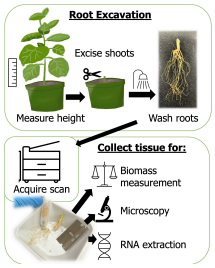
Aug 08, 2023 Version 1

Processing and maintenance of Nicotiana benthamiana tissue for phenotypic, histological, and ribonucleic acid analysis V.1



DOI
dx.doi.org/10.17504/protocols.io.261gedpqwv47/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.261gedpqwv47/v1

Protocol Citation: Brandon G Roy, Marc Fuchs, Marc Fuchs 2023. Processing and maintenance of Nicotiana benthamiana tissue for phenotypic, histological, and ribonucleic acid analysis. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.261gedpqwv47/v1

Protocol status: Working
We use this protocol and it's working

Created: June 15, 2023

Last Modified: August 08, 2023

Protocol Integer ID: 83506

Keywords: phenotyping, root, root system architecture, plant, virus, infection with grapevine fanleaf virus, grapevine fanleaf virus, model herbaceous plant for virus, acid analysis nicotiana benthamiana, root phenotyping software, nicotiana benthamiana tissue, maintenance of nicotiana benthamiana tissue, root tissue, images of whole root crown, borne virus, disease symptomology in leaf, plant host, present in most vineyard, utilization of other virus, other plant host, plant host below ground, other virus, adaptation to other plant host, whole root crown, most vineyard, leaf symptom, plant height, fruit

Funders Acknowledgements:
National Science Foundation Traineeship in Digital Plant Science
Grant ID: Award #1922551

Abstract

Nicotiana benthamiana is extensively used as a model herbaceous plant for virus-host interactions and sytems biology research. Here we describe a methodology to phenotype the root system architecture of *N. benthamiana* plants following infection with grapevine fanleaf virus, an economically important soil borne virus that is present in most vineyards worldwide. Upon completion of this protocol, images of whole root crowns can be analyzed with root phenotyping software. Following image acquisition, root tissue can be further processed for cross sectioning and ribonucleic acid extraction. Additional data (plant height, dry biomass, and leaf symptoms) were collected for correlation analysis. Documentation of root system architecture is an important first step to understand how a virus can manipulate its plant host below ground when most research is focusing on disease symptomology in leaves and fruits. This protocol allows for utilization of other viruses that infect *N. benthamiana* and adaptation to other plant hosts.

Image Attribution

Roy 2023

Materials

Plant growth

- Nicotiana benthamiana seed
- Soil/plant growth medium
- Pots
- Potting stick labels
- Fertilizer (Scott's Miracle Grow)

Inoculation

- Inoculum source of grapevine fanleaf virus (GFLV) in either infected plant tissue (Roy et al. 2023) or infectious clones of GFLV RNA1 and RNA2 in Agrobacterium tumefaciens (Osterbaan et al. 2018; 2019)
- Mortar and pestle
- Potassium dihydrogen phosphate (KH_2PO_4)
- Disodium phosphate (Na_2HPO_4)
- Deionized water

Excavation of crown root systems

- Bucket(s)
- Water
- Scissors
- EPSON 12000XL Scanner
- Clear scanning trays (12" X 18", 30.48 cm X 45.72 cm)
- Solid color background (we utilized a black felt on cardboard, 14” X 20”, 35.56 cm X 50.8 cm)

Root phenotyping

- Windows computer for Rhizovision Software, all other software is cross-compatible, including;
- EPSON Scanner 2 Software
- R and RStudio
- Microsoft Excel or Google Sheets

Tissue Collection

- 10% bleach
- 70% ethanol RNAse Away Sterilized steel BBs Razor blade Tweezers
- Nuclease-free or molecular grade water
- Nuclease-free 1.5 Eppendorf collection tubes
- 2 mL Eppendorf tubes
- Pipettes (P10, P20, P200, P1000)
- Omega E.Z.N.A. Plant RNA Mini Kit (cat no. R6827)

Troubleshooting

Plant production and growth

1 Seeds of *Nicotiana benthamiana* were sown in Cornell LM-2 soil, and seedlings were transplanted after ~14 days into individual pots. Throughout all growth stages, plants were maintained in controlled growth chamber conditions (16:8 light:dark photoperiod, 25 °C, 70% humidity). Plants were watered daily and provided Scotts Miracle-Gro® All Purpose Plant Food 24-8-16 once weekly. Experiments consisted typically of 100 plants.

Viral inoculation

2 Plants at the ~5 leaf stage were mechanically inoculated with GFLV using infected *N. benthamiana* leaf tissue as inoculum (Roy et al. 2023). Briefly, carborundum powder was dusted onto the leaves of each plant. The inoculum was prepared by macerating the infected leaf tissue with a mortar and pestle in a phosphate buffer (0.15 M KH_2PO_4, 0.35 M Na_2HPO_4, pH 7.0) and rub inoculated onto the two basal leaves. Water was generously applied to each plant using a spay bottle to eliminate the carborundum power and prevent additional abiotic stress. After the inoculation step, plants were monitored for phenotypical changes and tested for the presence of the virus in apical, uninoculated leaves. Mock inoculated plants, e.g., plants rubbed with phosphate buffer only, were used as controls.

2.1 Alternatively, *Agrobacterium tumefaciens*-mediated inoculation with infectious clones of grapevine fanleaf virus can be performed to infect *N. benthamiana* plants according to Osterbaan et al. (2018).

Citation

Osterbaan LJ, Schmitt-Keichinger C, Vigne E, Fuchs M (2018)
. Optimal systemic grapevine fanleaf virus infection in Nicotiana benthamiana following agroinoculation..

https://doi.org/10.1016/j.jviromet.2018.04.006 LINK

Testing viral infection status

3 Leaf and root tissue was used to test GFLV infection status by RT-PCR or RT-qPCR with specific primers beyond 4 days post-inoculation, and by DAS-ELISA with specific

antibodies beyond 14 days post-inoculation, as previously reported (Osterbaan et al. 2018; 2019, Roy et al. 2023).

Excavation of root systems

4 Gloves are recommended during all steps of root excavation.
Note: It is easier to excavate roots when the soil is dry.

1. **Excise shoots from roots.** Height of plants were measured prior to root excavation. The, the shoots were cut at the soil boundary line and samples were placed into a brown paper bag labeled with treatment and plant number.
2. **Remove soil.** Pots of *N. benthamiana* plant were squeezed on the side to loosen soil, and then inverted to release the soil and plant. The soil was then removed from the crown root system by gently rotating the root system in a vigorous motion.
3. **Wash root system**. Roots were submerged by hand in a bucket filled with distilled water to remove the remaining soil still attached to the more fiberous roots. The root system was then rotated within the water to shake off excess soil. Finally, any excess soil particles were removed by carefully massaging the root system.
4. (Optional) If soil was difficult to remove, a spray bottle with distilled water was used on troublesome areas, especially at junction points too small for hands to reach.
5. **Dry roots.** Roots clean of soil particles were placed on a towel to briefly remove water.

Root system architecture phenotyping

5

1. **Place clean roots on a scanning tray.** Clean roots were placed on clear scanning trays compatible with the EPSON 12000XL Scanner bed size. Between eight to fifteen *N. benthamiana* root systems could fit depending on respective size of roots. Pot labels were placed neatly beside each root system to document the order of individuals on each image acquired.
2. **Place tray and background on scanner.** Scanning tray was placed on the EPSON 12000XL Scanner bed and the black background (black felt and cardboard in our case) was placed on top of the scanning tray.
3. **Acquire image(s).** A computer with the EPSON Scan 2 software connected to the scanner was used with the following software parameters for image acquisition: ≥400 dpi and grayscale 16-bit. Roots were flipped 180 degrees to acquire images of the two sides of the root system.
4. **Save images for future analysis.** Files were saved in .tiff file format. Each image was saved with the date of acquisition, treatment, and side of the root system.
5. *Depending on the next step(s) of tissue preservation it is important to immediately complete step #6 for optimal results.*

Collection of tissue for histological or ribonucleic acid use

6 After confirmation of the viral infection status, the root system of a random subset of *N. benthamiana* individuals were used for the following three analyses:

1. Dry mass measurement
2. Tissue staining
3. Ribonucleic acid (RNA) extraction

For dry mass measurement, crown root systems were placed in the corresponding brown paper bag with the previously excised shoot system. The bags of shoots and/or roots were placed in a drying oven at 105 °C for 1 week and dry biomass measurements were taken. Shoot and root dry biomass were taken independently on a scale.

For root tissue staining, two methods were used in which the only difference was the final preservation step. In the instance of fresh tissue, whole root systems were placed into a 15 mL conical tube for immediate staining to detect reactive oxygen species (ROS). For preserving samples, the top half of the root system was excised with a razor blade and placed into a pre-labeled 2.0 mL Eppendorf tube filled with 70% ethanol. Only fresh samples were used in the ROS assay described below, however, preserved samples were used in histological stains specific to xylem and/or phloem. Root tissue was stained to detect ROS using diaminobenzidine (DAB) staining methods.

Citation

Vanacker H, Carver TL, Foyer CH (2000)
. Early H(2)O(2) accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction..

https://doi.org/10.1104/pp.123.4.1289 LINK

Citation

Daudi A, O'Brien JA (2012)
. Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves..

https://doi.org/10.21769/BioProtoc.263 LINK

For RNA extraction, the bottom half of the root system of individual plants was collected on a weighing tray using a razor blade. Collected root samples were placed into a pre-

labeled RNAse free 2.0 mL Eppendorf tube containing two sterile steel BBs using sterile tweezers. Samples were flash frozen in liquid nitrogen and stored at -80 °C until RNA extraction and processing. Total RNA was extracted from root samples as previously described for leaves of *N. benthamiana* (Roy et al. 2023). Briefly, tissue was flash frozen before maceration with sterile steel BBs in a Retsch Mixer Mill MM400 (Model 04182-09, Retsch USA, Verder Scientific, Inc.) at a frequency of 60 Hertz for two minutes, and then, total RNA was isolated by using an Omega E.Z.N.A. Plant RNA Mini Kit (cat no. R6827). A final elution was performed in molecular grade water.

Executing the above steps yields tissue suitable for cross section analysis under a microscope or purified total RNA isolation for viral diagnostic RT-PCR or RT-qPCR, or root transcript analyses by 3′RNA-Seq or RT-qPCR. Data from these analyses were used to complement root phenotypic information collected by excavating root systems in combination with image analyses with the Rhizovision Explorer software.

Citations

Step 6

Vanacker H, Carver TL, Foyer CH. Early H(2)O(2) accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction.
https://doi.org/10.1104/pp.123.4.1289

Step 6

Daudi A, O'Brien JA. Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves.
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