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Rhizoctonia solani Infection Assay of Young Sugar Beet and *Arabidopsis* plantlets

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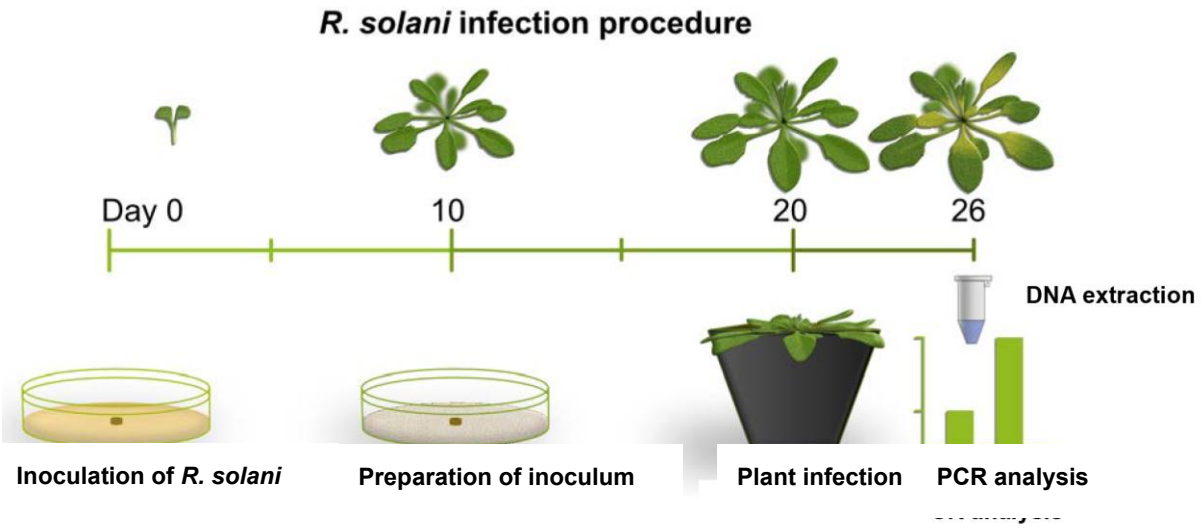
[Abstract] *Rhizoctonia solani* is a soil-borne fungus, which rarely produces any spores in culture. Hence, all inoculation procedures are based on mycelia, often as a coat on cereal kernels, placed in close vicinity to the plant to be infected. In this protocol, an inoculation method is described where the fungus is first allowed to infest a perlite-maize flour substrate for 10 days, followed by thorough soil mixing to generate uniform fungal distribution. Pre-grown seedlings are then replanted in the infested soil. Plant materials can be harvested, five (sugar beet) and ten days (*Arabidopsis*) post infection, followed by a rapid cleaning step ahead of any nucleic acid preparation. Commercial DNA or RNA extraction kits can be used or, if higher DNA yield is required, a CTAB extraction method. Our purpose was to develop a reliable and reproducible protocol to determine the infection levels *in planta* upon infection with *R. solani*. This protocol is less laborious compared to previous ones, improves the consistency of plant infection, reproducibility between experiments, and suits both a root crop and *Arabidopsis*.

Graphic abstract:



Overview of the *R. solani* infection procedure.

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Keywords: *Arabidopsis thaliana, Beta vulgaris*, DNA, *Rhizoctonia solani*, RNA

[Background] Sugar beet (*Beta vulgaris*) is a biennial plant species grown for its high sugar content in the taproot. In temperate regions where this crop is mainly grown, it takes about six months from sowing to harvesting (Draycott, 2006). This long timespan exposes the root to several soil-borne pathogens, such as *Rhizoctonia solani*. *Rhizoctonia solani* can cause damping-off disease on seedlings or root and crown rot disease on older roots, and this has developed into a major and increasing problem in sugar beet growing areas (Kluth and Varrelmann, 2010). The isolates of this fungal basidiomycete are divided into hyphal anastomosis groups (AGs). AG2-2IIIB is the subgroup that mainly incites disease on sugar beet, but such strains can also infect maize, resulting in problems where these two crops are used in the same crop rotation scheme (Führer Ithurrart *et al*., 2004).

Preparing materials for more advanced molecular analyses have become problematic due to the absence of asexual spores (conidia) and the sexual stage being very rare (Parmeter, 1970), which limits more precise inoculation procedures using specified spore concentrations. Hence, inoculation of sugar beets in the greenhouse is traditionally performed by replacing soil around the plants in the pots with infected millet seeds or infected ground barley kernels, followed by disease scoring (Scholten *et al*., 2001; Bolton *et al*., 2010). Under field conditions, infected ground barley kernels are spread in furrows or broadcast before sugar beet seeds are drilled. Alternatively, the barley kernels are placed in the crown of the plant, at the eight-leaf growth stage (Strausbaugh *et al*., 2013). These procedures can cause variation in terms of amount of fungal DNA being established on the host plant surface, thereby affecting the observed plant responses. There are methods available for measuring fungal DNA concentration in soil (Budge *et al*., 2009; Abbas *et al*., 2014). One of the problems, is that DNA concentration is rarely correlated with disease symptoms. Other factors that influence disease development is soil composition, soil humidity, and temperature (Bolton *et al*., 2010). Here, we describe in detail how to generate diseased plants under controlled growth conditions, followed by extraction of reproducible amounts of RNA or DNA. This protocol has been used to identify defense genes against *R. solani* in sugar beet and *Arabidopsis* (Holmquist *et al*., 2021). Our plant infection method was instrumental to identify the *R. solani* virulence factor *RsLysM* (Dölfors *et al*., 2019), the protease inhibition effector *RsRlpA* (Charova *et al*., 2020), and the mitochondria and chloroplast targeting effector *RsCRP1* (Tzelepis *et al*., 2021). RNA and DNA obtained through this protocol can be used to monitor plant transcript responses to *R. solani* and measure fungal colonization *in planta,* including fungal activities during plant pathogen interaction.

Materials and Reagents

A. Materials
   1. Plant pots, small pots (6 × 6 × 5 cm) and big pots (13 × 13 × 13 cm) (SW Horto, catalog number: 700-245)
   2. Plant trays (34 × 22 × 4 cm) (Nelson Garden, catalog number: 5770)

3. Soil (S-soil, Hasselfors Garden, Örebro, pH 5.5-5.6) composed of sighted light peat, black peat, perlite, sand, and lime
4. Miracloth Calbiochem® (Merck, catalog number: 475855-1)
5. Glass beakers (250 mL VWR, catalog number: 213-0014)
6. Laboratory bottles (500 mL SARSTEDT, catalog number: 3607507)
7. Plastic petri dishes (Ø × H: 92 × 16 mm, SARSTEDT, catalog number: 82.1473.001)
8. Glass Petri dishes (Ø × H: 92 × 16 mm, Fisher Scientific, catalog number: 1201333)
9. Eppendorf micro-tubes (SARSTEDT, catalog numbers: 72.690.001 [1.5 mL]; 72.695.500 [2.0 mL])
10. Spoon (sterile)
11. Aluminum foil
12. Scalpel and blades (Fisher Scientific, catalog numbers: 12348019 and 12398009)
13. Mortar and pestle (80 mm × 92 mm, VWR, catalog number: 470148-960)
14. Parafilm (10.2 cm × 38.1 m, VWR, catalog number: 52858-000)
15. Filtropur sterilization filter S0.2 (SARSTEDT, catalog number: 83.1826.001)

B. Plants
1. *Beta vulgaris* seeds (DLF Beet Seed)
2. *Arabidopsis thaliana*, Col-0 (*Arabidopsis* Information Resource, TAIR)

C. Pathogen

The *Rhizoctonia solani* AG2-2IIIB BBA 69670 isolate (Wibberg *et al*., 2016) was used throughout the work.

D. Molecular biology working kit
1. DNeasy plant mini kit (Qiagen, catalog number: 69104)
2. RNeasy plant mini kit (Qiagen, catalog number: 74903)

E. Other reagents

Inoculum medium (see Recipes)
1. Potato dextrose agar, PDA (Applichem, catalog number: A5838)
2. Distilled water
3. Perlite, 0-6.5 mm (SW Horto AB, Hammenhög, Sweden)
4. Maize flour (Risenta, Sollentuna, Sweden)

CTAB DNA extraction
1. Liquid nitrogen
2. Chloroform, EMSURE® ACS, ISO, Reag. Ph. (VWR, catalog number: 1.02445.1000, CAS number: 67-66-3)

3. Isopropanol (2-Propanol, EMSURE ACS, ISO, Reag. Ph. Eur. for analysis, VWR, catalog number: 1.09634.5000)
4. Ethanol (70%)
5. Sodium chloride (NaCl) (Saveen Werner AB (Duchefa), catalog number: 31434)
6. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S8045)
7. Ethylenediaminetetraacetic acid (EDTA) (VWR, catalog number: BDH9232, CAS number: 60-0-04)
8. Tris base TRIS-RO ROCHE (Sigma-Aldrich, CAS number: 77-86-1)
9. Hydrochloric acid (37%) (Sigma-Aldrich, catalog number: H1758)
10. Distilled water
11. Hexadecyltrimethylammonium bromide (CTAB) (Sigma-Aldrich, catalog number: H6269)
12. Tris-EDTA buffer solution (Sigma-Aldrich, catalog number: T9285)
13. Optional: RNase A (17,500 U) (Qiagen catalog number: 19101)
14. Potato dextrose agar (PDA) (1 L) (see Recipes)
15. 3% CTAB extraction buffer (50 mL) (see Recipes)
16. 1 M Tris-HCl (pH8) (1 L) (see Recipes)
17. 5 M NaCl (100 mL) (see Recipes)
18. 0.5 M EDTA (pH8) (1 L) (see Recipes)
19. 1× TE buffer (100 mL) (see Recipes)

Equipment

1. Growth chamber (Percival AR82L2/Split) or greenhouse
2. Analytic balance (Mettler Toledo, model: AE100)
3. Autoclave
4. Laminar flow hood
5. Heating cabinet
6. Water bath (Sigma-Aldrich, model: Julabo TW12, catalog number: Z615498)
7. Heating plate
8. Liquid nitrogen container
9. Safety glasses, gloves, and lab coat

Procedure

A. Preparation of infested soil

1. Dissolve 39 g PDA powder in 1 L distilled water and autoclave for 15 min at 125°C (135 kPa). Once the media cools down (at approximately 50°C), pour approximately 20 mL per Petri plate and let it solidify. Grow *R. solani* on PDA plates for 10 days at room temperature. Use sterile conditions. Store cultures at 4°C for up to one month.

2. Calculate how many grams inoculum will be needed for the experiment (depending on pot size, number of biological replicates, and experimental set up). For instance, 1 g inoculum per 20 g soil for *Arabidopsis* and 1 g inoculum per 10 g soil for sugar beet.
3. Mix perlite, maize flour, and distilled water (1:1:5) in a beaker (see inoculum medium recipe).
4. Place 40 g of perlite mixture in clean glass Petri dishes, seal with aluminum foil, and autoclave for 15 min at 125°C (135 kPa).
5. Work under sterile conditions and cut 1 × 1 cm pieces from PDA plates with *R. solani*. Place one piece upside down in the middle of each glass Petri dish filled with Perlite media (step 2) using a sterile scalpel. Seal the glass Petri dishes with Parafilm and incubate for 10 days at room temperature in the dark, to generate fungal infested substrate (FIS).
6. Add pre-weighed FIS to soil with a spoon and mix thoroughly. The mix should contain 1 part FIS for every 10 parts of soil for sugar beet inoculation and 1:20 (FIS:soil) for *Arabidopsis* inoculation.
   Note: It is critical to mix very thoroughly. Weigh and mix all soil/inoculum needed all at once to avoid soil batch variation.
7. Use the soil-FIS mix in the same day it was prepared.

B. Preparation of diseased sugar beet or *Arabidopsis* plants

1. Wrap sugar beet seeds in Miracloth and dip the “bag” in cold water in a glass beaker for 30 min.
2. Transfer the seeds to a 57°C water bath for 5 min.
3. Dip the seeds in cold water again and let them dry overnight at room temperature.
4. Transfer sugar beet seeds to soil and cultivate for three weeks under greenhouse conditions (16 h light/8 h dark cycle with 22°C/18°C). Add nutrients (2 mL of Blomstra, Cederroth, Upplands Väsby/L water) once a week,
5. Alternatively, incubate *Arabidopsis* Col-0 seeds in sterile water overnight or up to four days at 4°C.
6. Plant *Arabidopsis* seeds in soil and cultivate for four weeks in short day conditions (16 h dark at 18°C/8 h light at 22°C cycle, and 60% relative humidity). Keep the soil moist but not wet.
7. Carefully lift the plants from the soil with tweezers. Gently wash away the soil from the roots with distilled water.
   Note: If the soil has become compact, loosen the soil around the plant by pressing lightly on the sides of the pot to avoid damaging the root.
8. Transfer clean plantlets to infested soil. Monitor plants for symptom development (Figure 1; Figure 2). Symptoms on sugar beet seedlings may occur on the hypocotyl after two days. At day eight, susceptible plants start damping-off. Symptoms are diffuse on *Arabidopsis* seedlings, until the rosette leaves start showing signs of chlorosis and/or necrosis around day five.

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Figure 1. Sugar beet plantlets, 26 days post germination.

A. Tolerant genotype, 5 days post *R. solani* infection. B. Tolerant genotype, grown in non-infested soil. C. Susceptible genotype, 5 days post *R. solani* infection. D. Susceptible genotype, grown in non-infested soil.

Figure 2. *Arabidopsis thaliana* Col-0, 38-days post germination.

Plants in *R. solani* infested soil at five different inoculum ratios and control plants grown in H_2O-treated soil. Plant phenotypes at 10 dpi. The red rectangle marks the optimal infestation level.

C. Harvest of plant material and isolation of total RNA and DNA

1. Harvest roots and hypocotyls of sugar beet seedlings at 5 dpi and *Arabidopsis* plantlets, including roots, at 10 dpi, using a clean scalpel (harvest timing and number of plants per biological replicate depend on your project). Wash away soil and other contaminants using sterile distilled water. Let dry on paper towels. Wrap in aluminum foil and freeze immediately in liquid nitrogen.
   Note: It is important that roots are completely clean. Work fast. Avoid piling up materials ahead of freezing.
   Grind the samples in liquid nitrogen with a mortar and pestle (autoclaved or heat sterilized). Keep the plant material frozen until all samples have been ground to a fine powder.
   Note: Do not let the plant material thaw before DNA or RNA extraction.
2. Proceed with RNA and/or DNA isolation from 100 mg sample, using commercially available kits, such as RNeasy- and DNeasy-plant mini kit (Qiagen) following the manufacturer's manual. If higher DNA yield is preferred, the modified CTAB DNA extraction method can be used.

D. CTAB DNA extraction (Möller *et al*., 1992)

1. Add 500 µL of 3% CTAB extraction buffer to 100 mg powdered plant material in a 2.0 mL microcentrifuge tube and vortex vigorously.
   Optional: Add 4 µL of RNAse A (100 mg/mL) and mix by inverting.
2. Incubate the samples in a 65°C water bath for 30 min. Mix by inverting the tubes once every 5-10 min.
3. Centrifuge the samples for 10 min at 13,000 × *g* and transfer the supernatant to a new 1.5 mL tube.
4. Add 600 µL of chloroform and vortex.
5. Centrifuge at 13,000 × *g* for 10 min. Transfer the upper phase of the supernatant to a clean 1.5 mL Eppendorf tube.
6. Add 1 volume of chloroform and vortex.
7. Centrifuge for 10 min at 13,000 × *g* and transfer the upper phase to a clean 1.5 mL tube.
8. Add 1 volume isopropanol (2-propanol) and mix by pipetting up and down. Precipitate the mix at -20°C for 30 min.
9. Centrifuge for 20 min at 13,000 × *g*. Discard the supernatant by pipette (avoid disturbing the DNA pellet) and let the pellet dry briefly.
10. Add approximately 150 µL of chilled 70% ethanol and centrifuge at 13,000 × *g* for 5 min to wash the DNA pellet. Discard the ethanol and let the pellet dry.
11. Dissolve the pellet in 100 µL of 1× TE buffer.

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Data analysis

Infection levels are best evaluated by comparing fungal DNA in the different plant materials at 5- or 10-days post infections.

1. Wash the roots vigorously in sterile water, to eliminate excess soil and external fungal growth.
2. Repeat root washing until totally clean. This can be determined under a stereo microscope.
3. Use at least five sugar beet or *Arabidopsis* roots and hypocotyls in each replicate, for a minimum of four replicates.
4. Prepare total DNA according to Möller *et al.* (1992). See section C and D.
   The amount of fungal DNA (*RsG3PDH*) can be determined with qPCR and normalized to the amount of plant DNA (*Actin2*), using an iQ5 qPCR System (Bio-Rad, Hercules, CA). Each 20 µL reaction contained 2 µL of gDNA template (50 ng), 150 nM of each primer and 10 µL of SYBR Green PCR Master Mix (Fermentas, St. Leon-Rot, Germany). The amplification program consisted of: 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. Melt curve analysis was conducted to confirm a single amplification product.
5. Statistical analysis may be performed by applying Student's *t*-test using at least four replicates.

Recipes

1. Potato dextrose agar (PDA) (1 L)
   Mix 39 g/L PDA powder in 1 L distilled water.
   Adjust to pH 5.6.
   Autoclave for 15 min at 125°C (135 kPa).
2. Inoculation medium (1 kg)
   Mix 143 g perlite, 143 g maize flour, and 714 mL distilled water.
   Wrap glass Petri dishes in aluminum foil.
   Autoclave for 15 min at 125°C (135 kPa).
3. 3% CTAB extraction buffer (50 mL)
   Dissolve 1.5 g CTAB in 16.5 mL of distilled water on a heating plate.
   Add 7.5 mL of 1 M Tris-HCl (pH 7.4), 26 mL of 5 M NaCl, and 0.2 mL of 0.5 M EDTA (pH 8).
   Filter sterilize using a 0.2 µm filtropour filter.
4. 1 M Tris-HCl (pH8) (1 L)
   Add 121.1 g Tris base to 800 mL of distilled water.
   Adjust to pH 8 with concentrated HCl (approximately 42 mL).
   Adjust to 1 L with distilled water.
   Autoclave for 15 min at 125°C (135 kPa).
5. 5 M NaCl (100 mL)
   Add 29.22 g NaCl and adjust to 100 mL of distilled water.
   Autoclave for 15 min at 125°C (135 kPa).

6. 0.5 M EDTA (pH 8) (1 L)
   Add 186.1 g EDTA to 800 mL of distilled water.
   Adjust to pH 8 with NaOH (approximately 20 g).
   Adjust to 1 L with distilled water.
   Autoclave for 15 min at 125°C (135 kPa).
7. 1× TE buffer (100 mL)
   10 mM Tris-HCl
   0.1 mM EDTA
   Add distilled water up to the desired final volume.
   Autoclave for 15 min at 125°C (135 kPa).

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Competing interests

The authors declare no conflict of interest.

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