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Proceeding Paper

Induction of Immune Response in *Arabidopsis thaliana* Treated with Phytopathogen Filtrates †

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Abstract: We address the application of phytopathogen filtrates to induce an immune response on plants that may protect them from disease. We exposed *Arabidopsis thaliana* plants to filtrates of necrotrophic and biotrophic phytopathogens, and evaluated whether these triggered an inmmune response correspondent to each pathogen's infection pathway. We show filtrates induce a systemic immune response on plants, but this was not specific to the infection type of phytopathogens. When facing a real infection, however, filtrates enhanced the immune response compared to control plants. Moreover, filtrates increased plant growth by acting either as fertilizers or chemical inducers. Our study demonstrates the biotechnological potential of phytopathogen filtrates.

Keywords: Arabidopsis; filtrate; *Fusarium oxysporum;* immune response; phytopathogen; *Pectobacterium carotovorum; Pseudomonas syringae; Pythium irregular; Ralstonia solani; Sclerotinia sclerotiorum*

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1. Introduction

Phytopathogen infection on crops decrease the yield and quality of agricultural production, generating considerable economic losses and reducing food security worldwide [1–3]. Considerable efforts have been made to counteract phytopathogens with chemical compounds (i.e.; bactericides and fungicides), but, besides often being deleterious to ecosystems, these have the disadvantage of generating resistance in pathogens over time [4]. A valuable alternative may be making plants less susceptible to pathogens by an induced immune resistance [5,6]. Induced resistance consists of sensitizing the plant to activate its defense mechanisms by an elicitor agent, and preparing the plant for the pathogen arrival, infection, and colonization [7,8]. Pathogen filtrates may be able to activate the defense system in plants because they contain specific molecules, such as proteins, oligosaccharides, oligopeptides, toxins, and others, which are detected by receptors in the plant cuticule and trigger a microorganism recognition signature[4,8–12].

The induced resistance response to filtrates should be specific to the microorganism biology [13] and their interaction with the host [14]. For instance, biotrophic pathogens suppress the host immune system and derive nutrients from living cells, whereas necrotrophic pathogens secrete toxins to rapidly kill host tissues, and thrive on dead tissues. Hemibiotrophic pathogens combine both strategies of nutrient acquisition, starting with a biotrophic phase followed by a necrotrophic phase [15]. Plants can fight back biotrophic or necrotrophic pathogens through the balanced interaction between the phytohormones of the signaling pathways that mainly include salicylic acid (SA) against biotrophic pathogens and jasmonic acid (JA) against necrotrophic [13,16].

Here, we assessed the immune response of Arabidopsis thaliana to filtrates of biotrophic and necrotrophic bacteria, fungi, and oomycetes. Filtrates from necrotrophic and biotrophic pathogens should elicit the expression of a specific immune pathway. We, thus assesed whether necrotrophic and biotrophic prompted the expression of genes associated with the JA and SA pathways respectively. The induced resistance should enhance the defensive response of plants when facing a real infection. Thus, we infected plants with the necrotic fungus *Botrytis cinerea* and expected the expression of defense genes to be highest in plants exposed to filtrates from necrotrophic microorganisms. Finally, we assessed whether inducing a sustained immune response with filtrates has the trade-off of reducing plant growth and production.

2. Materials and Methods

We used wild-type Arabidopsis thaliana Columbia background (Col-0) plants obtained from the Arabidopsis Information Service (AIS) (https://www.arabidopsis.org, accessed on October 2019). Seeds of A. thaliana were surface sterilized and plated on MS medium (Murashige & Skoog, 1962), solidified with 1% (*w*/*v*) agar and supplemented with 1% sucrose (*w*/*v*). Seeds were saved for 7 days at 22 °C with a long-day photoperiod (16 h of light) and 40-50% of relative humidity. Seedlings were then transferred to a solid substrate of peat and vermiculite (3:1) and were kept in the greenhouse with 22 °C and 60% of relative humidity.

We used the phytopathogens *Pseudomonas syringae* pv tomato, *Pectobacterium carotovorum*, *Fusarium oxysporum* f.sp. *conglutinens*, *Sclerotinia sclerotiorum*, *Ralstonia solani*, and *Pythium irregulare* as sources of microorganism filtrates. Strains were provided by the Centro Regional de Diagnóstico de Alderrubia (Junta de Castilla y León, Spain). Bacteria (*P. syringae* and *P. carotovorum*) were grown on solid LB (Luria-Bertani, Sambrook et al.; 1989) medium at 28 °C, while fungi (*F. oxysporum* f. sp. *conglutinens*, *S. sclerotiorum*, and *R. solani*) and oomycete (*P. irregulare*) were grown on potato dextrose agar (PDA) medium at 25 °C. After 7 days, cultures were diluted in 5 mL of distilled-sterile water to obtain a suspension with an optical density between 0.15 and 0.19, except for *F. oxysporum* with which we used and 2.3 × 10^3 spores/mL suspension. Bacteria suspensions were inoculated in were cultivated in LB liquid medium and fungi and the oomycete were cultivated in Potato Dextrose Broth (PDB) medium and cultured with orbital shaking at 180 rpm and 28 °C for 48 h. Mediums were then filtrated through 0.22 μm Milipore filters, sealed, and stored at −20 °C.

We used the necrotrophic fungus *Botrytis cinerea* B05.10 strain as an infection agent provided by the Phytopathology and Biological Control Group of the Instituto Hispano Luso de Investigaciones Agrarias (CIALE), Spain. *B. cinerea* was grown in potato dextrose agar (PDA) medium at 25 °C for 7 days, after which culture was diluted in 5 mL of distilled-sterile water to obtain a suspension with 2x10^7 spores/mL.

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We evaluated the effect of filtrates from six phytopathogens on the defense gene expression, defense gene expression under a *B. cinerea* infection, plant growth, seed production. We applied 400 μL of each phytophatogen filtrate on the substrate of to 30~2 cm-long *A. thaliana* plants 0.5 cm from the stem (30 plants × 6 filtrate types). We applied distilled water to another 30 plants, which served as controls. Ten days after filtrate application, we collected the roots and leaves of nine plants of each filtrate treatment and stored at −80 °C in liquid nitrogen. At the same time, six plants in each filtrate treatment were infected with *B. cinerea*. We applied 5 uL of *B. cinerea* spore suspension on three leaves of each plant and sealed inside a plastic box for 15 days in a growth chamber 22 °C, 40% RH and a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1}. Infected leaves were collected and stored at −80 °C in liquid nitrogen.

We assessed the induced immune response by estimating the expression of genes associated with the JA and SA signaling pathways. To do this, we extracted total RNA from stored leaf samples using the Trizol method (Thermo Fisher Scientifics, Waltham, MS, USA) and following the commercial protocol. We used PrimeScriptTM RT reagent kit to synthesize complementary DNA from RNA. We used real-time PCR using a StepOnePlus Applied Biosystems equipment with the KAPA SYBR® FAST qPCR Master Mix Kit (2X) ABI Prism and primers to amplify *ICS1* and *PR1* genes associated with SA pathway, *LOX1* and *VSP2* associated with JA pathway, and Actin endogenous gene to assess a baseline genetic expression (Table S1). We applied the PCR program as in Poveda The resulting threshold cycle values (Ct) of gene amplification were analyzed using the delta-delta Ct method to assess expression of SA and JA pathways' genes relative to the expression of the endogenous gene and relative to the control treatment [17].

Seventy days after filtrate application, we assessed the effects of filtrates on plant growth on seven plants per treatment. To do this 2019, removed plants from the substrate and cleaned the roots. We cut separate roots from leaves and measured their dry weight after being placed in an oven at 65 °C for 48 h. Finally, we waited until the eight remaining plants per treatment fructified (100 days after sowing), and we counted siliques to assess the effects of filtrates on plant yield. We compared the root weight, aerial weight, and silique number between filtrate treatments using three general linear models.

All statistical analyzes were carried out in the R software [18]. The packages ggplot2 [19], ggpubr [20] and lemon [21] were used for the design of figures.

3. Results

Phytopathogen filtrates induced the expression of defense genes in Arabidopsis thaliana up to ten times more than in control plants (Figure 1). However, not all phytopathogen filtrates enhanced gene expression to the same degree in roots and leaves. Most gene induction in leaves concentrated on *LOX1* (JA pathway) in plants exposed to filtrates of *Pectobacterium caratovorum*, *Fusarium oxysporum*, and *Pithyum irregulare* (Figure 1). In roots, most gene induction concentrated on *ICS1* (SA pathway) by *P. irregulare*, *Pseudomonas syringae*, and *F. oxysporum* (Figure 1). *A. thaliana* leaves infected with *Botrytis cinerea* mostly induced the gene expression of either *PR1* (SA pathway) or *VSP2* (JA pathway), whereas the expression of *ICS1* and *LOX1* genes was minimal. However, plants expressed either *PR1* or *VSP2*, but never both to the same degree (Figure 1).

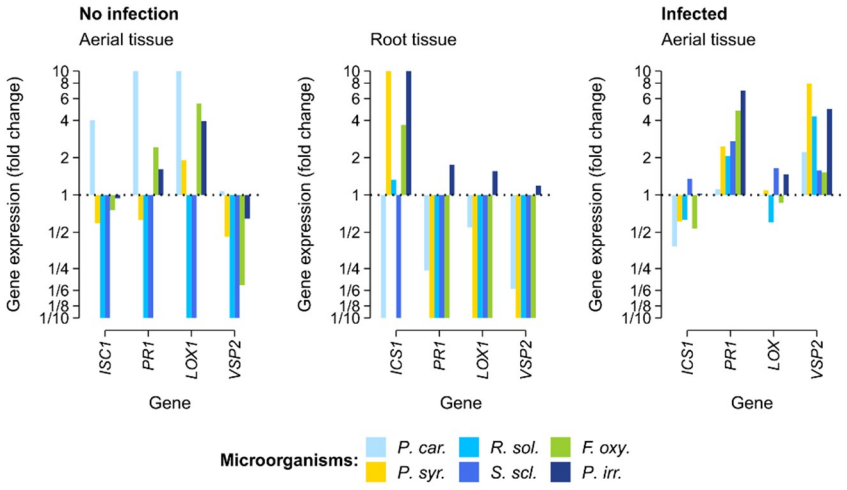

Figure 1. Expression of *Arabidopsis thaliana* defense genes in leaf and root tissue after exposure to phytopathogen filtrates (left and center), and after a subsequent infection with *Botrytis cinerea* (right). Gene expression is relative to endogenous gene expression and relative to control plants

using delta-delta Ct units log_{10} transformed representing proportional change. Microorganisms correspond as follows: P. car.: *Pectobacterium carotovorum*, P. syr.: *Pseudomonas syringae*, R. sol.: *Ralstonia solanacearum*, S. Scl.: *Sclerotinia sclerotiorum*, F. oxy.: *Fusarium oxysporum*, and P. irr.: *Pythium irregulare*.

Plants exposed to the filtrate treatments exhibited greater radicular and aerial biomass compared to control plants. However, plants exposed to *Sclerotinia sclerotiorum* filtrate were not different than control plants (Table S2, Figure 2). Regarding fruit production, there were no differences between plants treated with filtrates and control plants (Table S2, Figure 2).

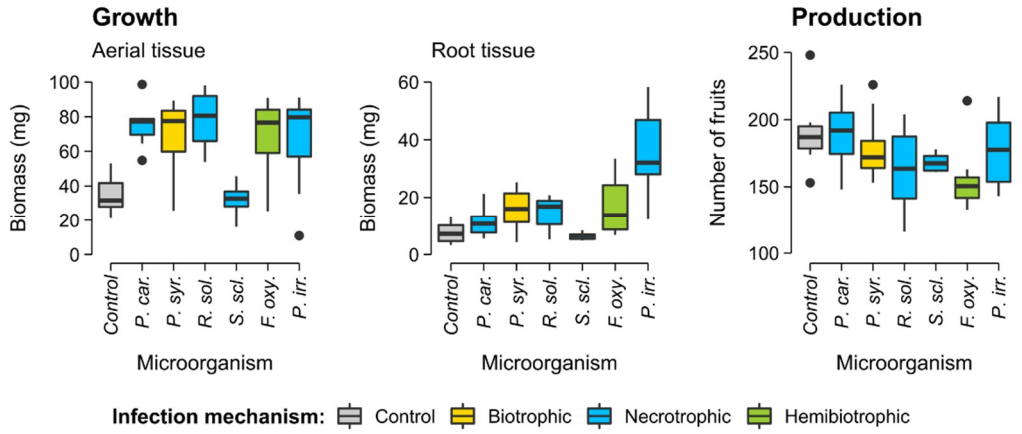

Figure 2. Biomass and silique production of *Arabidopsis thaliana* plants exposed to phytopathogens filtrates. Microorganisms correspond as follows: P. car.: *Pectobacterium carotovorum*, P. syr.: *Pseudomonas syringae*, R. sol.: *Ralstonia solanacearum*, S. Scl.: *Sclerotinia sclerotiorum*, F. oxy.: *Fusarium oxysporum*, and P. irr.: *Pythium irregulare*.

4. Discussion

We show that the inoculation of *Arabidopsis thaliana* rhizosphere with phytopathogen filtrates induced the gene expression of the SA and JA pathways, in roots and aerial tissue, suggesting the activation of systemic response. This demonstrates that the filtrates contain chemical compounds from the pathogens that the plants recognize, despite those living microorganisms are not present. Pathogen-derived elicitors trigger plant immune response by activating a signal cascade, and are now used to study the molecular mechanism of defense responses [12,22]. Moreover, the systemic response on plants exposed to filtrates demonstrate the signaling cascade stimulated by pathogen-derived elicitors is communicated throughout the plant during an infection. Local immune responses induce mobile signals that reach towards distal tissues to initiate a secondary immune response [23], conferring an enhanced resistance against subsequent infections, which has been referred to as systemic acquired resistance (SAR) [24].

We found, however, filtrates from necrotrophic and biotrophic did not induced an exclusive expression of JA and SA pathways respectively, suggesting plant induced immune response was not specific. This may result from phytopathogens being able to trigger SA and JA pathways as adaptive mechanisms to 'trick' plants into committing to a defense pathway not effective against the pathogen [25]. Filtrates likely contain a plethora of pathogen signals to trigger either JA and SA pathways causing a non-specific immune response [26]. Alternatively, but not exclusively, a non-specific immune response may result from plants being able to express both SA and JA pathways simultaneously in response to an infection that has not been fully identified [26].

In plants infected with *Botrytis cinerea*, we show that filtrates enhanced the immune response compared to control plants. Interestingly, *B. cinerea* triggered the expression of *PR1* and *VSP2* genes, which are associated with the SA and JA pathways respectively.

This may be associated with *B. cinerea* ability to 'trick' the plant to activate the SA pathway despite being a necrotrophic pathogen instead [27,28]. The immune response induced by filtrates was also more specific and directed to the infection pathway of the microorganism used for obtaining the filtrate. The necrotrophic phytopathogens *P. caraotovorum*, and *R. solani* induced the expression of *VSP2* –a precursor to the JA pathway. Interestingly, *P. syringae* also induced the expression of *VSP2* despite being a biotrophic phytopathogen. This likely results from its infection mechanism that secretes coronatine, which 'tricks' the host-plant into committing to a JA defense response instead of the corresponding SA response [29]. Filtrates induced the expression of either *PR1* or *VSP2*, but not both to the same degree. This evidences the crosstalk regulation between SA and JA pathways, where the expression of either pathway suppresses the expression of the other [26,30]. Thus, while the application of filtrates may enhance the immune response towards a real infection, the specificity of the response needs to be tailored to a target disease.

The application of filtrates did not influence fruit production, but did increase plant growth compared to the control. This suggests that the filtrates also contained molecules that the plants could use to promote their development. The increase growth may result from a "cocktail" of various organic molecules that may act either as fertilized or chemical inducers [31]. Free-living microbes including filamentous fungi and a variety of plant growth-promoting rhizobacteria (PGPR) are able to stimulate plant growth by different direct or indirect mechanisms, such as the production of phytohormones, decomposition, mineralization of organic material, and enhancing the bioavailability of mineral nutrients [32,33]. Further, these microorganisms may also contribute to plant immunity by producing elicitor molecules [34]. In addition, it can be shown that both growth and defense response occurred at a systemic level in plants.

Conclusions

Our study demonstrated that pathogen' filtrates contain chemical signals that can trigger a systemic immune response in plants. This response, however, was not specific to the infection mechanism of the filtrate source phytopathogen. Still, filtrates did bolster plants' immune response when facing a real infection as long as the pathogen filtrate triggers the same defense pathway as that of the infection. Moreover, filtrates can also produce plant growth increase by acting either as fertilizer or by fostering growth inducing signals, and do not appear to cause a trade-off between growth and an immune response. Thus, our study provides evidence that phytopathogen filtrates may be tailored to enhance the immune response of plants with specific defense pathways against real infections. The specific responses that filtrates may trigger on plants (e.g.; activation of enzymes, crosstalk of phytohormones pathways, among others) may hold great agrobiotechnological potential.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Oligonucleotides used in gene expression analysis in Arabidopsis thaliana, Table S2: Statistical estimates of general linear models comparing the root dry-weight, aerial dry-weight, and silique production of Arabidopsis thaliana plants exposed to different phytopathogen filtrates. Estimate values reflect means for each filtrate treatment compared to the control treatment.

Author Contributions: Ávila A. C. conducted experiments, recorded data, performed analyses and wrote the manuscript. Poveda J. designed the experiments and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are openly available in Mendeley Data at doi:1017632/y3b226n5v3.1.

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

Supplementary material

Table S1. Oligonucleotides used in gene expression analysis in *Arabidopsis thaliana*.

Gene	Application	Sequences (5′-3′)
ICS1	SA synthesis	GATCTAGCTAACGAGAACGG
ICS1	SA synthesis	CATTAAACTCAACCTGAGGGAC
PR1	SA response	CAAAGTGAGGTGTAACAATGGTGGA
PR1	SA response	ATGGCTTCTCGTTCACATAATTCCC
LOX1	JA synthesis	TCAACGATTTCAATGCTTCGTTTCT
LOX1	JA synthesis	TCAGAGCTTACAAGACGAAGAGTG
VSP2	JA response	GTTAGGGACCGGAGCATCAA
VSP2	JA response	TCAATCCCGAGCTCTATGATGTT
Actin	Endogenous gene	CTCCCGCTATGTATGTCGCC
Actin	Endogenous gene	TTGGCACAGTGTGAGACACAC

Table S2. Statistical estimates of general linear models comparing the root dry-weight, aerial dry-weight, and silique production of Arabidopsis thaliana plants exposed to different phytopathogen filtrates. Estimate values reflect means for each filtrate treatment compared to the control treatment.

Response Variable	Fixed Factor	Estimate ± S.E.	F	*p* Value
Root dry-weight (log10 transformed mg)	Phytopathogen filtrate:		5.79	<0.01
	F. oxysporum	0.41 ± 0.11		
	P. carotovorum	0.15 ± 0.11		
	P. irregulare	0.46 ± 0.11		
	P. syringae	0.23 ± 0.11		
	R. solani	0.32 ± 0.11		
	S. sclerotiorum	−0.01 ± 0.11		
Aerial dry-weight	Phytopathogen filtrate:		8.91	<0.01
	F. oxysporum	0.03 ± 0.01		
	P. carotovorum	0.03 ± 0.01		
	P. irregulare	0.02 ± 0.01		
	P. syringae	0.02 ± 0.01		
	R. solani	0.03 ± 0.01		
	S. sclerotiorum	0.01 ± 0.01		
Number of siliques	Phytopathogen filtrate:		2.15	<0.06
	F. oxysporum	-34.2 ± 12.7		
	P. carotovorum	0.5 ± 12.7		
	P. irregulare	-13.2 ± 12.7		
	P. syringae	-10.6 ± 12.7		
	R. solani	-26.6 ± 12.7		
	S. sclerotiorum	-22.1 ± 12.7		

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