Bioassay *Phytophthora sojae*-soybean

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Inoculum: *Phytophthora sojae*

Materials

- Petri dishes containing clarified V8-gellan gum medium
- Mother culture[1] of *Phytophthora sojae*
- Small hermetic plastic containers (ca. 350 ml)

Equipment

- Autoclave
- Incubator
- Magnetic stirrer
- Rocking shaker
- Flame for sterilization

Day 1.

> Following good laboratory practices for microbiology, inoculate 2 Petri dishes containing V8-gellan gum (see recipes below) with the *Phytophthora sojae* isolate you want to test. Incubate at 28˚C for 6 days.

- V8 clarified

  Mix 14 g $CaCO_3$ with 946 ml of V8 juice. Stir for 15 minutes with a magnet.
  Centrifuge @ 4000*g* for 5 minutes
  The supernatant is harvested and frozen in 50 ml-aliquots if not immediately used.

- V8-gellan gum (or phytagel) 20%

  For 1 L
  In a 1-Liter bottle, mix 200 ml of clarified V8 with 12 g of gellan gum and add water up to a final volume of 1 L
  Autoclave @ 121˚C for 20 minutes

[1] Our mother cultures are simply pieces of mycelium in water kept in sterile tubes

Day 7.

> Cut the mycelium from the 6 day-old cultures with a cork borer to obtain mycelial plugs. Transfer about 60 plugs in a small hermetic plastic container. Add 15 ml of a cold soil decoction to 60 ml of sterile water. Close the container(s) tightly and put it(them) on a rocking shaker overnight at low/medium speed.

Materials

- 10 g of Promix
- 1 L distilled water
- Stir for 15 minutes on a magnetic stirrer
- Filter and sterilize the filtrate @121˚C for 20 minutes
- Store at 4˚C prior utilization

Day 7 and/or 8.

> After observation and zoospore counting under a microscope, harvest the liquid from the container(s) and use it for inoculation. (1 µl-drops of supernatant are placed on a microscope slide and, without cover slide, observed for counting under a microscope at 100X)

N.B. *If your count is below 2 zoospores/µl, harvest the supernatant and inoculate the hydroponic system(s) with it at day 7. However, with the same mycelial plugs, repeat a run of 15 ml soil decoction-60 ml sterile water-rock overnight and harvest a second inoculum. At day 8, this second inoculum is added to the hydroponic system(s).*

Plants: soybean seedlings for hydroponic bioassay

Materials

- Sterilized (or pasteurized) vermiculite
- Soybean seeds
- Sowing trays and plastic cover
- Dark plastic bags

Day 2.

> Sow the seeds in humid vermiculite at ca. 1 cm below the soil line. Place a cover on the sowing tray to keep a high humidity level during germination. Keep the tray in the dark with a black plastic bag for 3 days.

Day 5.

> Remove plastic bags and put the seedlings under light (in a window or under a neon light) for 3 days

D-day: bioassay

Materials

- Hyrdroponic systems (dish pans, polystyrene floater and baskets; see Annex I)
- Salts: 20-20-20, Epsom salt, Fe-EDTA
- Pipets
- Rockwool
- Nitrile gloves

Equipments

- Growth chamber

Day 7.

> Fill the hydroponic systems (grey dish pans) with 10 L of water and add the following ingredients to each system:
>
> 20-20-20 1.3 g
> $MgSO_4.7H_2O$ 1.5 g

OR

> Add 20 ml of a concentrate nutrient solution per 10 L-system (see below)

AND

> Add 3 ml of the iron solution (see below)

- Nutrient concentrates

  65 g 20-20-20
  75 g Epsom salt
  Complete the volume to 1 L with tap water

- Iron solution Fe-EDTA (13.2% Fe) (3333X)

  45 g Fe-EDTA (13,2% Fe)
  Complete the volume to 1 L with tap water

> Install each polystyrene floater in a dish pan. Rinse plantlet roots with tap water to remove vermiculite. Coat the roots with rockwool that has been

drenched in water beforehand. Transfer plantlets and rockwool in the 2”-baskets. Place baskets in the polystyrene floater according to the experimental design (if there is one).

Once plantlets are transferred, place each dish in a growth chamber under the following parameters:

Photoperiod: 12 h
Relative Humidity (%): 40%
Light: 440-500 µmoles
Day temp: 28˚C
Night temp: 16˚C

Harvesting and scoring

Day 21. Vertical resistance

> Plantlets are removed from the hydroponic systems, cleaned and pooled according to the line/cultivar tested. For example, if you used an "N" of 5, you must pool those 5 plants and compare them against the susceptible checks (usually cv. Harosoy).

NOTE: Usually, it is very easy to discriminate susceptibility from resistance when testing for vertical resistance. The susceptible plants are mostly dying after 2 weeks in culture. This is the type of results obtained when pathotyping (characterization of a new *Phytophthora sojae* isolate) or phenotyping for vertical resistance (characterization of Rps genes in new soybean lines/cultivars). Alternatively, you can use plant dry weight to discriminate resistant from susceptible lines.

Day 28. Horizontal resistance

> For horizontal resistance you can use plant dry weight and/or a susceptibility scale (see Annex III).

Assembly of the Hydroponic system

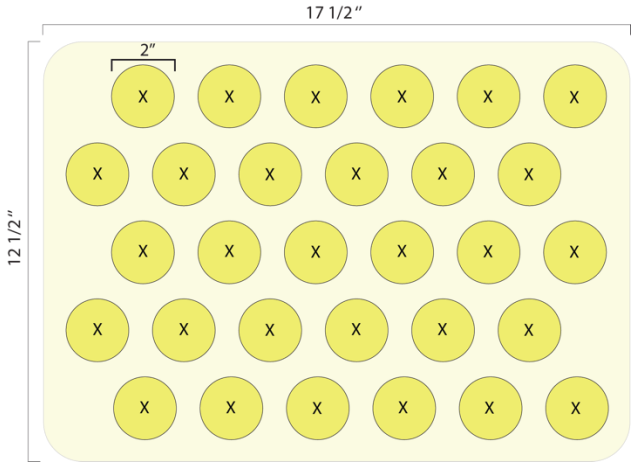
Materials

- Grey dish pan (5” x 15” x 20”)

- Extruded polystyrene panel 1,5” x 2’ x 8’

(Or any other material light enough to float)

- Template for hole drilling and polystyrene floater design



- Bench drill

Step 1. Cut polystyrene sheet in pieces of 12 ½" x 17 ½" (or according to the dimensions of the dish tray) with a bench drill

Step 2. With the help of the template (see above), make a mark for each hole with a clove and draw the corners with a marker.

Step 3. Drill the holes with the appropriate equipment (e.g. hole saw 2" *dia*. mounted on a bench drill)

Step 4. Cut the corners with a band saw.

Step 5. Install Styrofoam in the dish pan and insert 2”-baskets

ANNEX II

Susceptibility scale for *Phytophthora sojae* root rot (PRR) in soybean

Horizontal resistance classification

Classification	Symptoms	Susceptibility score	
Class I	Healthy root system with good density and white roots; good aerial growth.	1 to 1.5	
Class II	Root system slightly affected by discoloration; root density lower than the resistant check; aerial part healthy.	1.6 to 2.5	
Class III	Altered lateral root development; brown discoloration present on lateral roots (what is left); principal root still there and relatively healthy; aerial parts looking healthy	2.6 to 3.5	
Class IV	Root system heavily affected; brown, slimy, rotten roots. The plant still bearing green leaves looking but growth lower than Classes I to III.	3.6 to 4.5	
Class V	Root system totally black; plant dead or dying	4.6 to 5	

Warnings

If poor germination…
It magnifies the response and can lead to false positives.

If plantlets are too old…
If, for any reason, you inoculate seedlings that are older than 5-6 days, the response may be delayed and differences less discriminant, making the interpretation difficult.

Troubleshooting

The positive check is healthy

Your isolate has either lost its virulence or, more probably, has not produced enough inoculum. In either case, you must start over.

The negative check is diseased

There is possibly a contamination (caused or not by Phytophthora) in the system. This is why you should always use a control system i.e. without *Phytophthora sojae* addition.

Symptoms light and delayed

Plants were too old at the time of inoculation or inoculum too weak

Leaves turning yellow

You must fertilize a second time at day 10 if you wish to keep the bioassay for more than 14 days.

Pathotyping of *P. sojae* isolates

Panel of eight soybean differentials (Harosoy background)

Line (differential)	Rps gene
Haro (1-7)	*rps rps*
Haro 12	Rps1a
Haro 13	Rps1b
Haro 14	Rps1c
Haro 16-72	Rps1d
Haro 15	Rps1k
Haro 3272	Rps3a, Rps7
Haro 6272	Rps6, Rps7
Haro 72	Rps7

Panel of fourteen soybean differentials (mostly from Harosoy background)

Line (differential)	Rps gene
Haro (1-7)1	*rps rps*
Haro 12	Rps1a
Haro 13	Rps1b
Haro 14	Rps1c
Haro 16-72	Rps1d
Haro 15	Rps1k
L49-4091	Rps2
Haro 3272	Rps3a, Rps7
Haro 33	Rps3b
Haro 34XX	Rps3c
Haro 4272	Rps4, Rps7
Haro 52	Rps5
Haro 6272	Rps6, Rps7
Haro 72	Rps7