. It takes time for the plant to recognise whether a microorganism is pathogenic or beneficial. Higher plants possess many cell surface receptors to perceive various chemical and immunogenic signals [27, 28]. The central cell surface receptors consist of receptor-like proteins (RLPs) and receptor kinases (RKs) that were previously found in higher transcriptional levels in plants treated with HA isolated from vermicompost [15]. Pattern recognition receptors (PRRs) are RLPs and RKs that perceive extracellular immunogenic patterns in the cell surface. The complex formation between PRRs and co-receptors leads to phosphorylation events within the cytoplasmic kinase domains and to the activation of receptor-like cytoplasmic kinases, which directly phosphorylate and regulate target proteins in order to activate pattern-triggered immunity (PTI) [29]. These complexes activate downstream signalling, inducing PTI, of which Ca^{2+} release within a few minutes after ligand perception is one facet. A significant cytoplasmic Ca^{2+} increase has been reported to occur in *Arabidopsis* leaves starting at 1.5 h and peaking at about 2-h after infection with avirulent bacteria [29]. Increases in apoplast to cytoplasm Ca^{2+} fluxes in response to exposure of rice seedlings to HA were reported [30]. Activation of either layer of the immune system triggers numerous overlapping cell signalling events, including Ca^{2+} fluxes, transcriptional reprogramming, and phytohormone biosynthesis [32, 33]. The transcriptional reprogramming of different plants treated with HA

(See figure on next page.)

Fig. 2 Differential transcription of the PAL (phenylalanine ammonia-lyase) PR3, PR7 and PR11 genes (pathogenesis-related protein) measured by RT-qPCR according to the treatments: untreated plants (control); humic substances (HS); humic substances plus plant growth-promoting bacteria (HS + PGPB). The expression was normalised concerning the control treatment (control = 0). The data represent the mean; the bars are the three independent plants' standard deviation ($n = 3$)

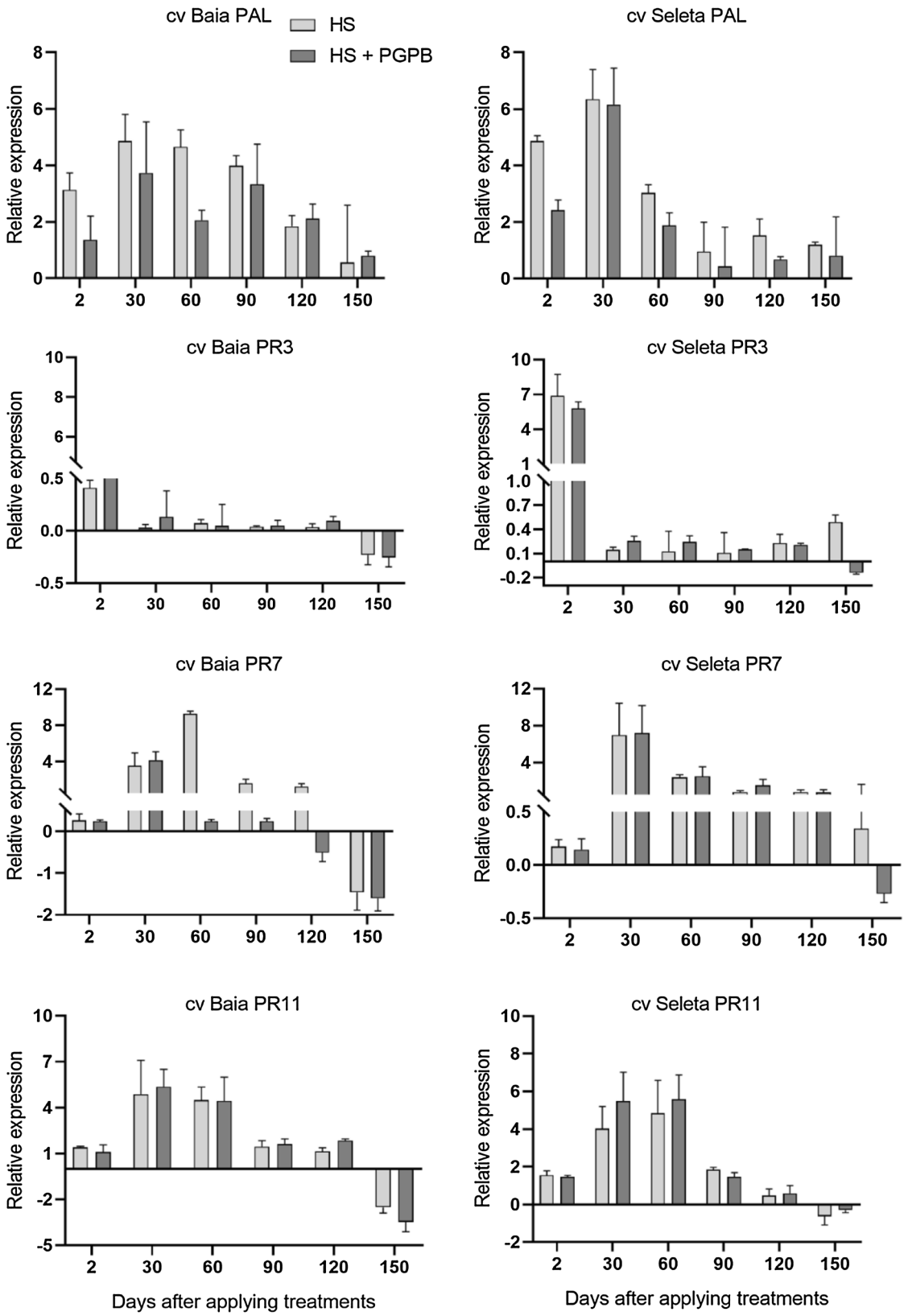


Fig. 2 (See legend on previous page.)

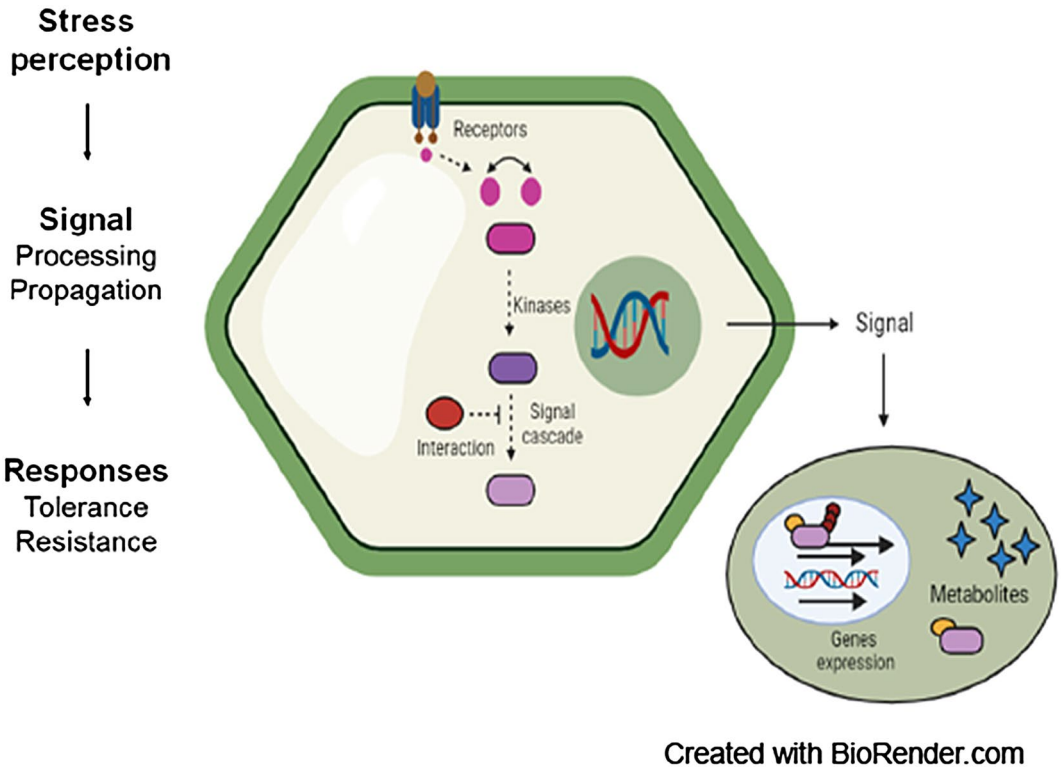


Fig. 3 Schematic representation of the action of SH triggering immune responses in plants

has already been described [33], along with the adjustment of hormonal balance [34].

Plants produce defence hormones ethylene, jasmonates and salicylic acid (SA) to modulate defences. SA plays an essential role in resistance against biotrophic and hemibiotrophic pathogens. In addition, SA has profound importance in amplifying PRR- and NLR-mediated immune signalling [35]. The SA biosynthesis uses the shikimiate pathway, where PAL catalyses the first committed step in the biosynthesis of phenolics by converting phenylalanine to trans-cinnamic acid. Schiavon and colleagues [21] showed for the first time that HS promote phenylpropanoid metabolism by induction of PAL enzyme activities and expression, increasing total phenolic concentration in plants. Interestingly, this stimulation was similar to other studies in which fungal elicitors enhanced phenylpropanoid synthesis at a transcriptional level [36]. Phenolic products are compatible solutes that protect plants against biotic and abiotic stresses [37].

The enhancement of antioxidative metabolism by HS is critical to prevent cell damage. HS applied to plants has been shown to up-regulate the production of antioxidant enzymes (e.g. superoxide dismutase, catalase) and non-enzymatic antioxidants (e.g. ascorbic acid, glutathione) that quench ROS, which eliminates or minimises oxidative damage [13, 16, 38]. The effect of HS on peroxidase activities was also observed [39]. POX reduces the H_2O_2 level inside the cell by oxidation of phenolic compounds and ultimately producing phenolic polymer [40]. Therefore, on the upstream, the ability to reduce ROS level makes them an efficient part of the antioxidant system against stresses, whereas, on the downstream, the production and the deposition of phenolic polymer give strength to the cell wall and inhibit pathogen entry. The effect of HS on POX activity was significant during all evaluation periods but especially two days after application (Fig. 1).

Among the plant defence mechanisms against pathogens is the increase in expression of a considerable group of genes, including those that express proteins related to pathogenesis, the PR proteins [41]. The most studied PR protein is β-1,3-glucanase (PR-2), whose activity is increased when plants are treated with elicitor of defence responses, proving useful in studies regarding biotechnological approaches or citrus resistance markers [42]. The use of HS as a plant defence elicitor was reviewed recently [25]. Here we showed that HS enhance the activity of PR2, including changes on the transcriptional level (Figure 4) together or not with PGPB. We had already observed this induction in passion fruit plants treated with bacteria consortia associated with HA isolated from vermicompost [24].

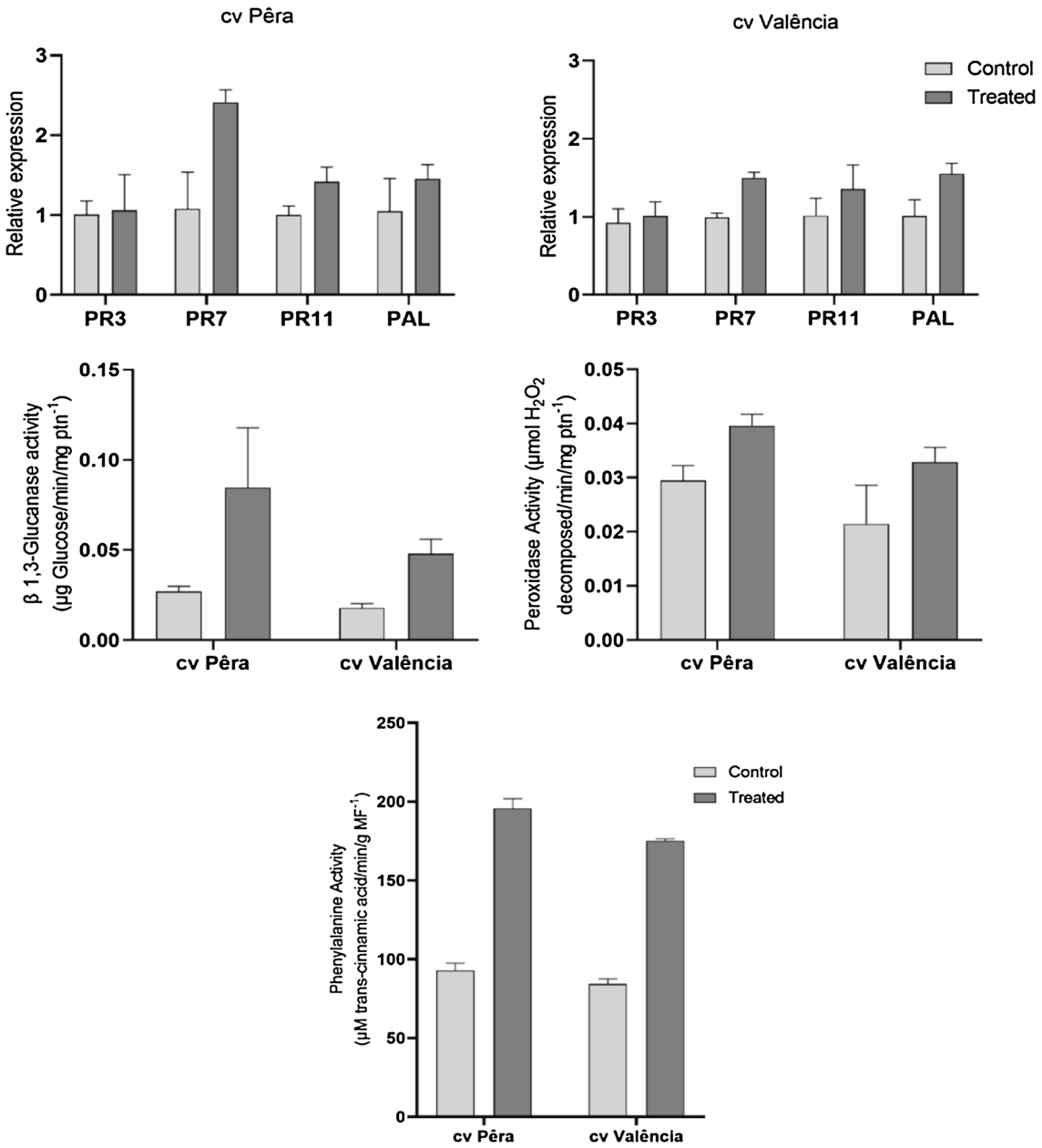


Fig. 4 Phenylalanine ammonia-lyase (PAL), Peroxidase (POX) and β-1,3-glucanase (B GLUC) activities in leaflets of two commercial orchards treated continuously for 12 (cv Valencia) and 6 (cv Pera Natal) years with humic substances. The activities concerning control plants in adjacent areas were normalised and not treated with humic substances. The orchards are located in São Paulo state, Brazil

All defence response markers used in this work (PAL, POX, β-1,3-glucanase) were significantly modified in oranges treated with HS. The variation of this response over time was different for each enzyme, but considering it together, the global enzymatic activities point to a state of attention of the citrus plants, mobilised and stabilised at a higher level at 90-d. Therefore, a possible infection would find the plants previously prepared, with a more accelerated immune response. In other words, HS trigger the plant's immune response.

The commercial orchards were used to collect leaf samples in randomised sampling, and the results of enzymatic activities were similar in qualitative response compared to the field experiment (Figs. 1, 2 and 4). Commercial crops showed apparent symptoms of greening, but even so, they continued to produce commercially,

living with the incidence of the disease. This is indicative that HS can be used in an integrated disease management programme and, at least, attenuate symptoms and their effects on production since they activate the plant defence system monitored by the activity of the three enzymes used as markers of the immunological action of HS in field trials. In summary, HS can mitigate ROS via antioxidant mechanisms and promote new growth, allowing productive coexistence with the disease.

Previously we primed maize seedlings with HS isolated from vermicompost and submitted to different stresses (drought, salinity, heavy metal) and observed that primed seedlings showed more considerable growth, with significant attenuation of stress symptoms concerning untreated plants [15]. In addition, RNAseq of primed seedlings showed the presence of two genes related to disease response at a high transcriptional level, such as HOPZ-activated resistance and DZC (disease resistance, zinc finger). The first one was considered vital to the surveillance system against plant pathogens [43, 44], while the second one is also a classical resistance (R) gene type of defence, previously involved in defence against necrotrophic fungal pathogens, including *Pseudomonas* [43]. These disease gene responses are linked to leucine-rich (LR) TFs highly induced by HA isolated from vermicompost. As shown in the first part of the discussion, the LR-TFs work as a central hub to integrate plant PTI, and it is possible to speculate that oranges can be stimulated to trigger immunity response by HS, resulting in a broad aspect of responses against abiotic stress, including production of antimicrobial compounds and activation of the salicylic acid mechanism by PAL, activation of antioxidant enzymes (POX) and pathogen response genes (PR2).

Abbreviations

ETI	Effector-triggered immunity
HLB	Huanglongbing
HS	Humic substances
HA	Humic acids
ISR	Induced systemic resistance
NLRs	Nucleotide-binding, leucine-rich repeat receptors
PAL	Phenylalanine ammonia-lyase
PAMPs	Pathogen-associated molecular patterns
PGPB	Plant growth-promoting bacteria
PR	Proteins related to pathogenesis
PRx	Peroxidase
PTI	Pattern-triggered immunity
SAR	Systemic acquired resistance
ROS	Reactive oxygen species

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Author contributions
LPC and FLO conceived the concept. LPC and NOA carry out the field trial and did the field campaign. LPC write the first version. RMS did the biochemical and molecular assays. All the authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
This manuscript is an original paper and has not been published in other journals. The authors agreed to keep the copyright rule.

Consent for publication
The authors agreed to the publication of the manuscript in this journal.

Competing interests
DNagro Biotechnology do Brasil provided the commercial humic liquid product (HS) used in this experiment and had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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