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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 immune response correspondent to each pathogen's infection pathway. We show that 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, the filtrates enhanced the immune response compared to the control plants. Moreover, the filtrates increased plant growth by acting either as fertilizers or chemical inducers. Our study demonstrates the biotechnological potential of phytopathogen filtrates.

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

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1. Introduction

Phytopathogen infection on crops decreases 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). However, besides often being deleterious to ecosystems, chemicals have the disadvantage of inducing pathogen resistance 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]. The pathogen filtrates may be able to activate the defense system in plants because they contain specific molecules, such as proteins, oligosaccharides, oligopeptides, and toxins,, which are detected by receptors in the plant cuticle and trigger a microorganism recognition signature [4,8–12].

The induced resistance response to filtrates should be specific to the microorganism's biology [13] and their interaction with the host [14]. For instance, biotrophic pathogens suppress the host's immune system and derive nutrients from living cells, whereas necrotrophic pathogens secrete toxins in order to rapidly kill the host's 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 pathogens [13,16].

Here, we assessed the immune response of *Arabidopsis thaliana* to filtrates of biotrophic, hemibiotrophic, and necrotrophic phytopathogens. These filtrates elicit the expression of a specific immune pathway. We thus assessed whether the biotrophic, hemibiotrophic, and necrotrophic pathogens 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 the plants that were exposed to the filtrates from necrotrophic microorganisms. Finally, we assessed whether inducing a sustained immune response with filtrates had the trade-off of reducing plant growth and production.

2. Materials and Methods

We used wild-type *A. thaliana* Columbia background (Col-0) plants obtained from the *Arabidopsis* Information Service (AIS) (https://www.arabidopsis.org, accessed on 31 October 2019). Seeds of *A. thaliana* were surface sterilized and plated on Murashige and Skoog (MS) medium (Sigma-Aldrich, Darmstadt, Germany) [17], solidified with 1% agar (Chem-Lab, Zedelgem, BE) (*w*/*v*), and supplemented with 1% sucrose (Sigma-Aldrich, Darmstadt, Germany) (*w*/*v*). Seeds were incubated for 7 days at 22 °C with a long-day photoperiod (16 h of light) and 40–50% relative humidity (RH). Seedlings were then transferred to a solid substrate of peat and vermiculite (3:1) and were kept in the greenhouse at 22 °C and 60% RH.

We used bacteria, fungi, and an oomycete as sources of microorganism filtrates. *Pectobaterium carotovorum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Phytium irregulare* were the necrotrophic phytopathogens. Whereas the species *Pseudomonas syringae* pv. *Tomato* and *Fusarium oxysporum* f.sp. *conglutinans* were the biotrophic and hemibiotrophic phytopathogens, respectively. Microorganisms were wild strains isolated from infected crops by the Centro Regional de Diagnóstico de Aldearrubia (Junta de Castilla y León, Salamanca, Spain). The bacteria (*P. syringae* and *P. carotovorum*) were grown on solid Luria-Bertani (LB) medium (Sigma-Aldrich, Darmstadt, Germany) [18] at 28 °C, while fungi (*F. oxysporum* f. sp. *conglutinans*, *S. sclerotiorum*, and *R. solani*) and oomycete (*P. irregulare*) were grown on potato dextrose agar (PDA) medium (Oxoid, Hampshire, UK) at 25 °C. After 7 days, the cultures were diluted in 5 mL of sterile distilled water to obtain a suspension with an optical density between 0.15 and 0.19, except for *F. oxysporum*, with which we used a 2.3×10^3 spores mL^{-1} suspension. Bacteria suspensions were cultivated in LB liquid medium and fungi and the oomycete were cultivated in potato dextrose broth (PDB) medium (Neogen, Scotland, UK). Both cultures were incubated with orbital shaking at 180 rpm, and at 28 °C for 48 h. Mediums were then filtrated through 0.22 µm Millipore 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 PDA medium (Oxoid, Hampshire, UK) at 25 °C for 7 days, after which the culture was diluted in 5 mL of sterile distilled water to obtain a suspension with 2×10^7 spores mL^{-1}.

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We evaluated the effect of filtrates from six phytopathogens on the defense gene expression before and after an infection with *B. cinerea*, as well as plant growth and production. We applied 400 µL of each phytopathogen filtrate on the substrate of 30 *A. thaliana* plants (~2 cm-long), not further than 0.5 cm from the stem (30 plants × 6 filtrate types). We applied distilled water to another 30 plants, which served as controls.

We assessed the induced immune response by estimating the expression of genes associated with the JA and SA signaling pathways. We collected leaves and roots of nine plants of each filtrate treatment ten days after filtrate application and stored each tissue separately at −80 °C in liquid nitrogen. At the same time, six plants in each filtrate treatment were infected with *B. cinerea*. We applied 5 µL of *B. cinerea* spore suspension on

three leaves of each plant and sealed them inside of a plastic box for 15 days in a growth chamber at 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 (n = 6 × 7). We pooled stored tissue samples of each filtrate and infection treatment (nine leaf/root samples in non-infected treatments, and 6 samples in infected treatments) to maximize the amount of genetic material analyzed. We extracted total RNA from the leaf and root samples using the Trizol method (Thermo Fisher Scientifics, Waltham, MS, USA) following the commercial protocol. We used PrimeScriptTM RT reagent kit (Takara, Göteborg, SWE) 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 (KAPABiosystems, Cape Town, South Africa) and primers to amplify *ICS1* and *PR1* genes associated with the SA pathway, and *LOX1* and *VSP2* associated with the JA pathway, and Actin endogenous gene to assess a baseline genetic expression (Table A1). We applied the PCR program as described in Poveda, 2018 [19]. The resulting threshold cycle values (Ct) of gene amplification were analyzed using the delta–delta Ct method to assess the expression of SA and JA pathway genes relative to the expression of the endogenous gene and relative to the control treatment [20]. We calculated the averages of three Ct value calculations, resulting in one unique value of gene expression per gene, tissue (leaves or roots), and filtrate/infection treatment.

We also evaluated the effect of filtrates on plant growth and seed production. Seventy days after filtrate application, we assessed the effects of the filtrates on plant growth on seven plants per treatment (n = 7 plants × 7 treatments = 49 aerial/root growth measurements). To do this, we removed the plants from the substrate and cleaned the roots. We cut separate roots from aerial tissue and measured their dry weight after being placed in an oven at 65 °C for 48 h. Finally, we waited until the eight plants per treatment fructified (100 days after sowing), and we counted the siliques to assess the effects of the filtrates on plant yield (n = 8 plants × 7 treatments = 56 production measurements). We compared the root weight, aerial weight, and silique number between the filtrate treatments using three general linear models.

All statistical analyzes were carried out in R software [21]. The packages ggplot2 [22], ggpubr [23], and lemon [24] were used for the design of the figures.

3. Results

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

The plants that were exposed to the filtrate treatments exhibited greater aerial and radicular biomass compared to the control plants. However, the plants that were exposed to *S. sclerotiorum* filtrate were not different from the control plants (Table A2, Figure 2). Regarding fruit production, there were no differences between the plants that were treated with the filtrates and the control plants (Table A2, Figure 2).

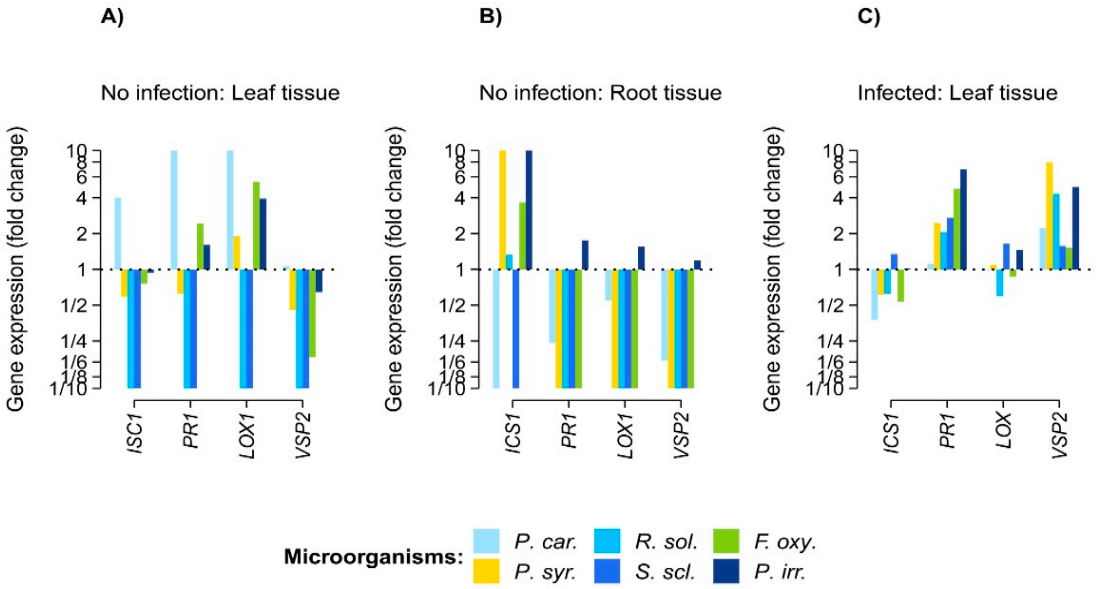

Figure 1. Effects of phytopathogen filtrates on the expression of *Arabidopsis thaliana* defense genes before (**A**,**B**) and after (**C**) infection by *Botrytis cinerea*. Gene expression is relative to endogenous gene expression and relative to control plants using delta–delta Ct units $\log_{10}$ transformed representing proportional change. This shows the necrotrophic phytopathogens in blue, hemibiotrophic in green, and biotrophic in yellow. Microorganisms correspond as follows: P. car.: *Pectobacterium carotovorum*, P. syr.: *Pseudomonas syringae*, R. sol.: *Rhizoctonia solani*, S. scl.: *Sclerotinia sclerotiorum*, F. oxy.: *Fusarium oxysporum*, and P. irr.: *Pythium irregulare*.

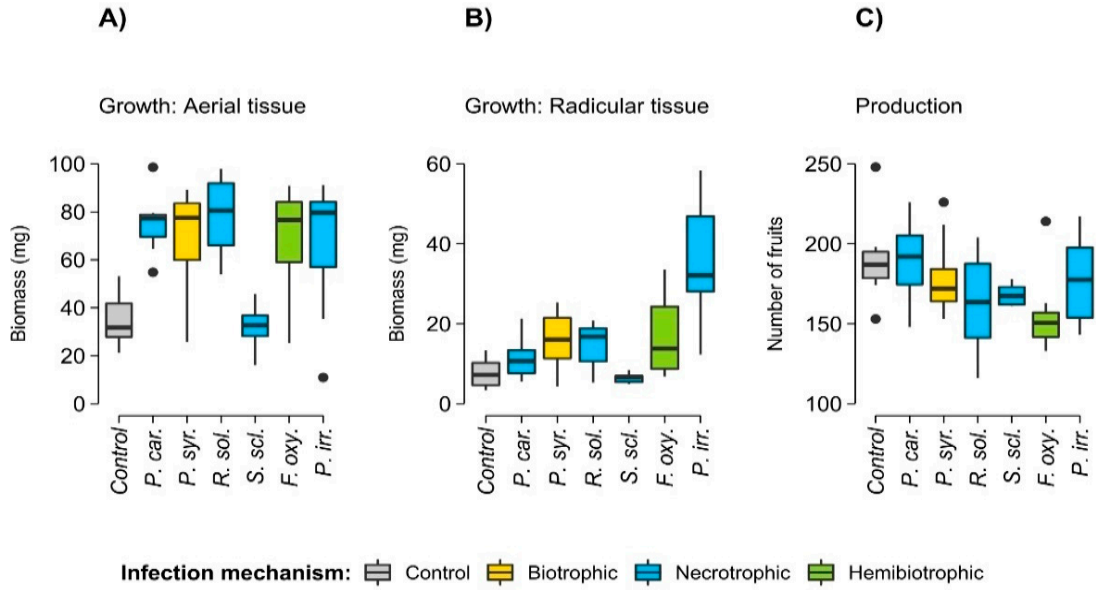

Figure 2. Effect of phytopathogen filtrates on the growth (**A**,**B**) and silique production (**C**) of *Arabidopsis thaliana* plants. Microorganisms correspond as follows: P. car.: *Pectobacterium carotovorum*, P. syr.: *Pseudomonas syringae*, R. sol.: *Rhizoctonia solani*, S. scl.: *Sclerotinia sclerotiorum*, F. oxy.: *Fusarium oxysporum*, and P. irr.: *Pythium irregulare*.

4. Discussion

We have shown that the inoculation of *A. thaliana* rhizosphere with phytopathogen filtrates induced the gene expression of the SA and JA pathways, in radicular and aerial tissue, suggesting the activation of a systemic defensive response [25]. This adds to the body of literature showing that the filtrates contain chemical compounds from the pathogens that

the plants recognize despite the absence of the living microorganisms [26,27]. Pathogen-derived elicitors can trigger a plant's immune response by activating a signaling cascade communicated throughout the plant [25]. Here, local immune responses induce mobile signals that reach towards the distal tissues in order to initiate a secondary immune response [28], conferring an enhanced resistance against subsequent infections, which has been referred to as systemic acquired resistance (SAR) [29]. For instance, we have shown that the filtrates enhanced the immune response against an infection from *B. cinerea* compared to control plants. Thus, triggering this process through chemical elicitors in microorganism filtrates may be used as a tool to prepare a plant's immune system against real disease, akin to a vaccination [30].

We have found, however, that filtrates from necrotrophic and biotrophic microorganisms did not induce an exclusive expression of JA and SA pathways respectively, suggesting a plant-induced immune response by microorganism filtrates was not specific. This may result from plants being able to express both SA and JA pathways simultaneously, as a preventive strategy against an infection, that has not been fully identified [31]. Alternatively, but not exclusively, a non-specific immune response may be a consequence of phytopathogens infection strategies, some of which 'trick' plants into committing to a defense pathway that is not effective against the pathogen [32]. For instance, *P. syringae* induced the expression of *VSP2* despite being a biotrophic phytopathogen, which may result 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 [33]. Filtrates likely contain an array of pathogen signals that may prompt a non-specific immune response [31], and it has been suggested that specific defense pathways can only be triggered through the complex interaction between the pathogen and its host [25]. Paradoxically, studying microorganism filtrates is a promising tool to understand the molecular mechanism of plant defensive responses [12,34,35], which may help developing microorganism-derived filtrates that are specifically tailored to fight a target disease.

The exposure to the phytopathogen filtrates enhanced plant growth, on top of inducing an immune response, despite a well-known trade-off between plant growth and the defensive response [36]. Free-living microbes, 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 [37,38]. For instance, the volatile components in phytopathogen filtrates have been shown to increase plant growth and fruit production in pepper plants [39]. However, we did not find an increase in production in our study, which may be caused by the specific signaling pathways of each phytopathogen and each plant [40,41].Thus, the mixture of components constituting phytopathogen filtrates may act simultaneously as immune response elicitors, as well as fertilizer [42].

5. Conclusions

Our study demonstrated that phytopathogen 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 phytopathogen from which the filtrate was extracted. Still, filtrates did bolster the plants' immune response when facing a real infection. Moreover, we observed that the filtrates stimulated plant growth without causing a trade-off with their immune response. Thus, our study provides evidence that phytopathogen filtrates may be used to enhance the immune response of plants against a real infection. Further research could focus on developing filtrates that are tailored to elicit specific defense pathways. The specific responses that filtrates may trigger in plants (e.g., activation of enzymes and crosstalk of phytohormones pathways, among others) may hold great agrobiotechnological potential.

Supplementary Materials: The video presentation can be downloaded at the following address: https://www.mdpi.com/article/10.3390/IECPS2021-11974/s1.

Author Contributions: A.C.Á. conducted experiments, recorded data, performed analyses, and wrote the manuscript. J.P. 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 the following doi:1017632/y3b226n5v3.1.

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

Appendix A

Table A1. Oligonucleotides used in gene expression analysis in *A. 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 A2. Statistical estimates of general linear models comparing the root dry-weight, aerial dry-weight, and silique production of *A. 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) (n = 49)	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		

Table A2. *Cont.*

Response Variable	Fixed Factor	Estimate ± S.E.	F	*p* Value
Aerial dry-weight (n = 49)	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 (n = 56)	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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