bio-101 www.bio-protocol.org/e2864 DOI:10.21769/BioProtoc.2864

Root Gall Formation, Resting Spore Isolation and High Molecular Weight DNA Extraction of *Plasmodiophora brassicae*

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[Abstract] Isolation of DNA from obligate biotrophic soil-borne plant pathogens is challenging. This is because of their strict requirement of living plant tissue for their growth and propagation. A soil habitat further imposes risk of contamination from other microorganisms living in close vicinity of the plant roots. Here we present a protocol on how to prepare DNA suitable for advanced molecular analysis on the soil-borne pathogen *Plasmodiophora brassicae,* a peculiar unicellular plant pathogenic organism, causing disease on Crucifers. First, it is important to grow *Brassica* or *Arabidopsis* plants in infested soils below a temperature of 25 °C under moist conditions to promote root gall formation. Root galls should be harvested ahead of initiation of the decomposing process, no later than four or nine weeks post inoculation of *Arabidopsis* or *Brassica* plants, respectively. Resting spores with reduced numbers of soil organisms are achieved by gradient centrifugations of homogenized gall tissues. Treatments with 70% alcohol and a suit of different antibiotics promote *P. brassicae* purity. A CTAB-based procedure allows isolation of high quality DNA suitable for massive parallel sequencing analysis.

Keywords: *Arabidopsis*, *Brassica*, Clubroot, DNA, *Plasmodiophora brassicae*, Resting spores, Rhizaria

[Background] *Plasmodiophora brassicae* is a soil-borne plant pathogen causing root galls (clubs) in the *Brassicaceae* family including *Arabidopsis*. The clubroot disease has a major impact on oilseed rape (canola) and cabbage cultivation worldwide. *P. brassicae* is an obligate biotroph (require a host for growth) assigned to the supergroup Rhizaria, one of the least studied organism groups of eukaryotes (Sierra *et al*., 2016; Sibbald and Archibald, 2017). Phylogenetically, *P. brassicae* belongs to a plant pathogenic group of protists in Phytomyxea (Neuhauser *et al*., 2011 and 2014; Adl *et al*., 2012). Few genomes of related species are available, a circumstance which has considerably delayed the molecular analysis and genome comparisons. *P. brassicae* forms hardy resting spores in the clubs, spores that have the capacity to remain dormant for decades in the soil, ready for new rounds of root infections if a host plant grow nearby. Here we describe how to generate diseased plants, isolate resting spores from root galls followed by extraction of large amounts of DNA. This protocol is a further improvement and clarification of the procedures described in Schwelm *et al*. (2015). The outlined work is substantial but yields high-quality DNA suitable for long-read massive parallel sequencing.

Materials and Reagents

A. Materials
1. Safety glasses, gloves and lab coat
2. Filter paper
3. Plant pots, small pots (6 x 6 x 5 cm) and big pots (13 x 13 x 13 cm)
4. Plant trays (34 x 22 x 4 cm)
5. Soil (S-soil, Hasselfors Garden, Örebro, pH 5.5-6.5) composed of sighted light peat, black peat, perlite, sand, and lime
6. Petri dishes 10 cm (ø)
7. Miracloth Calbiochem® (Merck, catalog number: 475855)
8. Tubes (Falcon tube 15 ml, SARSTEDT, catalog number: 62.554.001; Falcon tube 50 ml, SARSTEDT, catalog number: 62.547.004; Eppendorf micro-tube 2 ml, SARSTEDT, catalog number: 72.695.500)
9. Plastic and glass beakers
10. Scalpel
11. Mortar, pestle and spoon (sterile and pre-chilled)
12. Filtropur S0.2 (SARSTEDT, catalog number: 83.1826.001)

B. Plants
1. *Brassica rapa* cv. 'Granaat' (European Clubroot Differential Set ECD-05)
2. *Arabidopsis thaliana* Col-0

C. Plasmodiophorid

Plasmodiophora brassicae strain e3 (Fähling *et al*., 2004; the strain is available upon request to the authors)

Note: Not all strains incite disease on Arabidopsis.

D. Molecular biology working kit

Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, catalog number: STRN50)

E. Other reagents

*Note: *Those solutions are prepared with sterile distilled water.*

Spore isolation
1. Ethanol (70%, 500 ml*)
2. Sodium hypochlorite (1%, 500 ml*) (Commercial bleach)
3. Sterile distilled water 5 x 1L
4. Ficoll PM 400 (16% and 32%*) (Sigma-Aldrich, catalog number: F4375)

5. Rifampicin (100 mg/ml stock, prepared with methanol solvent) (Duchefa Biochemie, catalog number: R0146)
6. Streptomycin sulfate (100 mg/ml stock*) (Thermo Fisher Scientific, catalog number: 11860038)
7. Carbenicillin disodium (100 mg/ml stock, prepared with water solvent) (Duchefa Biochemie, catalog number: C0109)
8. Pimaricin (100 mg/ml stock*) (Merck, Sigma-Aldrich, catalog number: 1.07360)
9. Cefotaxime sodium (100 mg/ml stock*) (Duchefa Biochemie, catalog number: C0111)
10. Hygromycin B (50 mg/ml stock) (Duchefa Biochemie, catalog number: H0192)
11. Lysozyme from chicken egg white (4 mg/ml*) (Sigma-Aldrich, catalog number: L6876)
12. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 31434)
13. Potassium chloride (KCl) (Merck, catalog number: 104936)
14. di-Sodium hydrogen phosphate dihydrate (Na_2HPO_4) (Merck, catalog number: 119753)
15. Potassium dihydrogen phosphate (KH_2PO_4) (Merck, catalog number: 104873)
16. Tris-HCl (Trizma® hydrochloride solution) (1 M, pH 7.5) (Sigma-Aldrich, catalog number: T2694)
17. DNase I, RNase-free (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EN0521)
18. Proteinase K (20 mg/ml stock*) (Sigma-Aldrich, catalog number: RPROTK-RO)
19. EDTA (0.5 M) (VWR, catalog number: 20294.294)
20. N-lauroylsarcosine sodium salt solution (1%, v/v) (Sigma-Aldrich, catalog number: 61747)
21. 1x PBS buffer (see Recipes)
22. 1x TE buffer (pH 7.5) (see Recipes)
23. Termination buffer (see Recipes)

DNA extraction

1. Liquid nitrogen
2. Tris-HCl (Trizma® hydrochloride solution) (1 M, pH 7.5) (Sigma-Aldrich, catalog number: T2694)
3. EDTA (VWR, catalog number: BDH9232)
4. Sodium chloride (NaCl) (5 M stock *) (Sigma-Aldrich, catalog number: 31434)
5. Hexadecyltrimethylammonium bromide (CTAB) (Sigma-Aldrich, catalog number: H6269)
6. 2-Mercaptoethanol (VWR, catalog number: 436022A)
7. Phenol:chloroform:isoamyl alcohol 25:24:1 (pH 7.5-8.0) (Carl Roth, catalog number: A156.2)
8. RNAse A, DNase and protease-free (10 mg/ml) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EN0531)
9. Chloroform, EMSURE® ACS, ISO, Reag. Ph. (Merck, catalog number: 1.02445.1000)
10. Ethanol (95%)
    Note: Pre-chill at -20 °C before use.
11. Ethanol (70%)*
12. 0.1x TE buffer (see Recipes)

13. CTAB extraction buffer (see Recipes)

F. Media and buffers (see Recipes)
   1. 1x PBS buffer
   2. 1x TE buffer
   3. Termination buffer
   4. CTAB extraction buffer

Equipment

1. Growth chamber (Percival AR82L2/Split) or greenhouse
2. Analytic balance (Mettler Toledo, model: AE100)
3. Household mixer (Rusta, catalog number: 90951442, Max power 170 W)
4. Liquid nitrogen container
5. Water bath (JULABO, model: Julabo TW12, catalog number: 9550112)
6. pH meter (Mettler Toledo, model: SevenCompact S220, catalog number: 30019028)
7. Tabletop centrifuge for Eppendorf tubes (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Fresco™ 17, catalog number: 75002420)
8. Tabletop centrifuge for Falcon-tubes (Eppendorf, model: 5804/5804 R, catalog number: 5805000327)
9. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA)
10. Microscope (Zeiss, Axioplan; camera: Leica Microsystems, model: Leica DFC295)
11. Stereoscope (Lecia, model: MZ FL III)
12. Autoclave

Procedure

A. Preparation of infested soil
   1. Start preparing at least two rounds of clubroots of chosen strain or pathotype for soil infestation well in time before any larger experiments. This is achieved by crushing soft clubroots followed by mixing the tissue thoroughly with wet soil.
   2. Grow *Brassica* plants for 7 to 9 weeks or *Arabidopsis* plants for 3 to 4 weeks in the infested soil. Repeat this step. Clubs can also be left in the soil to decompose but a mixing step speeds up the procedure. Spores per gram soil should exceed 100,000 which can be determined by qPCR analysis (Wallenhammar *et al.*, 2012). Store infested soil at -20 °C for later use.

B. Preparation of diseased *Brassica* or *Arabidopsis* plants
   1. Place *Brassica* seeds on a wet filter paper in a Petri dish at room temperature, 8-10/14-16 h light/dark regime to germinate (1-2 days).

2. Transfer 2-3 germinated seeds to a large pot with infested soil. Cultivate the plants for eight to nine weeks under greenhouse conditions (16 h light/8 h dark cycle and 22 °C day/18 °C night temperature). Add nutrients (2 ml Blomstra, Cederroth, Upplands Väsby/l water) on a daily basis.
3. Collect clubs when just started to get soft (Figures 1A to 1D), rinse them carefully with water and store at -20 °C for later use.
   Note: Clubs younger than 7-9 weeks old are too firm for homogenization by a mixer, limiting spore isolation, while older clubs left in soil quickly start to decompose yielding few spores. Weekly monitoring of plants and club development by uprooting is important because club formation and 'rate of maturity' can vary between experiments.

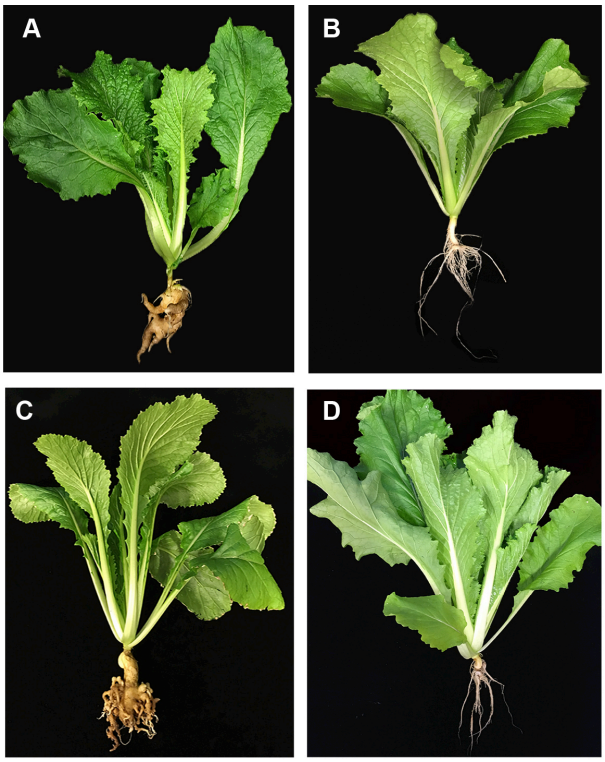

Figure 1. *Brassica rapa* cv. Granaat. A and B. Clubs harvested after 5 weeks in *P. brassicae* infested soil (A) and the corresponding control plant (B). C and D. Clubs harvested after 7 weeks in *P. brassicae* infested soil (C) and the corresponding control plant (D).

4. Alternatively, incubate *Arabidopsis* Col-0 seeds in sterile water overnight at 4 °C.
5. Plant five seeds per small pot of un-infested soil covered by transparent plastic top and keep them in short day conditions (16 h dark/8 h light cycle at 22 °C and 60% relative humidity (RH) for 14 days).
6. Transfer 24 plants into a tray with infested soil (Figure 2A) and cover the tray with a transparent plastic top. Keep the trays in short day conditions, at 15 °C, and 60% RH. Remove the lid after 4 days.

7. Galls are visible after 3 to 4 weeks (Figures 2B to 2H). Harvest and rinse the clubs carefully, and store at -20 °C for later use.
   Note: Clubs can be obtained from most crops, weedy and wild species in the Brassicaceae family.

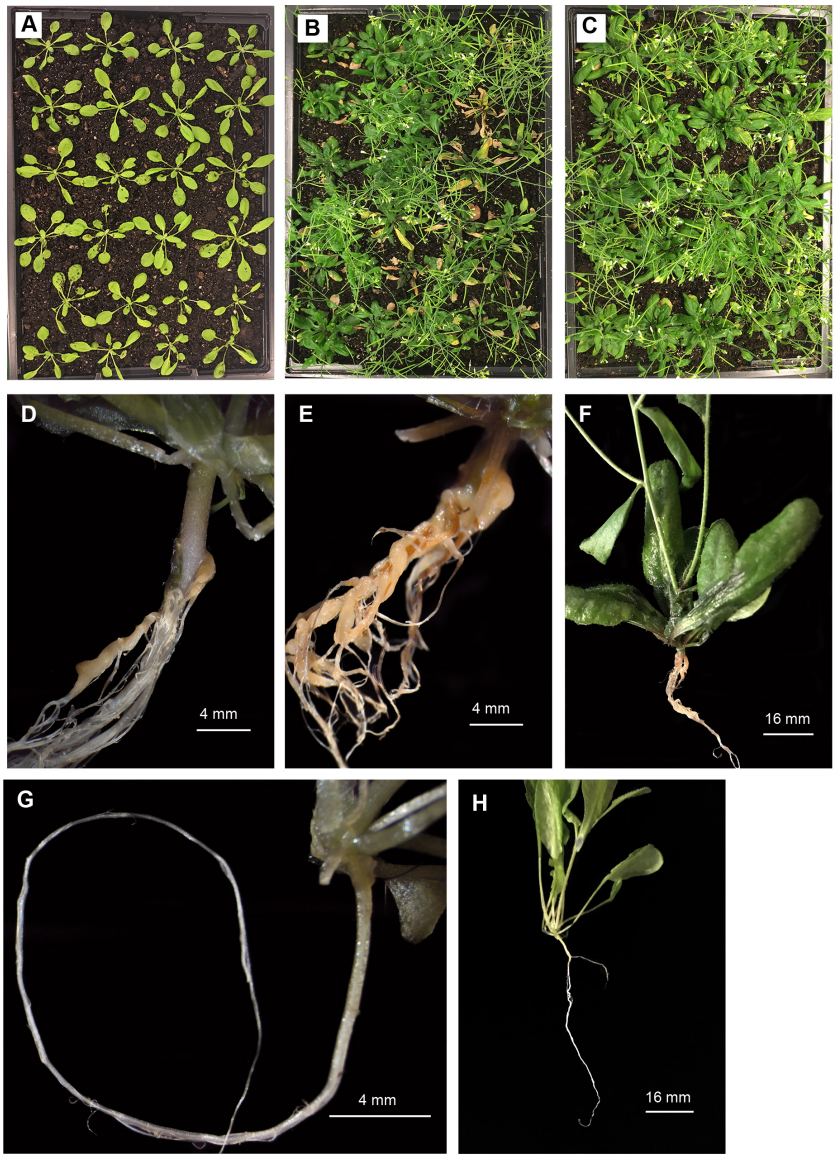

Figure 2. *Arabidopsis thaliana* Col-0. A. Healthy plants after two weeks; B. Diseased plants after four weeks in *P. brassicae* infested soil; C. Control plants, grown four weeks in H_2O treated soil; D-F. Gall formation on *Arabidopsis* roots; G and H. Control, *Arabidopsis* root grown in H_2O treated soil.

C. Isolation of resting spores, Day 1 (Steps C1 to C13) and Day 2 (Steps C14 to C20)

1. Use 250-300 g frozen clubs. Thaw shortly at room temperature. Surface sterilize the material in 70% ethanol for 2 min followed by 1% sodium hypochlorite for 5 min. Rinse 5 times with sterile H_2O. Repeat the entire surface sterilization step.

Note: Only use sterilized H_2O from now and onwards. Autoclave Miracloth and rinse the household mixer with 70% alcohol and sterile H_2O before use.

2. Homogenize galls in 500 ml H_2O using a household mixer. Avoid over-heating of the material by running the mixer for 30 sec followed by a 30 sec break. Repeat until a colloidal suspension has formed (Figure 3A).
   Note: Scalpel can be used to cut the root material into smaller pieces ahead of the mixing.
3. Filter the homogenized tissue through 4 layers of Miracloth to remove debris. Depending on the club size and amount of debris this step may require a new round of filtering (Figure 3A).
4. Transfer the filtered liquid into 50 ml Falcon tubes up to maximum volume and spin.
   Note: All centrifugations should be carried out using a tabletop centrifuge at 3,650 x g for 15 min if not otherwise is mentioned.
5. A pellet with two layers will form: a brown upper layer containing spores and a white layer with starch (Figure 3B). Carefully remove the supernatant with a pipet using a 1 ml tip. Transfer the spores to a new tube. Avoid starch contamination.
   Note: A dense spore layer may require a spoon to facilitate spore removal.
6. Wash the spores by re-suspending them in about 40 ml H_2O and repeat centrifugation (Figure 3C). Repeat washing and centrifugation if a white layer of starch is visible.
   Note: All volumes from now and onwards are adjusted to 2-4 ml of spores.

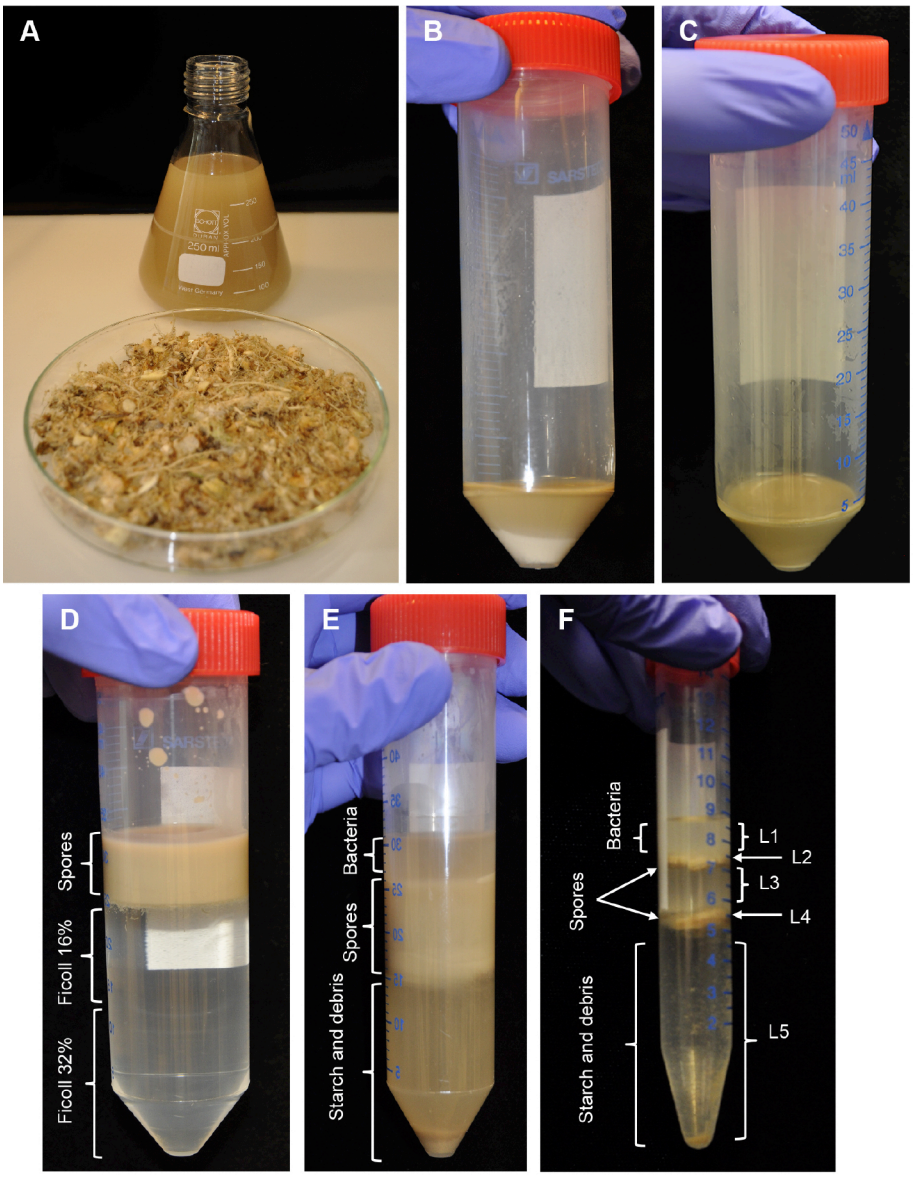

Figure 3. Spore isolation. A. Filtered liquid containing spores and the club roots left over after Miracloth filtering. B. A pellet with two layers: spores (brown) and starch (white). C. Spore layer after washing steps. D. Spores from Step C7 on top of two-step Ficoll gradient. E. Three layers after Ficoll gradient centrifugation (Step C9): upper layer with bacteria, middle layer mainly spores and bottom layers with starch and debris. F. Five layers (L1 to L5) after Ficoll gradient (Step C16), upper layer (L1) with bacteria, middle layers mainly spores (L2 and L4) and bottom layer (L5) with starch and debris.

7. Re-suspend the pellet in 5 ml H_2O. Check the purity and presence of the spores using a microscope (Figure 4A).

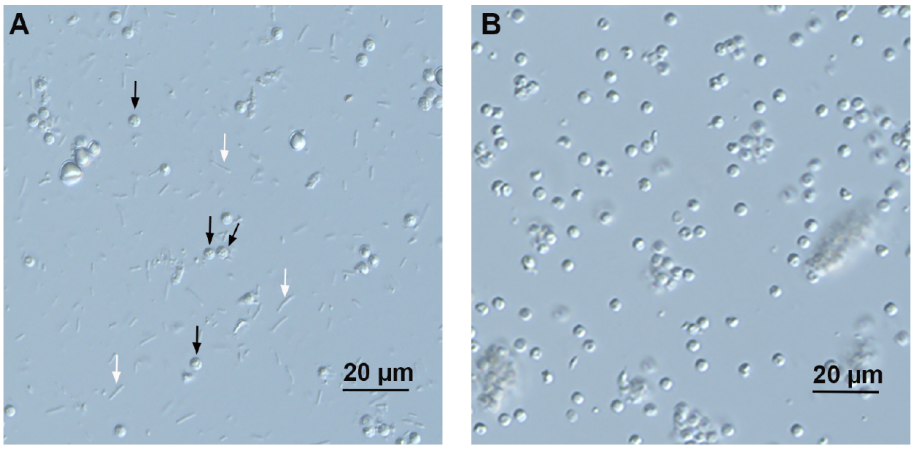

Figure 4. Different levels of spore purity. A. Spore preparation at Step C7. Spores are indicated with black arrows and debris is indicated with white arrows. B. Clean spores got at Step C20. Spore diameter, 2.75-3.75 µm.

8. Prepare a density gradient in a 50 ml Falcon tube. Add 32% Ficoll over which carefully layer 16% Ficoll and add the re-suspended spores from Step C7 on top (3v 32% Ficoll:2v 16% Ficoll:1v spores). Make sure the interphase between 3 layers is undisturbed (Figure 3D).
9. Centrifuge for 15 min at 400 *x g*. Avoid high-speed centrifugation. Several layers will form (Figure 3E).
10. Carefully aspirate and discard the upper layer. Transfer the spores to a 15 ml Falcon tube. Wash the spores using 10 ml of H_2O and centrifuge.
11. Dissolve the pellet in H_2O reaching a final volume of 5 ml. Add 5 µl of rifampicin and streptomycin each into the tube and incubate for 1 h at 37 °C. Pellet the spores and wash them with 10 ml of H_2O. Repeat centrifugation and remove supernatant.
12. Treat the spores with 5 ml ethanol (70%) for 2 min (not longer) and centrifuge. Wash the spores with 10 ml of H_2O and centrifuge. Repeat the washing-step with H_2O at least twice.
    Note: It is important to remove all ethanol.
13. Suspend the pellet in 5 ml H_2O. Add 5 µl carbenicillin, 5 µl pimaricin, 12.5 µl cefotaxime and 2.5 µl hygromycin B. Incubate the tube at room temperature overnight.
    Note: The antibiotics are active against various bacteria and fungal species.
14. Pellet the spores, wash with 10 ml of H_2O and repeat centrifugation. Re-suspend the spores in 5 ml lysozyme solution and incubate for 2 h at 37 °C.
15. Pellet the spores and re-suspend the pellet in 5 ml PBS buffer.
16. Repeat Step C8 and Step C9 with Ficoll density gradient centrifugation.
17. Collect the spores, layers 2 and 4 (Figure 3F) and wash them with 10 ml H_2O and centrifuge. Dissolve the pellet in 2 ml TE buffer containing 20 µl of DNase I and incubate for 2 h at 37 °C.
18. Add 3 ml termination buffer and incubate for 4 h at 37 °C.
19. Pellet and wash the spores 3 times each using 10 ml H_2O.
20. Dissolve the pellet in half volume of H_2O. Store the spores at -20 °C for future DNA or RNA

extractions. Clean spores should now have been achieved (Figure 4B).

Note: The amount of water to dissolve the pellet is dependent on the amount of spores. If we end up with 500 µl spores, we add 250 µl water.

D. DNA isolation

1. Add 7 ml of CTAB extraction buffer and 21 µl of 2-mercaptoethanoethanol (0.3% v/v) to a 15 ml Falcon tube.
2. Pre-heat the mixture in a water bath at 65 °C.
3. Grind the frozen spores to a fine powder in liquid nitrogen. Use mortar and pestle.
4. Add the spore-powder into the pre-heated extraction buffer (about 300 mg of spore-powder in 7 ml extraction buffer).
5. Incubate the solution at 65 °C and mix gently every 10 min for 1 h.
   Note: Avoid mechanical disruption of DNA by vortexing and excessive pipetting! This is very important throughout the procedure.
6. Add 1 volume of phenol:chloroform:isoamyl alcohol and mix gently for 5 min. Centrifuge and pipette the upper aqueous phase into a new Falcon tube.
   Note: Avoid to pipet aqueous/organic layer interface.
7. Add 5 µl RNase A to the solution and incubate for 45 min at 37 °C. Mix gently by inverting tubes 4-6 times.
8. Add 1 volume of phenol:chloroform:isoamyl alcohol and mix by inverting for 5 min. Centrifuge and pipette the aqueous phase to a new Falcon tube.
   Note: All centrifugations should be carried out on a tabletop centrifuge at 3,650 x g for 10 min if not otherwise mentioned.
9. Add 1 volume of chloroform and mix by inverting for 5 min. Centrifuge and pipette the upper aqueous phase to a new Falcon tube.
   Note: Repeat this step if phenol still is present in the solution.
10. Add 1/2 volume of 5 M NaCl to the sample and mix gently by inverting. Add 3 volumes of fresh and cold ethanol (95%) and mix gently by inverting. Keep at -20 °C for 1 h, not longer.
11. Centrifuge to pellet DNA. Carefully decant the supernatant and wash (do not re-suspend) the DNA pellet with 3 ml of 70% ethanol. Centrifuge again and carefully decant the supernatant.
    Note: Repeat washing to remove precipitated NaCl and other contaminants were seen as whitish crystals in the pellet.
12. Remove all visible ethanol from the final pellet. Let air-dry the DNA for 15 min at room temperature. Carefully suspend DNA in 200 µl (or less) of 0.1x TE buffer. Optional: dissolve DNA overnight at +4 °C.
    Note: Handle DNA with care, avoid vortexing and heavy pipetting. If DNA will be used for massively parallel sequencing, adjust elution buffer according to planned procedures.
13. Assess the quality and concentration of extracted DNA by using a NanoDrop or DropSense or a similar device (Figure 5).

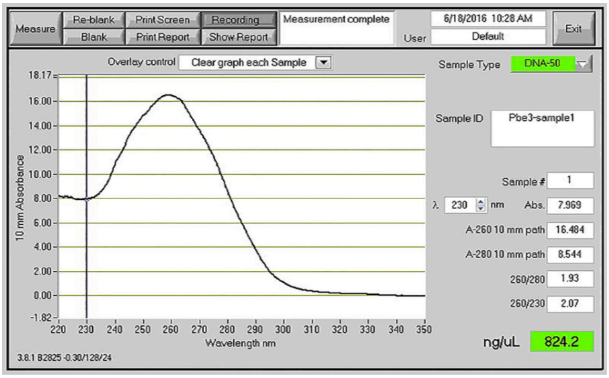

Figure 5. DNA quality check. NanoDrop measurement profile of *P. brassicae* DNA using this protocol.

E. RNA isolation

1. Grind the frozen spores to a fine powder in liquid nitrogen. Use a mortar and pestle.
2. Continue RNA extractions using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Notes

Root infection varies much due to the environmental conditions and experiments should first be run to ensure proper club formation. The yield of resting spores and consequently of *P. brassicae* DNA vary depending on the club size and stage of maturation.

Recipes

1. 1x PBS buffer (50 ml)
   137 mM NaCl
   2.7 mM KCl
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   Sterile water up to the final volume
   Adjust pH to 7.4 with HCl and autoclave
2. 1x TE buffer (30 ml)
   10 mM Tris-HCl
   0.1 mM EDTA
   Sterile water up to final volume, autoclave
   Store at 4 °C or room temperature
3. Termination buffer (50 ml)
   0.5 M EDTA
   1% N-lauroylsarcosine (v/v)

0.1 mg/ml Proteinase K

Note: The Proteinase K powder should be dissolved at a concentration of 20 mg/ml in sterile 50 mM Tris (pH 8.0), 1.5 mM calcium acetate and stored at -20 °C.

4. CTAB extraction buffer (CTAB) (50 ml)
   100 mM Tris-HCl (pH 7.5)
   25 mM EDTA
   1.5 M NaCl
   2 % (w/v) CTAB (cetyltrimethylammounium bromide)
   Sterile water up to final volume
   Filter sterilize using Filtropur and store at room temperature

Acknowledgments

We thank the plant growth facility staff at the Faculty of Natural Resources and Agricultural Sciences, Swedish University of Agricultural Sciences (SLU) for close attention of the plants. We also thank Dr. Arne Schwelm that started the extensive work of *P. brassicae* DNA preparation 2009. The authors declare that they have no conflicts of interest or competing interests. The work was funded by the Swedish Research Council (Formas) and SLU.

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