

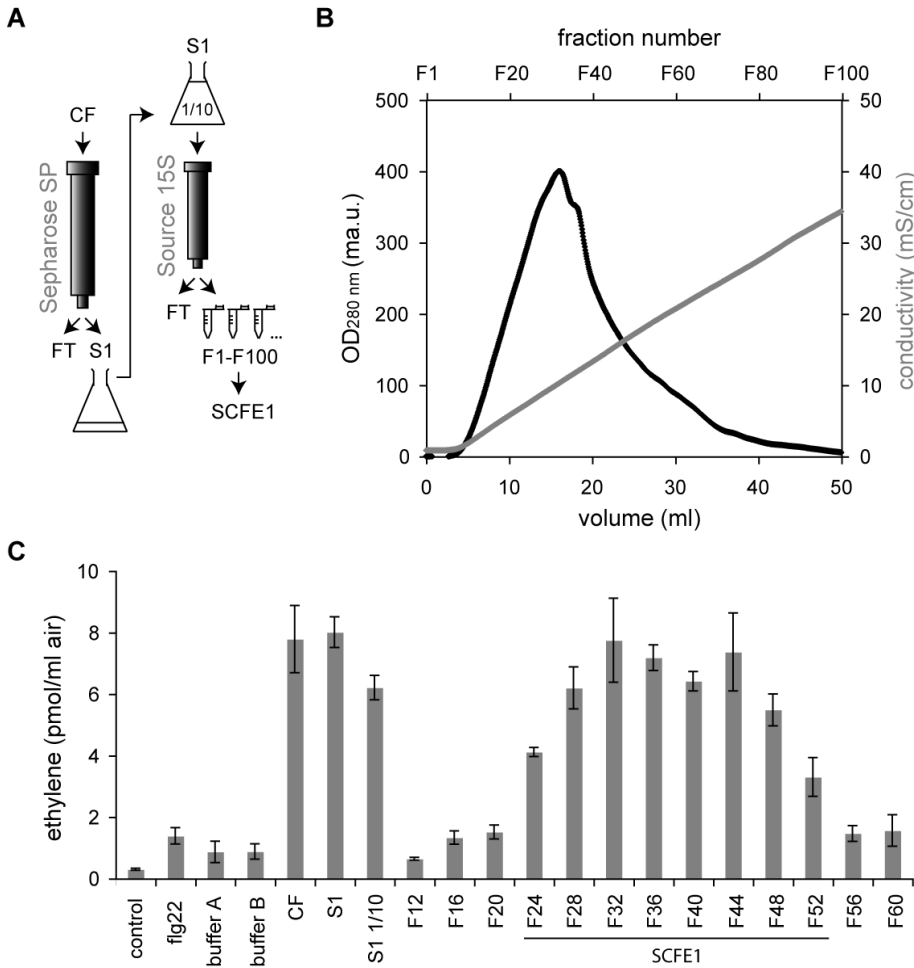
**Axenic Culture of *Sclerotinia sclerotiorum* and Preparation of *Sclerotinia* Culture Filtrate Elicitor 1 (SCFE1)-containing Fractions, Triggering Immune Responses in *Arabidopsis thaliana***

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**[Abstract]** The necrotrophic white mold fungus *Sclerotinia sclerotiorum* (*S. sclerotiorum*) is pathogenic to a broad range of plant species, including the Brassicaceae model plant *Arabidopsis thaliana* (Boland and Hall, 1994; Bolton *et al.*, 2006). In *Arabidopsis thaliana* (*A. thaliana*), the semi-purified proteinaceous *S. sclerotiorum* elicitor SCFE1 (*Sclerotinia* culture filtrate elicitor 1) is sensed at the plasma membrane by the receptor-like protein RLP30 and triggers strong immune responses (Zhang *et al.*, 2013), similar to the bacterial elicitor flagellin (Felix *et al.*, 1999). Elicitation of plant defenses with SCFE1 is a tool to dissect the signaling pathway involving RLP30 and to study immunity to necrotrophic fungi. Here, we describe a simple protocol to axenically grow *S. sclerotiorum*. Further, we present a two-step liquid chromatography-based method for the partial purification of SCFE1 from culture filtrate (Figure 1A-B). Measurement by gas chromatography of the emission of the plant stress hormone ethylene is proposed as a bioassay to monitor elicitor activity in the fractions throughout the purification procedure (Figure 1C).



**Figure 1. Two-step chromatographic fractionation of *S. sclerotiorum* culture filtrate to obtain semi-purified SCFE1.** A. Purification scheme of SCFE1. Crude filtrate (CF) is loaded onto a Sepharose SP cation exchange chromatography column. The eluate (S1) is diluted 10-fold and loaded onto a Source 15S cation exchange chromatography column. Elution is performed with a linear gradient of 0 to 0.3 M KCl and elution fractions of 0.5 ml (F1 - F100) are collected. FT = flow-through. B. Chromatogram of the SCFE1-containing fractions eluted from a Source 15S cation exchange chromatography column. The black line represents the protein elution profile monitored with OD<sub>280</sub> nm. The grey line shows the increasing conductivity of the elution buffer. ma.u. = milli-arbitrary units. mS = milli-Siemens. C. Ethylene response in *Arabidopsis* Col-0 to SCFE1-containing fractions obtained by two-step cation exchange chromatography. *Arabidopsis* Col-0 leaf pieces are treated with 15  $\mu$ l CF, undiluted and 10-fold diluted S1 as well as the Source 15S elution fractions (Fx). Treatment with 0.5  $\mu$ M flg22 is used as a positive control for ethylene production. No treatment and treatment with 15  $\mu$ l buffers A and B are used as negative controls. In this representative purification, SCFE1 is contained in fractions F24 to F52. Bars represent average values (n=2)  $\pm$  S.D.

## **Materials and Reagents**

1. *Arabidopsis thaliana* Col-0 purchased from 'Nottingham Arabidopsis Stock Centre'
2. *Sclerotinia sclerotiorum* strain 1946 [purchased from the biological resource center 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (DSMZ) GmbH, shipped in a sterile culture tube on agar medium, stored at 15 °C in the dark]
3. Malt extract (Carl Roth, catalog number: X976.2)
4. Peptone from casein (tryptic digest) (Carl Roth, catalog number: 8986.2)
5. Agar-agar (bacteriological) (Carl Roth, catalog number: 2266.3)
6. Ultrapure H<sub>2</sub>O (type 1) (filtered with MilliQ Reference purification system) (Merck KGaA)
7. MES [2-(*N*-morpholino) ethanesulfonic acid] (Carl Roth, catalog number: 4256.3)
8. KCl (Carl Roth, catalog number: P017.1) (2 M solution with ultrapure H<sub>2</sub>O)
9. Ethanol (Merck KGaA, catalog number: 100983) (diluted to 20% with ultrapure H<sub>2</sub>O)
10. XK16/20 chromatography column (GE Healthcare, catalog number: 28-9889-37)
11. Sepharose SP Fast Flow (GE Healthcare, catalog number: 17-0729-01) (stored at 4 °C)
12. Source 15S 4.6/100 PE column (GE Healthcare, catalog number: 17-5182-01) (stored at 4 °C)
13. GS90 soil (CL Ton Kokos) (Gebrüder Patzer, catalog number: 10-00800-40)
14. Vermiculite for plant culture (BayWa AG or Gebrüder Patzer)
15. Rubber stoppers (Carl Roth, catalog number EC93.1)
16. Flg22 peptide from *Pseudomonas aeruginosa* (Felix *et al.*, 1999) (synthesized, 50 µM, stored at -20 °C)
17. Malt-peptone-agar (see Recipes)
18. Malt-peptone medium (see Recipes)
19. Buffer A (see Recipes)
20. Buffer B (see Recipes)

## **Equipment**

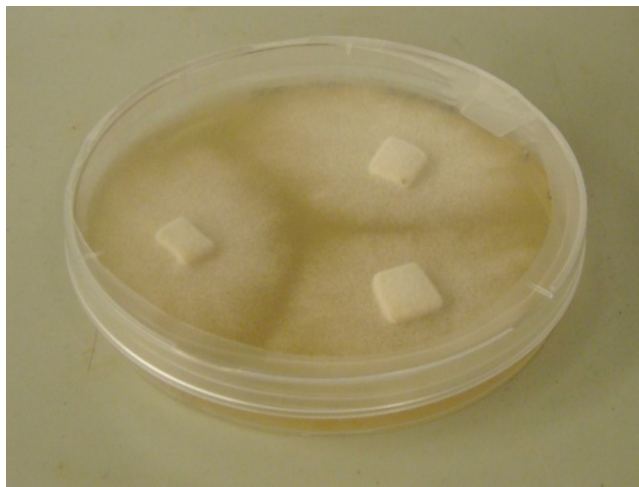
1. Petri dishes (94 x 16 mm, with vents) (Greiner Bio-One GmbH, catalog number: 633180)
2. Paraffin film (Parafilm M) (Sigma-Aldrich, catalog number: P7793)
3. Needle holder (Carl Roth, catalog number: 6189.1)
4. Lancets (Carl Roth, catalog number: 6181.1)
5. 1 L culture flasks (erlenmeyer-shaped, with straight neck and metal caps) (SCHOTT AG, DURAN<sup>®</sup>, catalog number: 21 771 54)
6. Laboratory funnel (minimum 15 cm diameter) (Carl Roth, catalog number: YA51.1)
7. Nylon mesh (60 µm pore size) (Carl Roth, catalog number: XA95.1)

8. 1 L laboratory bottles with screw-cap (SCHOTT AG, DURAN<sup>®</sup>, catalog number: 21 801 54 5)
9. Small silica gel bag
10. 50 ml Falcon conical centrifuge tubes (VWR International, catalog number: 21008-940)
11. 0.2 µm bottle-top filters for 500 ml (Carl Roth, catalog number: AC20.1)
12. Paper scissors
13. 6 ml thick-walled glass test tubes (Carl Roth, catalog number: HA75.1)
14. 1 ml syringe (B. Braun Melsungen AG, Omnifix-F Tuberculin, catalog number: 9161406V)
15. Syringe needle (27 Ga. x ¼ in., size 20) (B. Braun Melsungen AG, Sterican, catalog number: 4657705)
16. Spatula (Carl Roth, catalog number: YK51.1)
17. 1.5 ml reaction tubes with caps (VWR International, catalog number: 700-5239)
18. MilliQ Reference purification system (Merck KGaA, catalog number: Z00QSV001)
19. Laminar flow clean bench
20. 15 °C incubator
21. Front-loading lyophilizer of at least 10 L capacity with basins (e.g. L10, WKF-Gesellschaft für elektrophysikalischen Apparatebau, not produced anymore)
22. Floor-standing high-speed centrifuge (e.g. Sorvall RC-5B Plus Superspeed centrifuge and Sorvall SLA-1500 Superlite rotor with adapters for 50 ml Falcon conical centrifuge tubes, Thermo Fisher Scientific Inc., not produced anymore)
23. Vacuum pump (Gardner Denver, Welch, catalog number: 2522C-02)
24. 4 °C cold lab chamber or cold room
25. ÄKTA Explorer 10 liquid chromatography system (GE Healthcare, catalog number: 18-1300-00) equipped with:
  - a. Pump P-900
  - b. Sample pump P-960 (GE Healthcare, catalog number: 18-6727-00)
  - c. Monitor UV-900 (GE Healthcare, catalog number: 18-1108-35)
  - d. Monitor pH/C-900 (GE Healthcare, catalog number: 18-1129-74)
  - e. Fraction collector Frac-950 (GE Healthcare, catalog number: 18-6083-00)
  - f. Rack A for 18/30 mm tubes (GE Healthcare, catalog number: 18-6083-11)
  - g. Rack B for 12 mm tubes (GE Healthcare, catalog number: 18-6083-12)
  - h. Unicorn control software (version 4.12 or more)
26. Plant growth chamber (controlled light, temperature and humidity)
27. Gas chromatograph with Al<sub>2</sub>O<sub>3</sub>-column and flame-ionization detector (e.g. GC-14A with analysis unit C-R4AX Chromatopac, Shimadzu Deutschland GmbH, not produced anymore)

**Procedure**

A. Axenic culture of *Sclerotinia sclerotiorum*

1. Work under sterile conditions when handling *S. sclerotiorum* cultures.
2. Prepare fungal stock plate:
  - a. Cut 2 agar plugs (0.5 x 0.5 cm) from the initial *S. sclerotiorum*-containing culture tube using a lancet.
  - b. Place agar plugs in a Petri dish on malt-peptone-agar.
  - c. Seal the plate with paraffin film.
  - d. Let the fungus grow for 3 days at room temperature (RT) in the dark (Figure 2).  
*Note: The mycelium should cover almost the whole surface of the plate.*
  - e. Store plate at 15 °C in the dark and reuse it for up to 3 months.
3. Prepare fungal liquid culture:
  - a. Prepare 1 L culture flasks containing 400 ml malt-peptone medium (25 flasks for 10 L culture).
  - b. Cut agar plugs (0.5 x 0.5 cm) of fresh mycelium from the stock plate and inoculate each flask with 2 of them.
  - c. Let fungus grow without shaking for 2-3 weeks at RT in the dark until the liquid surface is completely covered with mycelium.



**Figure 2. *S. sclerotiorum* growing on a malt-peptone-agar plate**

B. Freeze-drying of *S. sclerotiorum* culture filtrate

4. Put a nylon mesh into a laboratory funnel. Filter the culture medium through the mesh and collect it.
5. Discard the mycelium.

6. Pour the culture filtrate into basins and freeze-dry it in a front-loading lyophilizer for 3 to 4 days.
7. Store freeze-dried material at RT in a hermetically closed 1 L laboratory bottle. Add a silica gel bag to keep the material dry.

C. Re-suspension of the culture filtrate

8. Re-suspend freeze-dried culture filtrate (e.g. 15 g dry weight) in a minimal volume (approx. 6 ml/g) of ice-cold buffer A in a 50 ml Falcon conical centrifuge tube.
9. Centrifuge sample for 20 min at 10,000 x g and 4 °C.
10. Collect the supernatant and keep on ice.
11. Repeat steps C9-10 if necessary to remove all insoluble particles.
12. Keep an aliquot of culture filtrate for subsequent activity assays in plants.
13. Re-suspended culture filtrate may be stored at -20 °C.

*Note: Frozen concentrated culture filtrate is stable for at least one year. Repeated freeze-thaw cycles do not damage the sample.*

D. Isolation of SCFE1: Rough fractionation by cation exchange chromatography

14. For the isolation of SCFE1 from re-suspended culture filtrate, work with the ÄKTA Explorer liquid chromatography system cooled at 4 °C (in a cold lab chamber or cold room).
15. All solutions for the chromatography should be prepared with ultrapure H<sub>2</sub>O, filtered through a bottle-top filter into clean, dust-free bottles and cooled at 4 °C.
16. Pack a XK16/20 chromatography column from GE Healthcare with Sepharose SP Fast Flow matrix from GE Healthcare for cation exchange chromatography to around 15 ml column volume (CV).

*Note: For a small-scale purification (less than 5 g dry weight), 1 or 5 ml pre-packed Sepharose SP Fast Flow columns from GE Healthcare may be used.*

17. Wash column with 5 CV ultrapure H<sub>2</sub>O at a flow rate of 3 ml/min.
18. Equilibrate column with 5 CV buffer A at a flow rate of 3 ml/min.
19. Load re-suspended culture filtrate via the P-960 sample pump at a flow rate of 3 ml/min.
20. Register flow, pressure, pH, conductivity, OD<sub>280</sub> nm and OD<sub>215</sub> nm during the run.
21. Collect flow-through once OD<sub>280</sub> nm increases and until loading is completed.
22. Wash column with buffer A until OD<sub>280</sub> nm and OD<sub>215</sub> nm are back to the base line and stable.
23. Elute in one step with 100% buffer B.

24. Collect eluate into a 50 ml Falcon conical centrifuge tube using rack A for the fraction collector. Start collecting once OD<sub>280</sub> nm exceeds 300 ma.u. (milli-arbitrary units) and stop when the end of the elution peak is reached.
  25. Wash column subsequently with 5 CV 2 M KCl, 5 CV ultrapure H<sub>2</sub>O, then 2 CV 20% ethanol for storage.
  26. Before proceeding to the second purification step, test activity of culture filtrate (CF), flow-through (FT) and eluate (S1) with the ethylene assay in *Arabidopsis* (see step F).  
*Note: S1 should have the highest activity and contain most of SCFE1.*
  27. Store S1 at 4 °C until next day or at -20 °C for more than 1 year. The flow-through may be discarded if the activity is successfully recovered in S1.
- E. Isolation of SCFE1: Refined fractionation by cation exchange chromatography
28. Dilute S1 10-fold with buffer A.
  29. Install a pre-packed Source 15S 4.6/100 PE column from GE Healthcare for cation exchange chromatography.
  30. Wash column with 5 CV ultrapure H<sub>2</sub>O at a flow rate of 1 ml/min.
  31. Equilibrate column with 5 CV buffer A at a flow rate of 1 ml/min.
  32. Load total volume of diluted sample via the P-960 sample pump at a flow rate of 1 ml/min.  
*Note: The pressure on the column increases with time. The flow rate may be reduced progressively.*
  33. Register flow, pressure, pH, conductivity, OD<sub>280</sub> nm and OD<sub>215</sub> nm during the run.
  34. Collect flow-through once OD<sub>280</sub> nm increases and until loading is completed.
  35. Wash column with buffer A until OD<sub>280</sub> nm and OD<sub>215</sub> nm are back to the base line and stable.
  36. Elute with a linear gradient from 0% to 60% buffer B in 40 CV at a flow rate of 0.5 to 1 ml/min (see Figure 1B for a typical elution profile).
  37. Collect 0.5 ml fractions in 1.5 ml reaction tubes using rack B for the fraction collector (approx. 100 fractions, labeled F1-F100). Create a program for automated fractionation with the method wizard of the Unicorn software.  
*Note: SCFE1 should elute at a salt conductivity value between 8 and 16 mS/cm.*
  38. Wash column with 5 CV 100% buffer B at a flow rate of 1 ml/min.  
*Note: A small elution peak may appear. It does not need to be collected, as it does not contain any activity.*
  39. Wash column with 5 CV water, then 2 CV 20% ethanol for storage.  
*Note: If the pressure on the column remains higher than initially, it means that it was slightly clogged during sample loading and requires additional washing before reuse. In*

*this case, follow the cleaning-in-place (CIP) procedure for Source 15S columns as described by the manufacturer.*

40. Test activity of flow-through (FT) and elution fractions (F1-F100) with the ethylene assay in *Arabidopsis* (see step F).

*Note: It is sufficient to test 1 fraction out of 5 for those with low OD<sub>280</sub> nm and 1 fraction out of 2 for those corresponding to the elution peak.*

41. Store active fractions containing semi-purified SCFE1 at 4 °C for maximum 1 day or at -20 °C for more than 1 year. The flow-through may be discarded if the activity is successfully recovered in the elution fractions.

#### F. Ethylene assay

42. Grow *Arabidopsis* Col-0 plants on soil (GS90/vermiculite mixture 3.5:1) for 5 to 6 weeks in a growth chamber: 8 h light/16 h dark, 23 °C, 40-60% relative humidity, 100 μmol/m<sup>2</sup>/s light intensity (short day conditions).

43. Choose the biggest leaves from the plants. Cut each leaf into 6 rectangular pieces using paper scissors. Omit the edges, tip and stalk. Float leaf pieces on dH<sub>2</sub>O in a Petri dish (Figure 3).

*Note: Prepare the leaf pieces 12 to 20 h before elicitation. Leaf pieces left for a longer time will not respond well to elicitation anymore.*

44. Leave leaf pieces over night in the closed Petri dish at RT or in an air-conditioned room.
45. Pipet 0.5 ml dH<sub>2</sub>O into glass test tubes. Use 2 or 3 tubes per sample to test (for duplicate or triplicate measurements).
46. Put 3 leaf pieces into each test tube using a spatula.

*Note: Leaf pieces may rest for a few hours in the test tubes or may be elicited immediately.*

47. Add 5 to 15 μl of the samples to test (CF, FT, S1 and/or selected fractions of F1-F100). Include an untreated control and a positive control (5 μl of 50 μM flg22 = 0.5 μM final concentration).
48. Close test tubes with rubber stopper and carefully shake tube rack to mix.

*Note: Leaves should stay in the water or on the surface but not stick to the glass.*

49. Incubate for 3 to 4 h at RT.
50. Measure the ethylene content in each tube:
  - a. Prick through the rubber stopper of a tube using a 1 ml syringe with a needle.
  - b. Mix the air inside the tube by moving up and down several times.
  - c. Withdraw 1 ml air and inject it into the gas chromatograph.
  - d. Record retention time and peak area to determine the amount of ethylene in pmol/ml air (see Figure 1C for representative data sets).



*Note: For the untreated control, an average value of 0.60 +/- 0.21 pmol/ml air is expected. The mean values for buffer A and B are 0.81 +/- 0.23 and 0.81 +/- 0.23, respectively. With 1.30 +/- 0.20 pmol/ml, the ethylene production in the flg22 control is expected to be around 2x higher than in the non-treated and 1.5x higher than in the buffer controls. The values for culture filtrate (CF) can range from 2.5 to 15 pmol/ml. As culture filtrate yields a higher ethylene response than flg22, an active culture filtrate sample from a previous purification (stored at -20 °C) may be used as additional positive control.*



**Figure 3. Cutting and floating of *A. thaliana* leaf pieces**

### Notes

1. The ethylene assay is a very reliable and fast method to detect and quantify the immune-eliciting activity of the SCFE1-containing fractions. It also allows testing a large number of samples in a row. Other immune assays may however be used, such as the luminol-based oxidative burst assay to measure the production of reactive oxygen species (requires dialysis of the SCFE1-containing fractions to remove buffer), immunoblotting to detect activated MAP kinases or qRT-PCR for transcriptional profiling of defense-related genes (Zhang *et al.*, 2013).
2. To confirm that in the selected chromatographic fractions truly contain SCFE1 and no other or additional elicitor, the fractions may be tested on the *Arabidopsis RLP30* knock-out mutants *rlp30-1*, *-2* or *-4*. These mutants are completely insensitive to treatment with SCFE1-containing fractions, but react normally to flg22 challenge. Alternatively proteinase K sensitivity and heat stability of the immune-eliciting activity are good indicators for the identity of SCFE1 (Zhang *et al.*, 2013).

## **Recipes**

1. Malt-peptone-agar (autoclaved)
  - 10 g malt extract
  - 2.5 g peptone from casein, tryptic digest
  - 15 g agar-agar
  - dH<sub>2</sub>O qs. to 1 L
2. Malt-peptone medium (autoclaved)
  - 10 g malt extract
  - 2.5 g peptone from casein, tryptic digest
  - dH<sub>2</sub>O qs. to 1 L
3. Buffer A
  - 0.1 M MES
  - Ultrapure H<sub>2</sub>O
  - Adjust pH to 5.4
  - Stored at 4 °C
4. Buffer B
  - 0.1 M MES
  - 0.5 M KCl
  - Ultrapure H<sub>2</sub>O
  - Adjust pH to 5.4
  - Stored at 4 °C

## **Acknowledgments**

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## **References**

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