1. Experimental procedures

1.1. Induced systemic resistance bioassays in *Arabidopsis thaliana*

1.2. Method used in: "Bacterial elicitors and plant signaling in induced systemic resistance"

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1.3. Equipment and materials

A. Equipment

Equipment	Type	Producer
Autoclave	Omega bench top	Prestige medical
Centrifuge	RC5C plus	Sorval
Freezer (-80 °C)	Polar 550H	Angelantoni
Growth chamber	Walk in	Weiss
Greenhouse (air-conditioned)	Large walk in units	
Haemocytometer	Bürker	Optik Labor
Microscope	Standard 25	Zeiss
Spectrophotometer	DU-64	Beckman

B. Chemicals

Product name	Product number	Supplier
Chemicals		
Agar granulated	214530	Bacto
Bentonite	B-3378	Sigma
$Ca(NO_3)_2$	C-1396	Sigma
Glycerol	13,487-2	Aldrich
Glycine	G-88898	Sigma
K_2HPO_4	1.05104	Merck
KH_2PO_4	1.04873	Merck
KNO_3	5061	Merck
Lactic acid	1.00366	Merck
$MgSO_4$	1.05886	Merck
Phenol	8.22296	Merck
Potato Dextrose Agar (PDA)	213400	Difco

Proteose peptone	211693	Bacto
Silwet L-77		Van Meeuwen Chemicals
Trypan-blue	302643	Aldrich
Tween 20	822184	Merck

Plant materials

Different accessions of *Arabidopsis thaliana* (L.) Heynh. including Columbia (Col-0) and Dijon (Di-0), were used in this study. Seeds were stored in the dark at 4 °C.

Plant Growth-Promoting Rhizobacteria (PGPR)

- *Pseudomonas putida* WCS358r
- *P. fluorescens* WCS374r
- *P. fluorescens* WCS417r (Geels and Schippers, 1983; Lamers *et al.*, 1988)

Pathogenic bacteria

- *Pseudomonas syringae* pv. *tomato* DC3000 (Whalen *et al.*, 1991)
- *Xanthomonas campestris* pv. *armoraciae* (Sahin and Miller, 1996)

Fungal/oomycete pathogens

- *Hyaloperonospora parasitica* strain WACO9,
- *Alternaria brassicicola*
- *Botrytis cinerea*

Pathogenic viruses

- Turnip crinkle virus (TCV)

Solutions

Half-strength, modified Hoagland solution: 5mM $Ca(NO_3)_2$, 2mM KNO_3, 1mM KH_2PO_4, 1mM $MgSO_4$, trace elements, 10 µM Sequestreen, pH =7
King's medium B (KB): Per liter distilled water, 20 g of proteose peptone, 10 g glycerol, 1.5 g $MgSO_4$, 1.2 g KH_2PO_4, 13 g agar
Lactophenol trypan-blue: 10 mL of lactic acid, 10 g of glycerol, 10 g of phenol, and 10 mg of trypan-blue dissolved in 10 mL distilled water.

1.4. Steps of the procedure

1.4.1. Growing *A. thaliana*

1. Autoclave river sand for 20 min, 121 °C.
2. Add 300 mL of half-strength, modified Hoagland solution to small plastic trays (300×200×50 mm, L×W×H) (Pieterse *et al.*, 1996) and fill up with the autoclaved river sand.
3. Sow seeds in the filled trays. Take care that the seeds are well spread out over the entire surface of the tray and not exceeding 300 seeds per tray (100 seeds weigh approximately 1.5 mg).

4. Place seeded trays in larger containers (450×300×80 mm, L×W×H), cover with a transparent plastic lid (450×300×80 mm, L×W×H), resulting in a close to 100 % relative humidity.
5. Grow seedlings in these closed containers in a greenhouse, or a plant growth chamber with a short day regime (8 hr light [24 °C] and 16 hr dark [20 °C]) for 14 days. Keep the lid of the container closed.
6. Autoclave a potting soil – river sand mixture (12:5 v/v) twice at 121 °C for 20 min with a 24-hr interval. Prepare pots containing approximately 60 cm^3 of the mixture.
7. After flooding the sand in the small plastic trays with tap water, gently remove seedlings from the river sand with tweezers and transfer into individual pots.
8. Cultivate plants with an 8-hr day (24 °C) and a 16-hr night (20 °C) cycle at a relative humidity of 70 % in a greenhouse or plant growth chambers. In the greenhouse the temperature can be maintained by air conditioning, allowing accurate setting of 24 °C even in the summer time. Complete darkness can be created during the 16 hr night period by covering the tables, on which the plants are grown, with an automatically controlled light-impermeable curtain. During dark daytime periods, the photoperiod of 8 hr can be guaranteed with artificial light (Philips HPI lamps). In the growth chambers the 8-hr photoperiod can be established by setting the duration of artificial light (Philips HPI, 200 $\mu E/m^2/sec$).
9. Use 20-25 plants for each treatment.

1.4.2. Induced Systemic Resistance (ISR) treatment

1. Grow the PGPR on King's medium B (KB) agar plates (King *et al.*, 1954) for 24 hr at 28 °C. Store all bacteria at -80 °C in 17 % glycerol in King's B medium.
2. Scrape the bacteria off the agar surface and suspend the cells in 10 mM $MgSO_4$.
3. Centrifuge the suspension at 8,000×*g* for 10 min. Resuspend the pellet in 10 mM $MgSO_4$. Measure the density of the bacterial suspension at 660 nm and adjust to 10^9 colony forming units (CFU) per mL. For the *Pseudomonas* spp. strains used, OD_{660} =1 equals 10^9 CFU/ mL.
4. Mix 50 mL bacterial suspension thoroughly with 1 kg of potting soil – river sand mixture This should result in a final density of 5×10^7 CFU per gram of soil.
5. Prepare control treatment for ISR by mixing 50 mL sterile 10 mM $MgSO_4$ through the soil.
6. Transfer seedlings into the bacteria-treated soil and grow for 17 days before challenge inoculation.

1.4.3. Systemic Acquired Resistance (SAR) treatment

1. Culture *P. syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2* (Kunkel *et al.*, 1993) overnight at 28 °C in liquid KB medium. Add kanamycin 25 mg/L to the medium for selection of the avirulent strain. For the virulent bacterial pathogens, use rifampicin 50 mg/L.
2. Collect bacterial cells by centrifugation at 8,000×*g* for 10 min.
3. Resuspend the pellet in 10 mM $MgSO_4$ and measure at 660 nm, adjust to a density of 10^7 CFU/mL.

4. Induce SAR 3 days before the challenge inoculation in plants grown in the autoclaved potting soil-river sand mixture for 14 days. Pressure infiltrate the bacterial suspension into three lower leaves of each plant by using a 1-mL syringe without a needle, as described by Swanson *et al.* (1988). Briefly, the leaves are gently turned and while the upper surface is pressed against the index finger, the suspension is infiltrated by firmly placing the syringe against the lower surface while gently pressing the plunger.

1.4.4. Challenge inoculation and scoring of disease

X. campestris pv. armoraciae* or *Pseudomonas syringae* pv. *tomato

1. Culture the bacterial pathogen overnight at 28 °C in liquid KB medium.
2. Collect the bacterial cells by centrifugation and resuspend in 10 mM $MgSO_4$, containing 0.015 % (v/v) Silwet L-77, to a final density of $2.5\text{-}5\times10^7$ CFU/mL.
3. Inoculate the *Arabidopsis* plants by carefully dipping the leaves into the bacterial suspension. Turn the pot upside down and dip all the leaves in the suspension while avoiding contact of the suspension with the soil.
4. After 30 min, collect and weigh replicate leaf samples from 5 plants per treatment. After briefly rinsing in sterile water, homogenize leaves in 10 mM $MgSO_4$.
5. Determine percentage of leaves with symptoms for each plant after 3-4 days. Count leaves with necrotic or water-soaked lesions surrounded by chlorosis as diseased ones. Collect another set of leaf samples from 5 plants per treatment, weigh, rinse in water and homogenize in 10 mM $MgSO_4$.
6. Plate serial dilutions of the $MgSO_4$ suspensions on KB agar plates containing 100 mg/L cycloheximide and 50 mg/L rifampicine. Incubate the plates for 2 days at 28 °C. Determine the number of CFU/g leaf tissue and calculate proliferation over the 3 day time interval.

Hyaloperonospora parasitica

1. The obligate pathogen *H. parasitica* WACO9 can be maintained on susceptible *Arabidopsis* plants according to Koch and Slusarenko (1990).
2. Wash sporulating *Arabidopsis* leaves in 10 mM $MgSO_4$, and collect the sporangia by centrifugation.
3. Resuspend the sporangia in 10 mM $MgSO_4$ to a final density of 5×10^4/mL as described by Ton *et al.* (2002).
4. Spray the shoots with the sporangial suspension.
5. Keep the inoculated plants at 17 °C and 100 % relative humidity.
6. Score disease symptoms 7 days after inoculation. Disease rating is expressed as the intensity of disease symptoms and pathogen sporulation on each leaf:

I	No sporulation
II	Trailing necrosis
III	<50 % of the leaf area covered by sporangiophores
IV	>50 % of the leaf covered with sporangiophores, with additional chlorosis and leaf collapse

7. Wash leaves in 10 mM $MgSO_4$ and collect conidiospores by centrifugation (1,500×*g*, 2 min), resuspend in 10 mM $MgSO_4$. Determine the number of conidiospores microscopically in a haemocytometer.
8. To determine leaf colonization, stain infected leaves with lactophenol trypan-blue and examine microscopically at 9 days after inoculation, as described by Koch and Slusarenko (1990).

Alternaria brassicicola* and *Botrytis cinerea

1. Grow *A. brassicicola* strain MUCL 20297 on Potato Dextrose Agar (PDA) for two weeks at 22 °C (Broekaert *et al.*, 1990; Ton *et al.*, 2002). Alternatively, grow *B. cinerea* B0510 on half strength PDA for two weeks at 22 °C under TL-light. Store the fungal isolates at -80 °C in 50 % glycerol.
2. Harvest the *A. brassicicola* conidia as described by Broekaert *et al.* (1990), by flooding plate cultures with sterile water and rubbing with a sterile spatula. The spore suspensions were filtered through glass wool, and washed twice with water by centrifugation (4000×g for 15 min). For *B. cinerea*, scrape the conidia from the plate and suspended in half-strength potato dextrose broth (½ PDB).
3. The wild-type *Arabidopsis* Col-0 plants can be challenge inoculated with *B. cinerea* but not with *A. brassicicola*, as they are resistant. The phytoalexin-deficient mutant *pad3-1* is susceptible (Thomma *et al.*, 1999) and should be used instead. Grow the *Arabidopsis* plants (n=20 per experiment) for five weeks, as described under 1.4.1.
4. Apply 3-µL droplets of suspension containing conidia of *A. brassicicola* in 10 mM $MgSO_4$ at density of $5x10^5$/mL on the second, third and fourth pair of true leaves. Apply *B. cinerea* similarly in 3-µL droplets of ½ PDB containing 5.10^5 conidia/mL to eight well-developed leaves, as described by Thomma *et al.* (1998).
5. Keep the inoculated plants at 100 % relative humidity (place them in the larger plastic containers (450×300×80 mm, L×W×H) and tightly cover with the transparent plastic lids (450×300×80 mm, L×W×H). Record disease symptoms 3 to 7 days after inoculation. Express the disease ratings on the basis of extent of symptoms and lesion size:

	A. brassicicola	***Botrytis cinerea***
I	No visible disease symptoms	No visible disease symptoms
II	Non-spreading lesion with a diameter <1.5 mm	Non-spreading lesions and spreading lesions less than 50 % of the leaf surface
III	Spreading lesion with a diameter between 1.5 and 7 mm	Extended lesions equal or between 50 % to 75 % of the leaf surface
IV	Spreading lesion >7 mm with extensive tissue maceration and sporulation by the pathogen.	Spreading lesions with extensive tissue maceration

For *A. brassicicola*, isolate and count the newly formed spores as described by Thomma *et al.* (1999). Take batches of 15 leaves from five plants and place them in 9 ml of 0.1 % (v/v) Tween 20 in a test tube. After vigorous shaking, remove the leaves and centrifuge the spore suspension at 3 200×*g* for 15 min. Resuspend the spores in 100 µL of 0.1 % (v/v) Tween 20 and count in a haemocytometer.

Turnip crinkle virus (TCV)

1. Bioassays with TCV should be performed as described by Ton *et al.* (2002). The TCV inoculum can be produced by *in vitro* transcription from plasmid pT7TCV66 according to Oh *et al.* (1995).
2. The inoculum should be adjusted to a concentration of 0.1 mg RNA/mL.
3. TCV is avirulent on Di-0, leading to a necrotic lesion (Dempsey *et al.*, 1997; Simon *et al.*, 1992). Inoculate Di-0 plants (n =20) by applying 3-µL droplets of the viral inoculum in 0.05 M glycine, 0.03 M K_2HPO_4, and 0.02 g/mL bentonite on three leaves per plant.
4. Rub the droplets across the leaf surface with a glass rod and mark the inoculated leaves.
5. After four days, record the number and diameter of the lesions under a dissection microscope.

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