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Trichoderma hamatum can act as an inter-plant communicator of foliar pathogen infections by colonizing the roots of nearby plants: A new inter-plant "wired communication"

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ABSTRACT

Trichoderma is a genus of filamentous fungi widely studied and used as a biological control agent in agriculture. However, its ability to form fungal networks for inter-plant communication by means of the so-called inter-plant "wired communication" has not yet been addressed. In our study we used the model plant *Arabidopsis thaliana*, the fungus *Trichoderma hamatum* (isolated from Brassicaceae plants) and the pathogens *Sclerotinia sclerotiorum* and *Xanthomonas campestris* (necrotrophic fungus and hemibiotrophic bacteria, respectively). We performed different combinations of isolated/neighboring plants and root colonization/non-colonization by *T. hamatum*, as well as foliar infections with the pathogens. In this way, we were able to determine how, in the absence of *T. hamatum*, there is an inter-plant communication that induces systemic resistance in neighboring plants of plants infected by the pathogens. On the other hand, the plants colonized by *T. hamatum* roots show a greater systemic resistance against the pathogens. Regarding the role of *T. hamatum* as an inter-plant communicator, it is the result of an increase in foliar signaling by jasmonic acid (increased expression of *LOX1* and *VSP2* genes and decreased expression of *ICS1* and *PR-1* genes), antagonistically increasing root signaling by salicylic acid (increased expression of *ICS1* and *PR-1* genes and decreased expression of *LOX1* and *VSP2*). This situation prevents root colonization by *T. hamatum* of the foliarly infected plant and leads to massive colonization of the neighboring plant, where jasmonic acid-mediated systemic defenses are induced.

1. Introduction

Trichoderma (teleomorphic stage: *Hypocrea*) is a genus of filamentous fungi, belonging to the Hypocreales order, initially described as ubiquitous mycotrophic and phytosaprotrophic fungi (Cai and Druzhinina, 2021). Currently, the genus *Trichoderma* includes several species widely studied and used as beneficial microorganisms in agriculture, primarily as biological control agents against fungi plant pathogens, due to different mechanisms of action, such as mycoparasitism, antibiosis, space and nutrients competition (Poveda, 2021a). Indirectly, *Trichoderma* will be able to reduce the plant disease produced by different fungi plant pathogens due to the activation of a systemic resistance in the plant, mediated by the hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), when interacting with its roots (Poveda et al., 2020a). Both directly and indirectly, *Trichoderma* reduces the damage produced by other organisms harmful to plants, such as nematodes (Poveda et al., 2020b) or arthropod pests (Poveda 2021b). Other mechanisms of action of *Trichoderma* as a beneficial microorganism in agriculture include its use as a plant growth promoter biofertilizer (Adetunji and Anani, 2020; Poveda and Eugui, 2022) and as biostimulant with the ability to increase plant tolerance against abiotic stresses such as drought or salinity (Poveda, 2020a).

Although several of the beneficial mechanisms of action for plants described can be carried out by *Trichoderma* when colonizing the rhizosphere, the behavior of the fungus as a root endophyte is necessary for several of them. The colonization of the roots by *Trichoderma* will be limited to the outermost layers, without reaching the vascular bundles, thanks to the modulation of a local defensive response by the plant, mediated by SA and the accumulation of callose, preventing the fungus from behaving like a systemic pathogen (Alonso-Ramírez et al., 2014;

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Poveda et al., 2020c).

On the other hand, the ability of plants to emit and intercept chemical signals that allow them to communicate with each other is a field of research with great development in recent decades. The first description of the communication between plants was made in 1983, reporting how the foliar mechanical damage caused by herbivores in willows and poplars induces the emission of chemical signals by the attacked plant that activate the defenses of its neighboring plants (Baldwin and Schultz, 1983; Rhoades, 1983). In this regards, the first review on this subject was carried out in 2000, highlighting the role of different derivatives of SA and JA as volatile organic compounds (VOCs) involved in inter-plant communication after an attack by a herbivore or pathogen (Agrawal, 2000). Today, several mechanisms involved in inter-plant communication have been described, in the aerial part, by the emission of VOCs, and underground, through the production of root exudates, root-root contact and through hyphal networks (Sharifi and Ryu, 2021).

By emitting different VOCs, plants will inform their neighbors of pathogen attack, insect attack, mechanical damage, touch stress, temperature stress, air pollutants, drought stress, salt stress or soil microorganisms (Ninkovic et al., 2021). The main plant-VOCs described as inter-plant communicators are the green leaf volatiles (hexenal, hexenol, hexanal, acetaldehyde), sesquiterpenoids (caryophyllene, dimethyl-nonatriene), monoterpenes (ocimene), octadecanoid derivatives (methyl jasmonate) or aromatic compounds (methyl salicylate, indole) (Meents and Mithöfer, 2020). Inter-plant communication by root exudates involves signaling chemicals that convey information on local conditions, including chemical compounds such as ET, JA, strigolactones, loliolide and allantoin (Wang et al., 2021). This communication between roots of different plants can also be carried out through physical contact between them, root-root contact, being a mechanism that is still little studied (Falik et al., 2012).

As far as inter-plant communication through hyphal networks is concerned, the most studied and widely publicized aspect in society is the ability of mycorrhizal fungi, through what are known as common mycorrhizal networks (CMNs), to communicate to the forest trees with each other, as if it were "internet" (Simard, 2018). These connections between plants by CMNs can also occur between species that are very distant taxonomically, such as herbaceous and trees (Gilbert and Johnson, 2017). Both signaling molecules and electrical signals may be involved in this inter-plant communication, favoring the increase of chemical defenses against biotic and abiotic stresses, and even the creation of a collective memory-based interactions among plants (Johnson and Gilbert, 2015; Simard, 2018). However, the ability of filamentous endophytic fungi to act as vehicles of inter-plant communication, by simultaneously colonizing the rhizosphere and plant roots, has been scarcely studied (Oelmüller, 2019). In this sense, there is a single study that describes how the colonization of *Arabidopsis thaliana* roots by the endophytic fungus *Piriformospora indica* leads to inter-plant communication after the leaf attack of the pathogen *Alternaria brassicae*. The authors reported how infection by *A. brassicae* causes a JA-mediated systemic resistance in *A. thaliana*, a signal that travels through the hyphae of *P. indica* to neighboring plants, where the signal is transformed into an abscisic acid (ABA)-mediated defensive plant response (Vahabi et al., 2018). Therefore, inter-plant communication through fungal networks is a field of study yet to be developed, especially with non-mycorrhizal fungi. This is allowing the development of a new field of study, colloquially referred to by researchers as inter-plant "wired communication" (Boyno and Demir, 2022).

The main objective of this work is to describe the possible capacity of the endophytic fungus *Trichoderma hamatum* to act as an inter-plant communicator after the foliar attack of different pathogens, in addition to trying to describe the plant-defense hormonal pathways involved in signaling.

2. Material and methods

2.1. Biological material

During all the work, the model plant *Arabidopsis thaliana*, ecotype Col-0, was used. This plant has been widely described and used as a model plant in plant-microorganism and plant-pathogen interactions (Poveda, 2022).

T. hamatum was isolated from roots of kale (*Brassica oleracea* var. *acephala*) in a previous work with different local populations from Galicia (Northwestern Spain), where it was described as an inducer of systemic resistance against the foliar attack of the phytopathogenic bacterium *Xanthomonas campestris* (Poveda et al., 2020d), and as a biostimulant, promoting plant growth and foliar accumulation of glucosinolates and antioxidants (Velasco et al., 2021). The fungus was routinely grown on potato-dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain) in the dark at 28 °C. Spores were harvested from 7-day-old PDA dishes, as previously described by Poveda et al. (2019).

The plant-pathogens used in the study were the hemibiotrophic bacterium *X. campestris* pv. *campestris* (Xcc) race 3 strain HRI5212, provided by Warwick HRI (Wellesbourne, UK) and the necrotrophic fungus *Sclerotinia sclerotiorum* (Ss) isolate MBG-Ss2, collected from a naturally infected plant of rapeseed (*B. napus*) in an experimental field at Biological Mission of Galicia (MBG).

2.2. Plant growth and Trichoderma inoculation

For the growth of *A. thaliana* and root inoculation with *T. hamatum*, the methodology previously described by Poveda (2021c) was followed, with the specific modifications of the study. *A. thaliana* seeds were surface-sterilized by vigorous sequential shaking in 70% ethanol and 5% sodium hypochlorite solutions for 10 min each and then washed thoroughly four times in sterile distilled water. After, seeds were grown on Murashige and Skoog (MS) (Duchefa, Haarlem, Netherlands) solid medium (agar 1%) with sucrose (1%) in greenhouse conditions, 14 h photoperiod, environmental temperature (12–30 °C) a relative humidity above 80%, for 2 weeks. *Arabidopsis* seedlings were transferred to 0.8 L-pots, containing a mixture of peat/vermiculite (3:1) sterilized by autoclave (twice, 24 h apart) and maintained again in greenhouse conditions. The seedlings were transferred to the pots following the criteria of 1 plant per pot (1 P) or 2 plants per pot (2 P) (Fig. 1), using 14 and 28 plants per condition, respectively.

One week after transplantation, the plants were individually inoculated with *T. hamatum*. 1 mL of a conidial suspension containing 2×10^7 spores/mL, determined using a haemocytometer, was used. The spores were inoculated 2 cm from the plant, inserting the pipette into the substrate 2 cm (Fig. 1).

2.3. X. campestris and S. sclerotiorum foliar infections

One week after *T. hamatum* inoculation, *A. thaliana* plants were foliarly infected with Xcc and Ss, as hemibiotrophic and necrotrophic pathogens, respectively. The infection with Xcc was carried out following the methodology previously described by Meyer et al. (2005), with some modifications. Leaves were infiltrated with 1 μl bacterial suspension of 10^8 CFU/mL. For Ss, all the mycelium formed in three PDA Petri dishes was collected, subsequently, 30 mL of sterile distilled water was added in a Falcon tube along with the mycelium and 0.5 g of Ballotini Glass Balls 0.15–0.25 mm diameter and others 0.5 g of 1 mm diameter (Potters, Saint-Pourçain-sur-Sioule, France), and vigorously shaking for 20 min. The resulting mycelium suspension was adjusted for inoculation to the absorbance of 0,17 per mL at 520 nm, infiltrating leaves with 1 μl.

To determine the effect of the pre-infection of a neighboring plant, the neighboring plant was inoculated in pots with two plants, as indicated, 72 h before the infection of the rest of the plants.

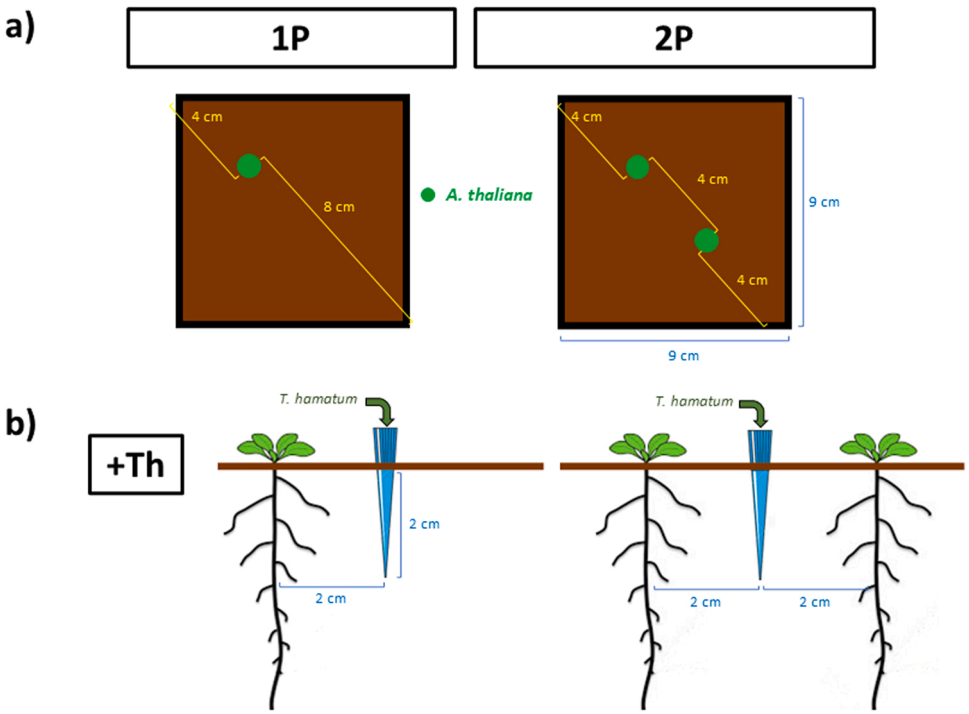

Fig. 1. Explanatory infographic of the methodology carried out for the growth and inoculation with *T. hamatum* (+ Th) of *A. thaliana* plants. The pots contained one or two plants (1 P and 2 P, respectively), leaving a distance of 4 cm between the two plants. (a) Method of planting *A. thaliana* seedlings into culture pots. (b) Method of inoculating the soil with a suspension of *T. hamatum* spores.

All the codes used throughout the work for the different conditions are summarized below:

- **1 P**: A single plant without *T. hamatum* inoculation and without pathogenic infection.
- **2 P**: Two neighboring plants without *T. hamatum* inoculation and without pathogenic infection.
- **1 P + Th**: A single plant with *T. hamatum* inoculation and without pathogenic infection.
- **2 P + Th**: Two neighboring plants with *T. hamatum* inoculation and without pathogenic infection.
- **1 P + Xcc**: A single plant without *T. hamatum* inoculation and foliar infected with *X. campestris*.
- **2 P + Xcc**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before.
- **2 P + Xcc(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before. **2 P + Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor.
- **1 P + Th+Xcc:** A single plant with *T. hamatum* inoculation and foliar infected with *X. campestris*.
- **2 P + Th+Xcc:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before. **2 P + Th-np+Xcc:** It refers specifically to the neighboring plant infected 72 h before its neighbor.
- **2 P + Th+Xcc(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before. **2 P + Th+Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor.
- **1 P + Ss**: A single plant without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*.
- **2 P + Ss**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before.
- **2 P + Ss(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before. **2 P + Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor.
- **1 P + Th+Ss:** A single plant with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*.
- **2 P + Th+Ss:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before. **2 P + Th-np+Ss:** It refers specifically to the neighboring plant infected 72 h before its neighbor.
- **2 P + Th+Ss(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before. **2 P + Th+Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor.

2.4. Pathogen effect analysis

2.4.1. Injuries measurement

Individual photographs were taken of the 14 infected leaves per condition, 10 days post-infection (d.p.i.). The area of the lesion produced on each leaf was quantified using ImageJ software (US National Institutes of Health, Bethesda U.S.A.).

From 14 leaves collected in each condition, 9 were used to form 3 pools of 3 leaves that were frozen with liquid nitrogen and pulverized with a mortar, and the remaining 5 leaves were used in the rest of the analysis of the subsection (2.4.2. and 2.4.3.). A summary infographic with the number of plants and tissues used in each experiment and analysis is shown in Fig. S1.

2.4.2. Vitality test in tissues

The vitality test of the *A. thaliana* leaves was carried out following the methodology described by Poveda (2021a). The reduction of triphenyltetrazolium chloride (TTC) by tissue to red-colored insoluble triphenylformazan (TF) is directly linked to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC

to TF. From 5 fresh-leaves 100 mg was transferred to 1 mL of 1% TTC and incubated for 72 h at 37 °C. After incubation, 200 mg of Ballotini Glass Balls (0.15–0.25 mm and 1 mm diameter) was added to each sample in 1.5 mL Eppendorf tubes and shaken vigorously by vortex. After centrifuging the samples for 15 min at 10,000 rpm, the supernatant was removed, and 1 mL of isopropanol was added to each tube. The samples were again agitated by vortex and centrifuged in the same way, and the supernatant was used to quantify the absorbance at 620 nm; this acted as an indirect measure of the vitality of the *A. thaliana* tissues.

2.4.3. Indirect quantification of reactive oxygen species (ROS) in tissues

Indirect ROS quantification was carried out following the methodology described by Poveda (2020b). The indirect quantification of reactive oxygen species (ROS) in A. *thaliana* tissues was carried out by measuring electrolyte leakage, which really measures cellular oxidative damage related to the production of ROS. From 5 fresh-leaves, 1 cm^2 of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water at room temperature for 6 h. The conductivity of the water was measured using a pH/conductivity meter Crison™ MM41 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the tissues (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total concentration of ions present in the tissues (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

2.5. Quantification of Trichoderma-root colonization

In order to quantify the differences in root colonization by *Trichoderma* according to the different conditions, its quantification was carried out by qPCR, following the methodology described by Poveda (2021c), with some modifications. From each condition, roots from 12 plants were pooled in 3 different pools (4 roots each). Roots were collected at the same time that the infected leaves were collected (10 d. p.i.). All root material was washed with water until there was no remaining substrate, immediately frozen with liquid nitrogen, and pulverized with a mortar. DNA was extracted using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific). A mix was prepared in a 15 μl volume using a Promega kit, 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. The *Actin* genes of *Trichoderma* and *Arabidopsis* were used as reference genes for the calculation of host plant and fungus DNA, and their corresponding primer pairs are indicated in Table 1. Amplifications were performed in a 7500 Real Time PCR System (Applied Biosystem, Forster City, CA, USA), programmed for 40 cycles under the following conditions, after denaturation at 95 °C for 10 min: 95 °C for 15 s and 60 °C for 60 s. Each PCR was performed in triplicate by using the DNA extracted from the roots collected. Cycle threshold values served to calculate the amount of fungal DNA using standard curves. Values of *Trichoderma* DNA were referred to the amount of *Arabidopsis* DNA in every corresponding sample, normalizing to 1 the value of 1 P + Th.

Table 1
Primers used in this work.

Code	Sequence (5′−3′)	Use	Reference
Act-T-F	ATGGTATGGGTCAGAAGGA	Endogenous *Trichoderma* gene	Poveda, 2021c
Act-T-R	ATGTCAACACGAGCAATGG		
Act-At-F	CTCCCGCTATGTATGTCGCC	Endogenous *Arabidopsis* gene	Poveda, 2022
Act-At-R	TTGGCACAGTGTGAGACACAC		
ICS1-At-F	GATCTAGCTAACGAGAACGG	Synthesis gene of SA in *Arabidopsis*	Poveda, 2022
ICS1-At-R	CATTAAACTCAACCTGAGGGAC		
PR-1-At-F	GGCTAACTACAACTACGCTG	Response gene to SA in *Arabidopsis*	Poveda, 2022
PR-1-At-R	GGCTTCTCGTTCACATAATTC		
LOX1-At-F	GTAAGCTCTGATGTTACTGATTC	Synthesis gene of JA in *Arabidopsis*	Poveda, 2022
LOX1-At-R	CTGCGGTTAACGACGTGATTG		
VSP2-At-F	GTTAGGGACCGGAGCATCAA	Response gene to JA in *Arabidopsis*	Poveda, 2022
VSP2-At-R	TCAATCCCGAGCTCTATGATGTT		

2.6. Defense-genes expression studies

The gene expression of different defense-related genes was analyzed in the already described root and leaf pools (3 pools of 3 leaves and 3 pools of 4 roots, per condition), following the methodology described by Sotelo et al. (2016), with some modifications. RNA was isolated from 100 mg of pools tissues using a Spectrum™ Plant Total RNA Kit (Quiagen, Valencia, CA, USA). To remove any traces of genomic DNA from extractions, the RNA was treated with RQ1 RNase-Free DNase (Promega, CA, USA) following the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA using a GoScript™ Reverse Transcription System, according to the manufacturer's instructions (Promega, Madison, WI, USA). RT-qPCRs were carried out on a 7500 Real Time PCR System (Applied Biosystem, Forster City, CA, USA), using a Promega kit in a total volume of 15 μl. After denaturation at 95 °C for 10 min, 40 cycles were performed under the following conditions: 95 °C for 15 s and 60 °C for 60 s. All PCR reactions were performed in triplicate. CT values were calculated using the *Arabidopsis Actin* gene as an endogenous control. The primers used are given in Table 1: genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and vegetative storage protein 2 (*VSP2*). Data were expressed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The statistical analysis of the data was carried out with the Statistix 8.0 software. Combined effects of *Trichoderma* inoculation and presence of neighboring plant were analyzed by two-way ANOVA followed by Sidak's multiple comparison test; the different letters indicate the significant differences ($P < 0.05$). One-way ANOVA using Tukey's multiple range test was used for pairwise comparisons; different letters indicate significant differences ($P < 0.05$).

3. Results

3.1. Effect of X. campestris and S. sclerotiorum foliar infections

Foliar infections of *A. thaliana* with Xcc produced significantly less lesions in the plants inoculated with *T. hamatum* (7.95 ± 3.18 mm^2), compared to the non-inoculated plants (31.10 ± 7.98 mm^2). In non-inoculated plants with *T. hamatum*, the infection of the neighboring plant with Xcc 72 h before caused a significant decrease in the area of the lesions (16.92 ± 3.74 mm^2) against the non-infection of the neighboring plant (27.31 ± 4.32 mm^2) and against the absence of a neighboring plant (31.10 ± 7.98 mm^2). On the other hand, in the plants inoculated with *T. hamatum*, no significant differences were quantified in lesions-area when the plant had another neighboring plant infected 72 h before (3.61 ± 2.72 mm^2), the neighboring plant had not been inoculated (7.20 ± 3.29 mm^2) or there was no neighboring plant (7.95 ± 3.18 mm^2) (Fig. 2).

The analysis of the viability of the tissues reported how the root inoculation of *A. thaliana* with *T. hamatum* significantly increased the viability of the leaves infected with Xcc (0.085 ± 0.028), compared to

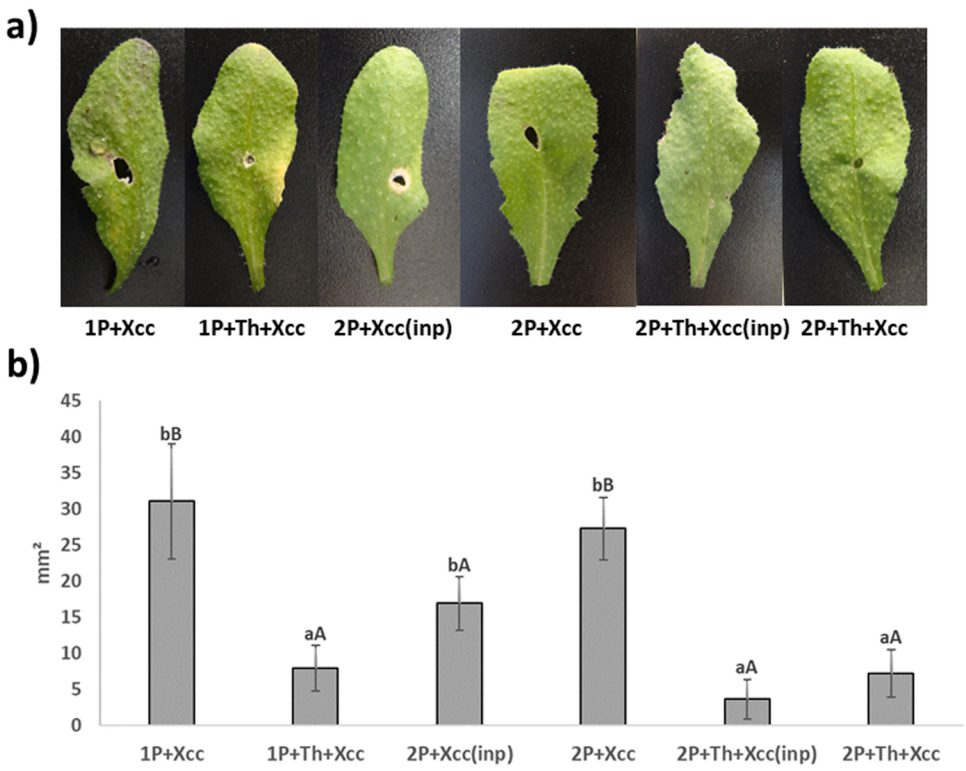

Fig. 2. *A. thaliana* leaves Xcc-infected (+Xcc) (a) and lesions area quantification (mm^2) (b). One plant (1 P) or two neighboring plants (2 P) were used. Data are the mean of 14 leaves for each condition. Two-way analysis of variance (ANOVA) was performed, followed by Sidak's multiple comparison test. Different letters represent significant differences ($p < 0.05$), for *Trichoderma* inoculation (+ Th) (identify by small letters) and for presence of infected neighboring plant (inp) (identify by capital letters). **1 P + Xcc**: A single plant without *T. hamatum* inoculation and foliar infected with *X. campestris*; **1 P + Th+Xcc:** A single plant with *T. hamatum* inoculation and foliar infected with *X. campestris*; **2 P + Xcc(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before; **2 P + Xcc**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before; **2 P + Th+Xcc(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before. **2 P + Th+Xcc:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before.

the non-inoculated plants (0.032 ± 0.016). With respect to the pre-infection of the neighboring plant 72 h before (0.066 ± 0.013), no significant differences were quantified compared to the non-inoculation of the neighboring plant (0.041 ± 0.020) (Fig. 3a). Similarly, plants inoculated with *T. hamatum* reported significantly lower levels of indirect ROS quantification (1.57 ± 0.52), compared to non-inoculated plants (3.24 ± 0.81). Infection of the neighboring plant with Xcc 72 h earlier (2.02 ± 0.34 and 0.44 ± 0.29) also did not lead to significant differences compared to plants whose neighboring plant was not infected (2.98 ± 0.27 and 0.89 ± 0.44) (Fig. 3b).

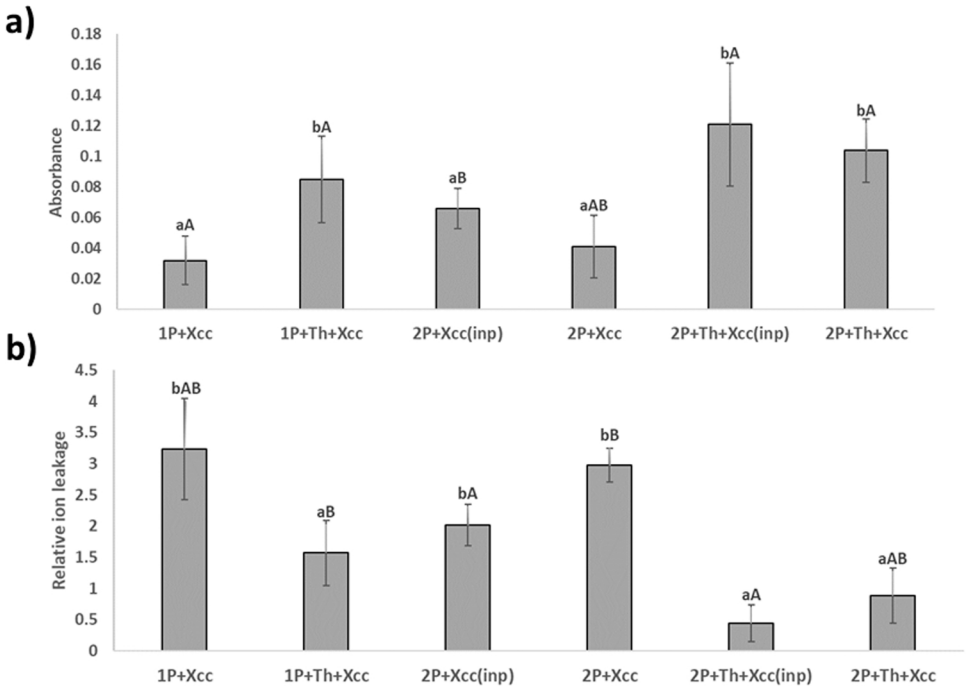

Fig. 3. Tissue vitality by TTC test (a) and indirect quantification of ROS (b) in *A. thaliana* leaves Xcc-infected (+Xcc), where the absorbance at 620 nm (TTC test) and the relative ion leakage (indirect ROS measurement) were analyzed. One plant (1 P) or two neighboring plants (2 P) were used. Data are the mean of 5 leaves for each condition. Two-way analysis of variance (ANOVA) was performed, followed by Sidak's multiple comparison test. Different letters represent significant differences ($p < 0.05$), for *Trichoderma* inoculation (+ Th) (identify by small letters) and for presence of infected neighboring plant (inp) (identify by capital letters). **1 P + Xcc**: A single plant without *T. hamatum* inoculation and foliar infected with *X. campestris*; **1 P + Th+Xcc:** A single plant with *T. hamatum* inoculation and foliar infected with *X. campestris*; **2 P + Xcc(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before; **2 P + Xcc**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before; **2 P + Th+Xcc(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before. **2 P + Th+Xcc:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before.

As far as Ss is concerned, foliar infection of *A. thaliana* plants inoculated with *T. hamatum* had significantly less lesions (8.51 ± 1.42 mm^2) than uninoculated plants (24.64 ± 6.16 mm^2). In addition, in the plants non-inoculated with *Trichoderma*, no significant differences were quantified between the plants with (15.06 ± 5.02 mm^2) and without (25.20 ± 8.40 mm^2) having infected the neighboring plant 72 h before. When the plants were inoculated with *T. hamatum*, the presence of a neighboring plant infected 72 h before (1.56 ± 1.04 mm^2) significantly reduced the area of the lesions caused by the pathogen, compared to plants without pre-infected neighboring plants (6.75 ± 2.25 mm^2) (Fig. 4).

In *A. thaliana leaves* infected with Ss whose plants had been root inoculated with *T. hamatum*, significantly higher levels of viability of their tissues were reported (0.053 ± 0.013), compared to plants without inoculation (0.018 ± 0.04). Plants not inoculated with *T. hamatum* did not show significant differences between having (0.034 ± 0.008) or not having (0.013 ± 0.009) a neighboring plant infected 72 h before. However, plants rooted inoculated with *T. hamatum* reported a significant increase in tissue viability when there was a neighboring plant infected 72 h before with the pathogen (0.101 ± 0.008) (Fig. 5a). Regarding the indirect quantification of ROS, the leaves whose plants had been root inoculated with *T. hamatum* had a significantly lower indirect measurement of ROS (2.58 ± 0.43). Among the plants not inoculated with *T. hamatum*, no significant differences were reported (3.56 ± 0.89 and 4.36 ± 0.54), whether or not there was a neighboring plant infected with Ss 72 h before. Pre-infection with Ss from a neighboring plant 72 h before in plants inoculated with *T. hamatum* reported a significant reduction (0.32 ± 0.21) compared to plants without an infected neighboring plant (2.05 ± 0.34) (Fig. 5b).

3.2. Trichoderma-roots colonization

The quantification of the *A. thaliana* root colonization by *T. hamatum* reported, after the foliar infection of the plant with each of the pathogens (Xcc and Ss), there was no significant increase in the levels of root colonization (1 P + Th+Xcc and 1 P + Th+Ss) against the non-infected plants (1 P + Th) (Fig. 6). The presence of two neighboring plants of *A. thaliana* (2 P) significantly reduced root colonization by *T. hamatum* (2 P + Th), compared to the presence of a single plant (1 P + Th) (Fig. 6). The foliar infection with Xcc of any of the neighboring plants (2 P + Th+Xcc(inp), 2 P + Th+Xcc(inp)np, 2 PTh-np+Xcc and 2 P + Th+Xcc) represented, in all cases, a significant increase in root colonization compared to uninfected plants (2 P + Th), not being significantly different levels from those reported in a root inoculated plant with the fungus and without foliar infection (1 P + Th) (Fig. 6a). In the case of foliar infection with Ss in two neighboring plants (2 P), very different levels of colonization were found. Infection of the neighboring plant 72 h before resulted in a significant increase in root colonization of the infected plant 72 h later (2 P + Th+Ss(inp)), compared to the other conditions. However, the neighboring plant inoculated 72 h before (2 P + Th+Ss(inp)np) did not report significant colonization differences compared to the levels quantified in two neighboring plants inoculated with the fungus and without foliar infection (2 P + Th). Lastly, foliar infection with Ss in one of the neighboring plants reported in these plants the same levels of root colonization by *T. hamatum* (2 P + Th-np+Ss and 2 P + Th+Ss) reported for a single plant without infection (1 P + Th) (Fig. 6b).

3.3. Defense gene expression in roots and leaves

At the root level, the *A. thaliana* inoculation with *T. hamatum* in a solitary plant (1 P + Th) and with a neighboring plant (2 P + Th) represented a significant increase in the expression of the SA- and JA-related genes, compared to plants without *Trichoderma* inoculation (1 P and 2 P) (Figs. 7 and 9). This same significant increase in the expression of the SA- and JA-related genes was also reported at the foliar level (Figs. 8 and 10).

In the system with a single *A. thaliana* plant, foliar infection with Xcc (1 P + Xcc) reported a significant reduction in the expression of SA-related genes and an increase in JA-related genes in roots (Fig. 7), and a significant increase in SA-related genes and a reduction in JA-related genes in leaves (Fig. 8), compared to the uninfected plants (1 P). When the plant was root inoculated with *T. hamatum* (1 P + Th+Xcc), an even more significant root expression of the JA-related genes (Fig. 7)

Fig. 4. *A. thaliana* leaves Ss-infected (+Ss) (a) and lesions area quantification (mm^2) (b). One plant (1 P) or two neighboring plants (2 P) were used. Data are the mean of 14 leaves for each condition. Two-way analysis of variance (ANOVA) was performed, followed by Sidak's multiple comparison test. Different letters represent significant differences ($p < 0.05$), for *Trichoderma* inoculation (+ Th) (identify by small letters) and for presence of infected neighboring plant (inp) (identify by capital letters).**1 P + Ss**: A single plant without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **1 P + Th+Ss:** A single plant with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **2 P + Ss(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before; **2 P + Ss**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before; **2 P + Th+Ss(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before; **2 P + Th+Ss:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before.

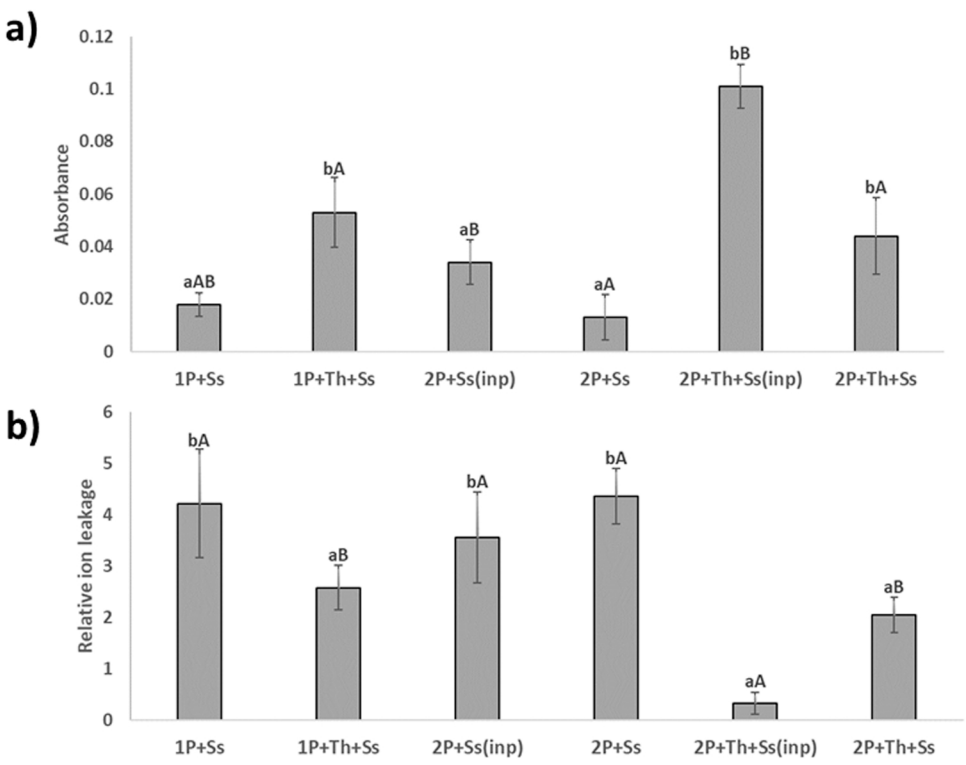

Fig. 5. Tissue vitality by TTC test (a) and indirect quantification of ROS (b) in *A. thaliana* leaves Ss-infected (+Ss), where the absorbance at 620 nm (TTC test) and the relative ion leakage (indirect ROS measurement) were analyzed. One plant (1 P) or two neighboring plants (2 P) were used. Data are the mean of 5 leaves for each condition. Two-way analysis of variance (ANOVA) was performed, followed by Sidak's multiple comparison test. Different letters represent significant differences ($p < 0.05$), for *Trichoderma* inoculation (+Th) (identify by small letters) and for presence of infected neighboring plant (inp) (identify by capital letters). **1 P + Ss**: A single plant without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **1 P + Th+Ss:** A single plant with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **2 P + Ss(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before; **2 P + Ss**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before; **2 P + Th+Ss(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before; **2 P + Th+Ss:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before.

and foliar expression of the SA-related genes (Fig. 8) was quantified. In the two-plants system, the infection with Xcc of any of the plants in isolation (2 P + Xcc(inp)np and 2 P + Xcc) reported the same results of expression of defense genes root and foliar than the infection of a solitary plant (1 P + Xcc) (Figs. 7 and 8). On the contrary, when the neighboring plant was infected 72 h before the analyzed plant (also infected with Xcc) (2 P + Xcc(inp)), a significant increase was reported at the foliar level in the expression of SA-related genes and a decrease in JA-related genes. (Fig. 8), no changes at the root level (Fig. 7), compared to the solitary infected plants (1 P + Xcc). Root inoculation with *T. hamatum* in the two-plants system (2 P + Th) reported a significant increase in the root expression of JA-related genes, compared to the same plants without *Trichoderma* inoculation (2 P) (Fig. 7). While at the foliar level, the presence of *Trichoderma* in the roots reported only a significant increase in SA- and JA-related genes in the neighboring plant infected with Xcc 72 h before (2 P + Th+Xcc(inp)np) and in its companion plant when its neighbor had not been pre-infected (2 P + Th+Xcc) (Fig. 8).

As far as Ss-infected *A. thaliana* plants are concerned, a significant increase in the root expression of SA-related genes and a reduction in JA related genes were reported in the single-plant system (1 P + Ss) (Fig. 9), compared to the uninfected plant (1 P). While in the leaves, a significant reduction in the expression of SA-related genes and an increase in that of JA-related genes was quantified (Fig. 10). Compared to the Ss-infected plant (1 P + Ss), root inoculation with *T. hamatum* (1 P + Th+Ss) reported a significant increase in the expression of the SA-related genes in the roots (Fig. 9) and JA-related genes in the leaves, along with a reduction in the SA-related genes expression (Fig. 10). In the two-plants system, the foliar infection with Ss did not report differences in the expression of defense genes in roots and leaves in any of the analyzed plants (2 P + Ss(inp)np and 2 P + Ss) (Figs. 9 and 10), compared to the infected plants of the single-plant system (1 P + Ss), except in plants whose neighbors had been inoculated 72 h before (2 P + Ss(inp)), where a significant increase in the foliar expression of JA-related genes and a reduction of SA related genes was quantified (Fig. 10). When these plants were root inoculated with *T. hamatum*, in the infected neighboring plant 72 h before (2 P + Th+Ss(inp)np) and in the plant without the inoculated neighbor (2 P + Th+Ss) a significant increase in the expression of the SA-related genes was reported in the roots (Fig. 9), together with a significant increase in the expression of SA- and JA-related genes in leaves, compared to the same plants without *Trichoderma* inoculation (2 P + Ss(inp)np and 2 P + Ss). In comparison with these plants infected with Ss and root inoculated with *T. hamatum* (2 P + Th+Ss(inp)np and 2 P + Th+Ss), the plants whose neighbor had been infected 72 h before, and both plants are colonized with the fungus (2 P + Th+Ss(inp)), reported a significant increase in the root expression of the SA-related genes and a reduction in JA-related genes (Fig. 9), in addition to a significant reduction in foliar expression of SA-related genes and an increase in JA-related genes (Fig. 10).

4. Discussion

The ability of mycorrhizal fungi to act as inter-plant communicators has been widely studied and reviewed by numerous authors, raising their clear similarities between the transmission of information through the fungal mycelium and the transmission of the nerve impulse by neurons (Johnson and Gilbert, 2015; Gilbert and Johnson, 2017; Simard, 2018; Oelmüller, 2019). However, there is little evidence of these processes in other root colonizing fungi that behave as endophytes and rhizospheric microorganisms. Only one previous work has been done, by Vahabi et al. (2018), where the capacity of the endophytic filamentous fungus *P. indica* to act as an inter-plant communicator between *A. thaliana* neighboring plants is analyzed, after foliar infection with *A. brassicae*. In this study, the fungus is capable of transmitting a plant signal of JA-mediated systemic resistance through its hyphae, inducing a similar defensive response in the neighboring plant (Vahabi et al., 2018). Through the use of the hemibiotrophic and necrotrophic pathogens *X. campestris* and *S. sclerotiorum*, respectively, the model plant

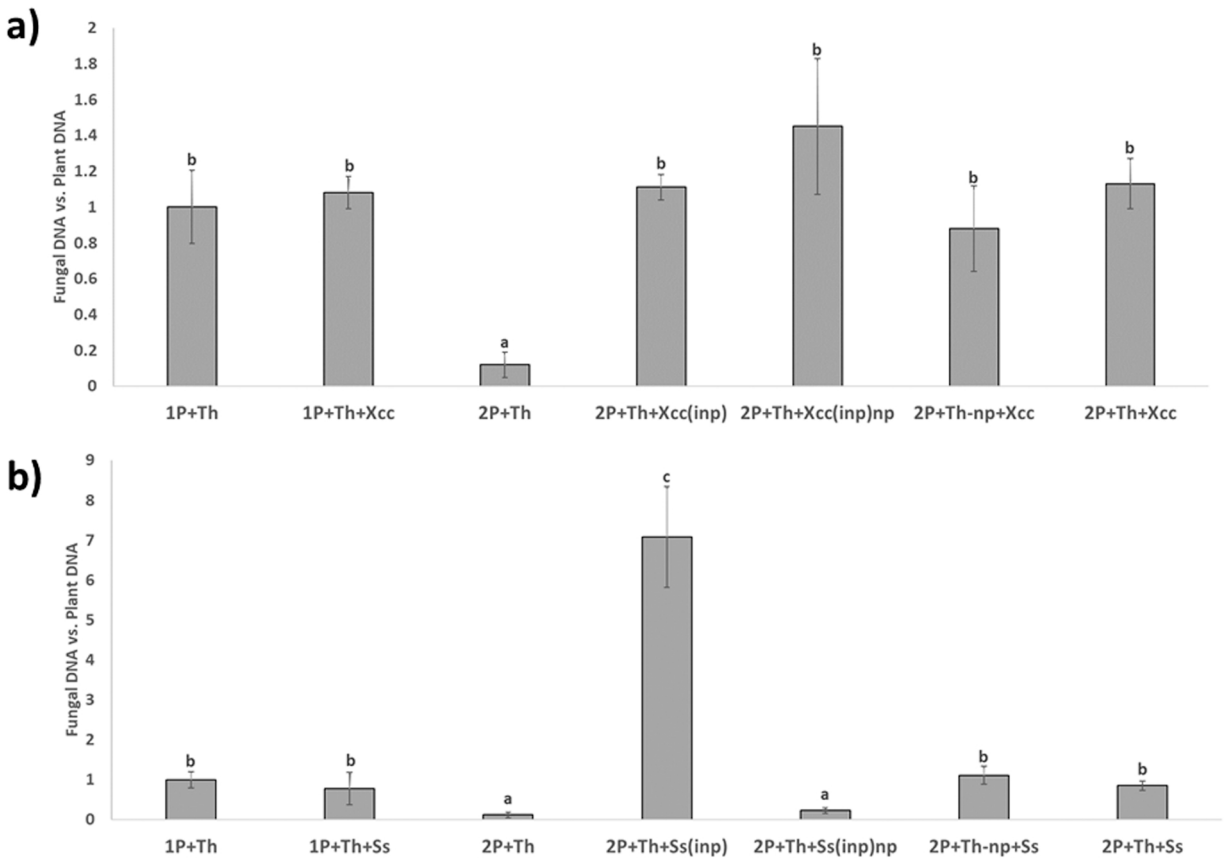

Fig. 6. Measurements of *Arabidopsis*-root colonization by *T. hamatum* (+Th) in one plant (1 P) or two neighboring plants (2 P), Xcc-infected (+Xcc) (a) or Ss-infected (+Ss) (b). When the neighboring plant was infected 72 h before (inp), the colonization in the neighboring plant (np) was also quantified, and when only the neighboring plant was infected, the colonizaton was analyzed in the non-infected plant (np+Xcc or np+Ss). To quantify *Arabidopsis*-root colonization, the DNA of the fungus was quantified by qPCR from radicular samples using the *Actin* genes from both the plants and the fungus. Fungal DNA/plant DNA ratio was normalized to 1 in the case of 1 P and was calculated based on this data for the rest of the lines. Data are the mean of nine plant-roots in 3 pools with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$). **1 P + Th**: A single plant with *T. hamatum* inoculation and without pathogenic infection. **1 P + Th+Xcc:** A single plant with *T. hamatum* inoculation and foliar infected with *X. campestris*; **2 P + Th**: Two neighboring plants with *T. hamatum* inoculation and without pathogenic infection; **2 P + Th+Xcc(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before (**2 P + Th+Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Th+Xcc:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before (**2 P + Th-np+Xcc:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **1 P + Th+Ss:** A single plant with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **2 P + Th+Ss(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before (**2 P + Th+Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Th+Ss:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before (**2 P + Th-np+Ss:** It refers specifically to the neighboring plant infected 72 h before its neighbor).

A. thaliana and the endophytic and rhizosphere-colonizer fungus *T. hamatum*, the present work attempts to expand the knowledge regarding the possible role of other fungi as inter-plant communicators.

After foliar infection with the hemibiotrophic bacterium *X. campestris*, we reported an increase in the expression of locally SA-related genes, a specific plant defensive response against biotrophic pathogens (Yang et al., 2015). Similarly, foliar infection with the necrotrophic pathogen *S. sclerotiorum* produced an increase in the expression of JA-related genes, a specific response against necrotrophs (Pandey et al., 2016). This activation of foliar defenses was related to an increase in the expression of genes of the contrary pathways in the roots. Antagonistic-spatial behavior previously reported for both defensive routes (Betsuyaku et al., 2018).

In the absence of pathogens, root colonization by *T. hamatum* causes an increase in the systemic expression of SA- and JA-related genes, a sign of the endophytic fungus's ability to induce systemic plant defensive responses before pathogens or pests attack, mechanism called priming, which has been previously described in different *Trichoderma* species (Gupta and Bar, 2020; Agostini et al., 2021; Morán-Diez et al., 2021). This systemic activation of plant defenses by *Trichoderma*-roots colonization can involve both SA- and JA/ET-related genes, against biotrophic or necrotrophic pathogens, therefore, through induced systemic resistance (ISR) and/or systemic acquired resistance (SAR) (Nawrocka and Małolepsza, 2013). When plants have been root-inoculated with *T. hamatum*, the foliar infection by both pathogens is reduced, an aspect previously reported against *Xanthomonas euvesicatoria* in tomato (Alfano et al., 2007) and *X. campestris* in kale (Poveda et al., 2020d), but never previously described for foliar infections by *S. sclerotiorum*, although *T. hamatum* has been reported as an efficient antagonist against the necrotrophic pathogen in the soil (Shaw et al., 2016). This reduction in foliar infection caused by both pathogens before *T. hamatum*-roots colonization is the consequence of a specific systemic increase in the expression of SA-related genes, in the case of *X. campestris*, and JA-related genes, in the case of *S. sclerotiorum*. This specific SA-mediated activation of systemic resistance by *Trichoderma*-roots colonization has been reported against other hemibiotrophic pathogens, such as *X. campestris* pv. *malvacearum* on cotton colonized by *T. harzianum* (Raghavendra et al., 2013), the fungus *Colletotrichum graminicola* in

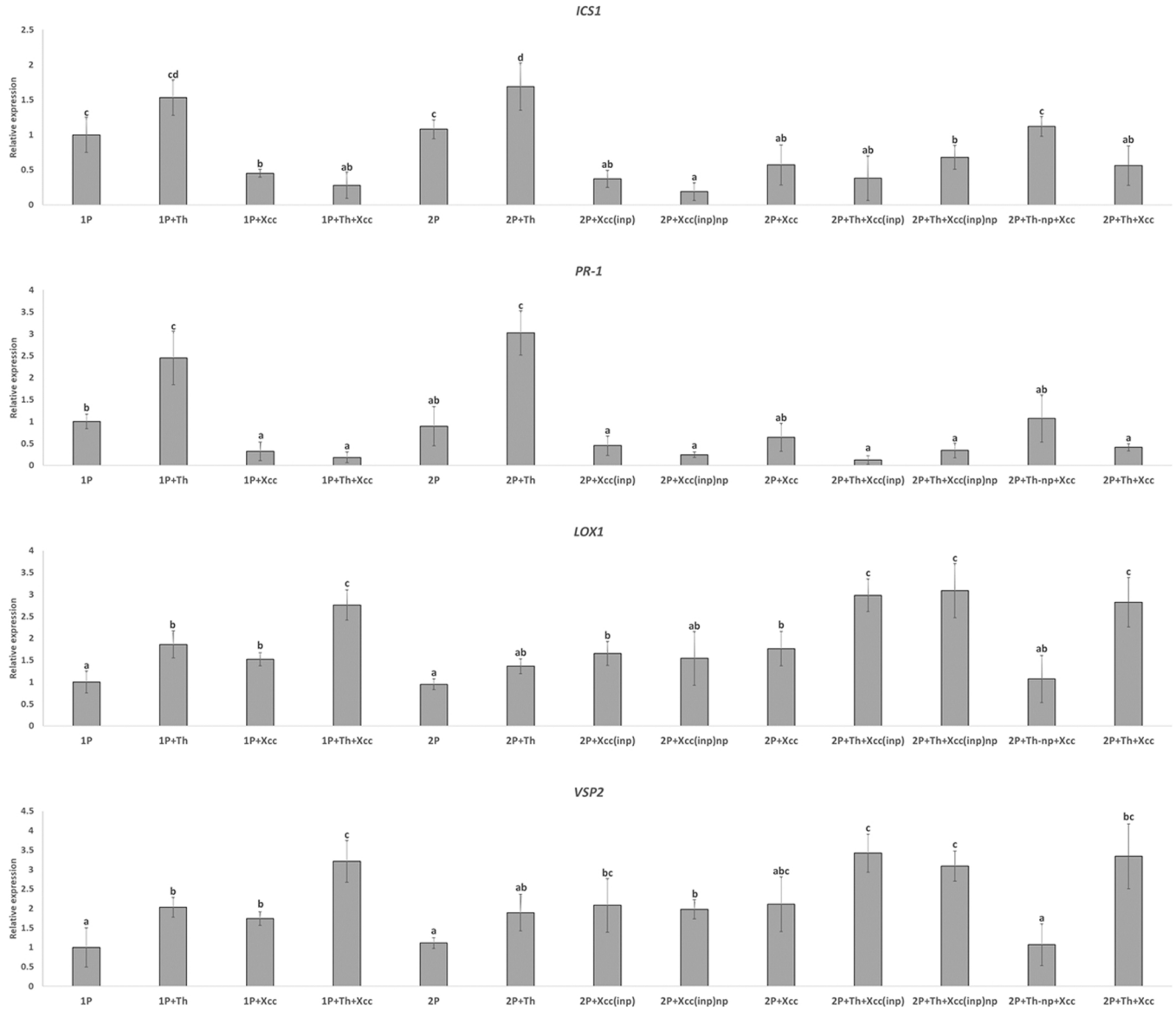

Fig. 7. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in *A. thaliana* roots foliar-infected with Xcc and root inoculated with *T. hamatum* (+Th). When the neighboring plant was infected 72 h before (inp), the gene expression levels in the neighboring plant (np) was also quantified, and when only the neighboring plant was infected, the expression was analyzed in the non-infected plant (np+Xcc). Genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and vegetative storage protein (*VSP2*). Values correspond to relative measurements against single plants without *Trichoderma*-roots inoculation ($2^{-\Delta\Delta Ct} = 1$). The *A. thaliana Actin* gene was used as an internal reference gene. Data are the mean of 3 pools of 4 leaves each with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$). **1 P**: A single plant without *T. hamatum* inoculation and without pathogenic infection; **1 P + Th**: A single plant with *T. hamatum* inoculation and without pathogenic infection; **1 P + Xcc**: A single plant without *T. hamatum* inoculation and foliar infected with *X. campestris*; **1 P + Th+Xcc:** A single plant with *T. hamatum* inoculation and foliar infected with *X. campestris*; **2 P**: Two neighboring plants without *T. hamatum* inoculation and without pathogenic infection; **2 P + Th**: Two neighboring plants with *T. hamatum* inoculation and without pathogenic infection; **2 P + Xcc(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before (**2 P + Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Xcc**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before; **2 P + Th+Xcc(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before (**2 P + Th+Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Th+Xcc:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before (**2 P + Th-np+Xcc:** It refers specifically to the neighboring plant infected 72 h before its neighbor).

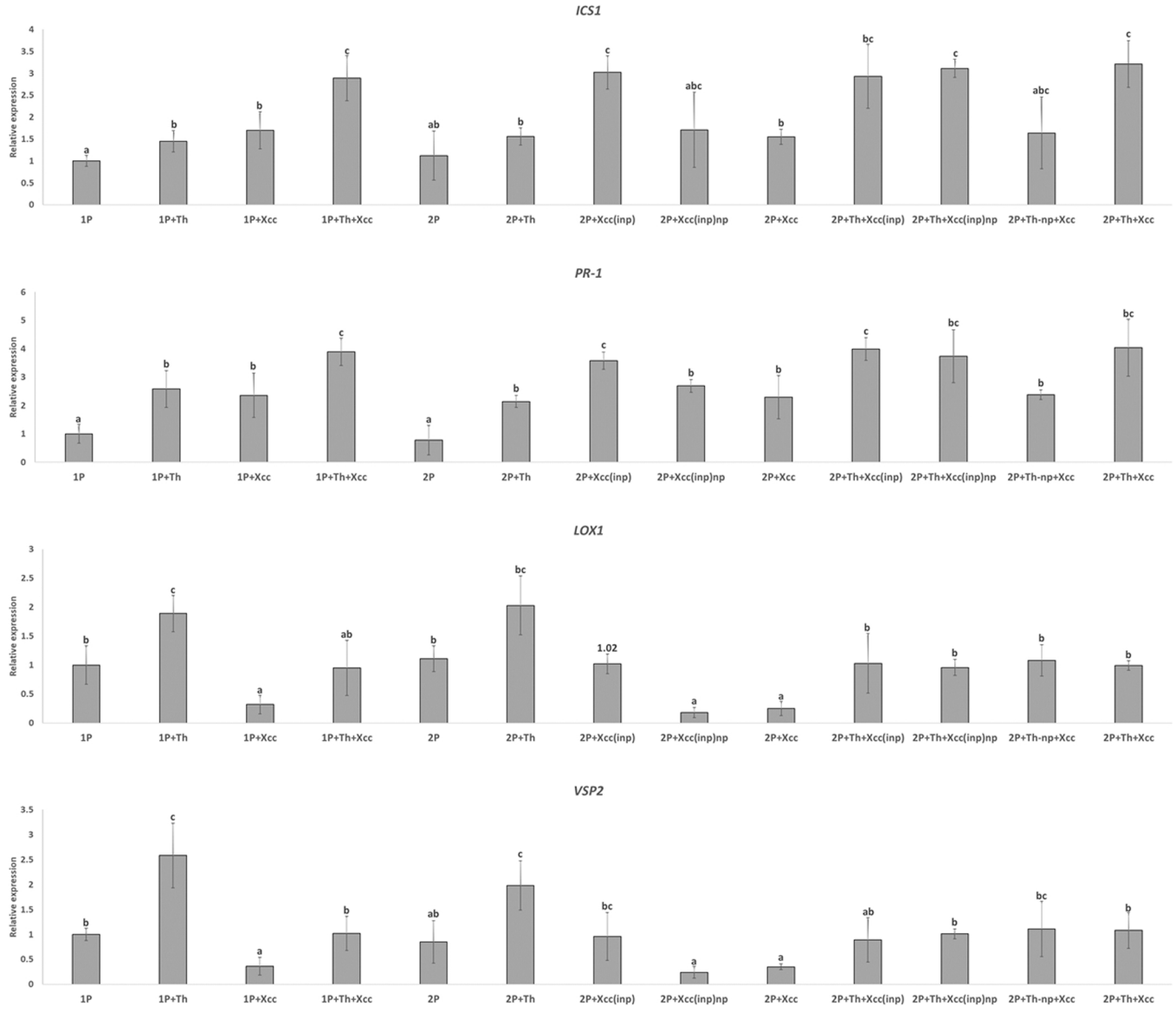

Fig. 8. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in *A. thaliana* leaves infected with Xcc and root inoculated with *T. hamatum* (+Th). When the neighboring plant was infected 72 h before (inp), the gene expression levels in the neighboring plant (np) was also quantified, and when only the neighboring plant was infected, the expression was analyzed in the non-infected plant (np+Xcc). Genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and vegetative storage protein (*VSP2*). Values correspond to relative measurements against single plants without *Trichoderma*-roots inoculation ($2^{-\Delta\Delta Ct} = 1$). The *A. thaliana Actin* gene was used as an internal reference gene. Data are the mean of nine plant-roots in 3 pools with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$). **1 P**: A single plant without *T. hamatum* inoculation and without pathogenic infection; **1 P + Th**: A single plant with *T. hamatum* inoculation and without pathogenic infection; **1 P + Xcc**: A single plant without *T. hamatum* inoculation and foliar infected with *X. campestris*; **1 P + Th+Xcc:** A single plant with *T. hamatum* inoculation and foliar infected with *X. campestris*; **2 P**: Two neighboring plants without *T. hamatum* inoculation and without pathogenic infection; **2 P + Th**: Two neighboring plants with *T. hamatum* inoculation and without pathogenic infection; **2 P + Xcc(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before (**2 P + Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Xcc**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before; **2 P + Th+Xcc(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, with neighbor plant infected 72 h before (**2 P + Th+Xcc(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Th+Xcc:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *X. campestris*, without neighbor plant infected 72 h before (**2 P + Th-np+Xcc:** It refers specifically to the neighboring plant infected 72 h before its neighbor).

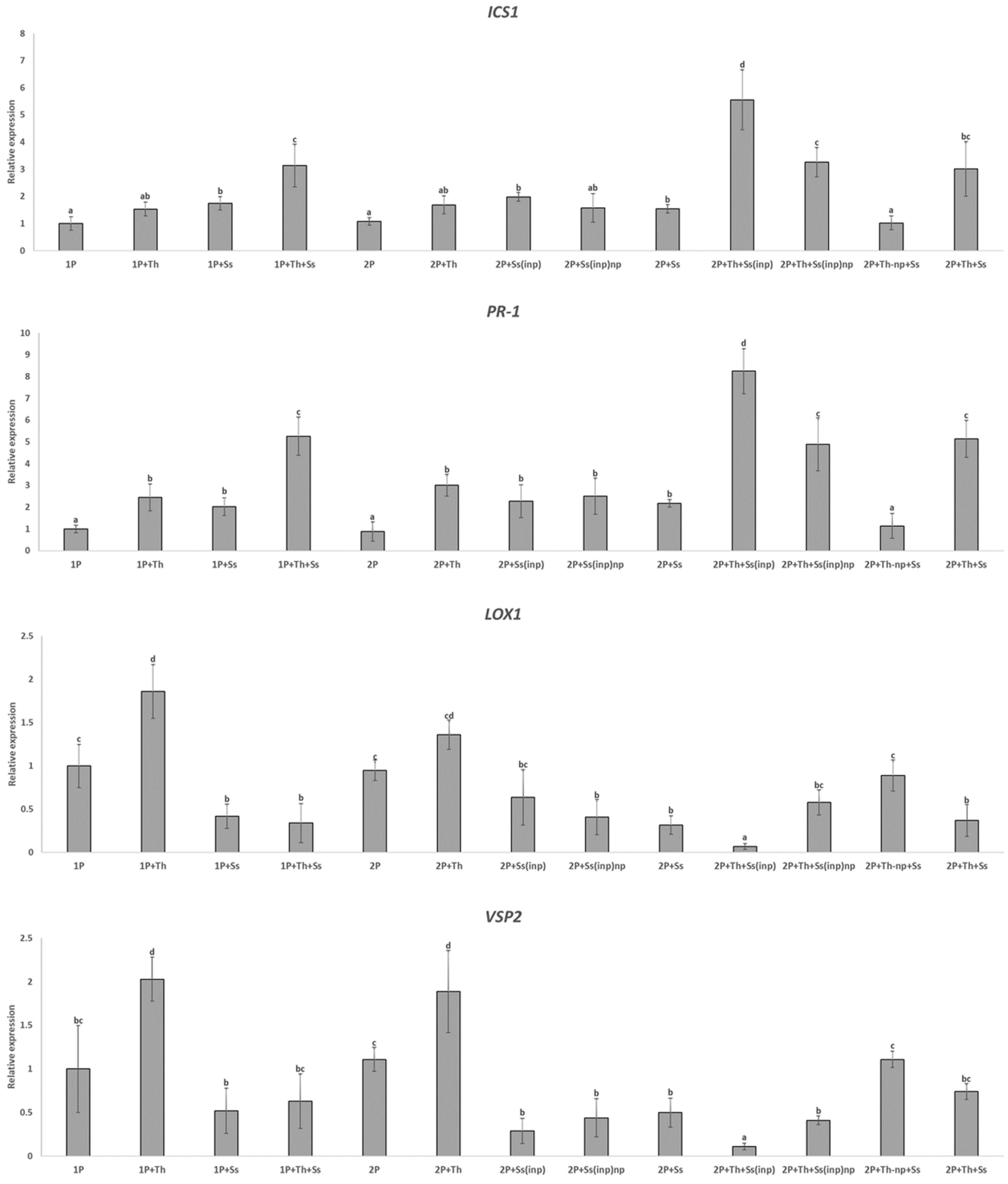

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Fig. 9. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in *A. thaliana* roots foliar-infected with Ss and root inoculated with *T. hamatum* (+Th). When the neighboring plant was infected 72 h before (inp), the gene expression levels in the neighboring plant (np) was also quantified, and when only the neighboring plant was infected, the expression was analyzed in the non-infected plant (np+Ss). Genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and vegetative storage protein (*VSP2*). Values correspond to relative measurements against single plants without *Trichoderma*-roots inoculation ($2^{-\Delta\Delta Ct} = 1$). The *A. thaliana Actin* gene was used as an internal reference gene. Data are the mean of 3 pools of 4 leaves each with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$). **1 P**: A single plant without *T. hamatum* inoculation and without pathogenic infection; **1 P + Th**: A single plant with *T. hamatum* inoculation and without pathogenic infection; **1 P + Ss**: A single plant without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **1 P + Th+Ss:** A single plant with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **2 P**: Two neighboring plants without *T. hamatum* inoculation and without pathogenic infection; **2 P + Th**: Two neighboring plants with *T. hamatum* inoculation and without pathogenic infection; **2 P + Ss(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before (**2 P + Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Ss**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before; **2 P + Th+Ss(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before (**2 P + Th+Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Th+Ss:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before (**2 P + Th-np+Ss:** It refers specifically to the neighboring plant infected 72 h before its neighbor).

maize colonized by *T. virens* (Wang et al., 2020), or the oomycete *Sclerospora graminicola* in pearl millet colonized by *T. hamatum* (Siddaiah et al., 2017). In the case of necrotrophic pathogens, the ability of *Trichoderma* to activate systemic defensive responses JA-mediated by roots colonization has been widely described against different necrotrophic pathogens, such as *Botrytis cinerea* in tomato and *A. thaliana* colonized by *T. harzianum* (Martínez-Medina et al., 2013; Poveda et al., 2019), or *Ascochyta rabiei* in chickpea colonized by *T. harzianum* and *T. koningii* (Poveda, 2021a).

In the 2 plants-system it was possible to determine how infection of the neighboring plant 72 h before with *X. campestris* reduced the area of the lesions in the other plant, results not observed in *S. sclerotiorum* infection. In turn, it was reported as in non-infected plants, whose neighboring plant had been infected with *X. campestris*, increased leaf expression of SA-related genes. These results would be the consequence of inter-plant communication through VOCs or root exudates, mechanisms described by several authors (Ninkovic et al., 2021; Wang et al., 2021). In this sense, the application of volatiles derived from SA, such as methyl salicylate (MeSA), is capable of activating systemic SA-mediated resistance in rice plants against infection by *Xanthomonas oryzae* pv. *oryzae* (Kalaivani et al., 2021).

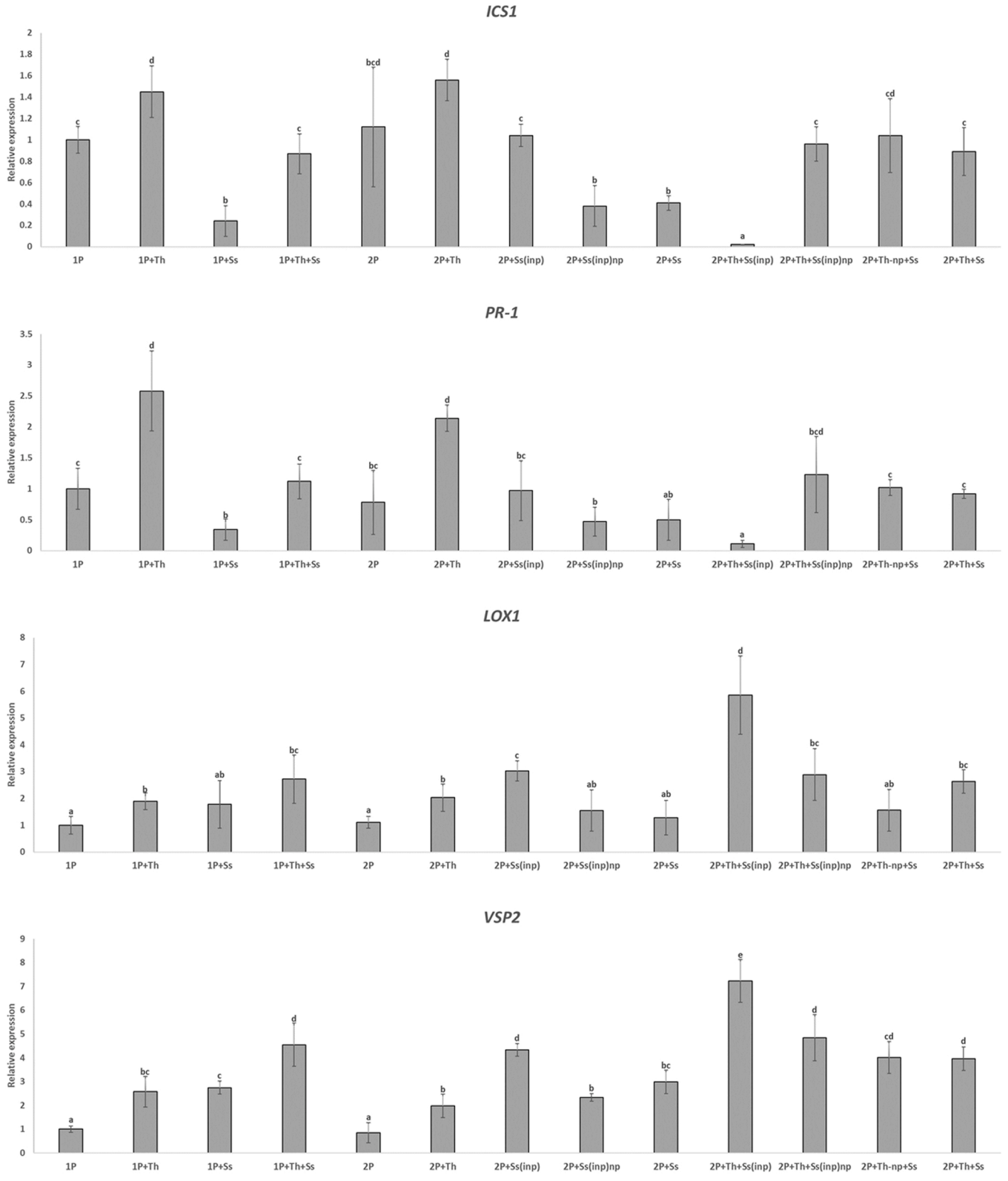
The inoculation of *T. hamatum* in the 2-plants system, in absence of pathogens, led to a reduction in the levels of root colonization. Although this aspect has not been studied previously in endophytic fungi and/or colonizers of the rhizosphere, it has been approached with arbuscular mycorrhizal fungi (AMF). However, the AMF obtained results are totally contrary to those reported in our study with *T. hamatum*. Root colonization by AMF increases with a greater number of neighboring plants (Šmilauer et al., 2020).

Infection of the neighboring plant 72 h before with *S. sclerotiorum* significantly reduced the damage caused by the pathogen when both plants were root colonized with *T. hamatum*. These results were not reported in the case of foliar infection with *X. campestris*. Although there is surely an inter-plant communication through other mechanisms, such as volatiles and root exudates, in infection with the necrotrophic pathogen, *T. hamatum* could be acting as an inter-plant communicator. Root colonization by *T. hamatum* of the plant whose neighbor had been infected with *S. sclerotiorum* 72 h before causes a very significant systemic increase in the expression of JA-related genes, a specific response against the necrotrophic pathogen, which would explain the great reduction in the adverse effects of infection.

Foliar infection with *X. campestris* increases the expression of SA-related genes in *A. thaliana* leaves and JA-related genes in roots, while infection with *S. sclerotiorum* causes exactly the opposite effect. In this sense, we believe that the key in the ability of *T. hamatum* to act as an inter-plant communicator in foliar infections would be the defensive pathway activated in the plant, specifically in the roots. The key role played by SA in plant roots to control colonization by *Trichoderma* has been described (Poveda et al., 2020c). This colonization is limited to the outer layers of the root and, thanks to callose deposits induced by SA-signaling, *Trichoderma* does not reach the vascular bundles, where it would behave as a systemic pathogen that would kill the plant (Alonso-Ramírez et al., 2014; Poveda et al., 2023). In foliar infection with *X. campestris*, there is a root increase in the expression of JA-related genes, which does not modify colonization by *T. hamatum*. However, foliar infection by *S. sclerotiorum* increases the expression of SA-related genes in the roots, reducing the levels of fungal colonization. When the neighboring plant is infected with *S. sclerotiorum* 72 h before, the activation of the JA-mediated foliar and SA-mediated root defenses causes a great reduction in the capacity of *T. hamatum* to colonize the roots. In this situation, *T. hamatum* massively colonizes the roots of another neighboring plant, which induces a very intense SA-mediated response in the roots to prevent *Trichoderma* from behaving as a pathogen. Antagonistically, the great activation of the SA-pathway in the roots is linked to a great systemic activation of the JA-mediated defenses, which, when *S. sclerotiorum* attacks, greatly reduce the damage caused by the necrotrophic pathogen. Previous results in *A. thaliana* with *T. harzianum* determined how greater root colonization increases the expression of SA-related genes in the roots and JA-related genes in the leaves, which reduces the damage caused by the necrotrophic pathogen *B. cinerea* (Poveda, 2021c).

The mechanism proposed for the behavior of *Trichoderma* as an inter-plant communicator presents several differences to that described before for *P. indica*. First, with *P. indica*, colonization is not analyzed, so the role is not yet known. On the other hand, it is assumed that *P. indica* perceives the activation of the defensive JA-pathway in the roots and transmits it to the neighboring plant, transforming the signal into an activation of the ABA-pathway (Vahabi et al., 2018). In our study with *Trichoderma*, root colonization plays a key role and the defensive pathway in the roots of both plants would be SA-mediated.

In conclusion, root colonization of *A. thaliana* plants activates systemic resistance against *X. campestris* and *S. sclerotiorum* by SA and JA pathways, respectively. The use of two nearby plants indicates that infection by *X. campestris* can be reported to neighboring plants through volatiles or root exudates, inducing a specific SA pathway systemic resistance against the hemibiotrophic pathogen. Regarding the possible role of *T. hamatum* as an inter-plant communicator, in Fig. 11 we have raised the possible mechanism involved, only in foliar infection with the necrotrophic pathogen *S. sclerotiorum*. Against foliar infection, the plant locally increases JA-mediated defenses, which leads to an increase in SA-related defenses in the roots. This forms a barrier to root colonization by *T. hamatum*, causing massive colonization of the neighboring plant. In order to control this root colonization, the plant increases the local defenses in the SA-mediated roots, which causes an activation of the systemic defenses in the JA-mediated leaves, which act more quickly and specifically against *S. sclerotiorum* infection. The possible SA-mediated defensive response against *X. campestris* in leaves could be implicated in the absence of the ability to inter-plant communication by *T. hamatum*



(caption on next page)

Fig. 10. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some defense genes in *A. thaliana* leaves infected with Ss and root inoculated with *T. hamatum* (+Th). When the neighboring plant was infected 72 h before (inp), the gene expression levels in the neighboring plant (np) was also quantified, and when only the neighboring plant was infected, the expression was analyzed in the non-infected plant (np+Ss). Genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and vegetative storage protein (*VSP2*). Values correspond to relative measurements against single plants without *Trichoderma*-roots inoculation ($2^{-\Delta\Delta Ct} = 1$). The *A. thaliana Actin* gene was used as an internal reference gene. Data are the mean of nine plant-roots in 3 pools with the corresponding standard deviation. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$). **1 P**: A single plant without *T. hamatum* inoculation and without pathogenic infection; **1 P + Th**: A single plant with *T. hamatum* inoculation and without pathogenic infection; **1 P + Ss**: A single plant without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **1 P + Th+Ss:** A single plant with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*; **2 P**: Two neighboring plants without *T. hamatum* inoculation and without pathogenic infection; **2 P + Th**: Two neighboring plants with *T. hamatum* inoculation and without pathogenic infection; **2 P + Ss(inp)**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before (**2 P + Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Ss**: Two neighboring plants without *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before; **2 P + Th+Ss(inp):** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, with neighbor plant infected 72 h before (**2 P + Th+Ss(inp)np:** It refers specifically to the neighboring plant infected 72 h before its neighbor); **2 P + Th+Ss:** Two neighboring plants with *T. hamatum* inoculation and foliar infected with *S. sclerotiorum*, without neighbor plant infected 72 h before (**2 P + Th-np+Ss:** It refers specifically to the neighboring plant infected 72 h before its neighbor).

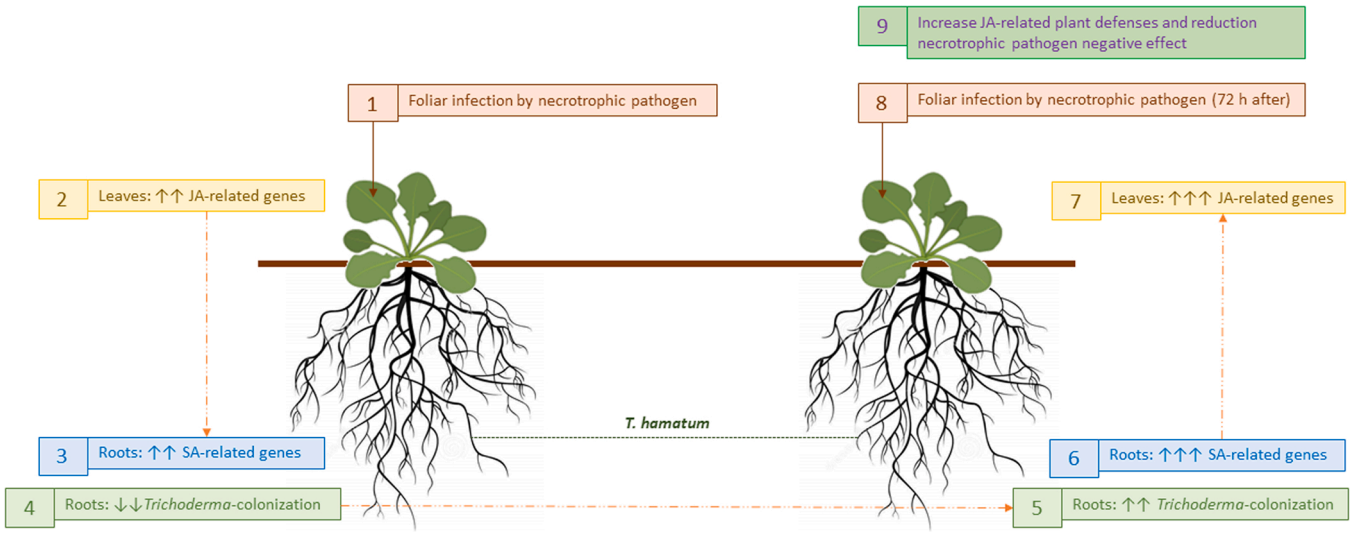


Fig. 11. Infographic summary the possible mechanism involved in the ability of *T. hamatum* to act as inter-plant communicator in foliar infections by necrotrophic pathogens. After foliar infection, the specific JA-mediated response against necrotrophic pathogens increases locally. This causes an antagonistic SA-mediated response in the roots, which reduces root colonization by *T. hamatum*. In response, the fungus massively colonizes the neighboring plant roots, inducing the activation of a local SA-mediated resistance. The defensive response activated in the roots induces the systemic activation of a JA-related antagonist response, specific against necrotrophic pathogens, which acts more quickly and effectively when the plant is infected, reducing the damaging effect of the necrotrophic pathogen.

in plants infected by the biotrophic pathogen.

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CRediT authorship contribution statement

JP conceived the study, carried out the experiments and wrote the initial manuscript. PV, JP and VMR discussed the results and performed the statistical analysis of the data. PV, VRM and RA performed the molecular analysis. All authors contributed to the article and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111664.

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