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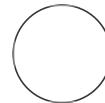
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Production of Rhizoctonia solani inoculum for sugar beet disease trials

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ABSTRACT

Rhizoctonia solani is an economically important plant pathogen resulting in substantial losses of crop production within the United States and worldwide. In order to properly study resistance to this pathogen, having a consistent, uniform disease pressure of correct intensity is essential to effectively screen plant cultivars for relevant disease traits. This procedure details the process of producing inoculum in order to create a uniform Rhizoctonia solani disease trials.

ATTACHMENTS

Colonized barley kernels.jpg colonized barley.jpg Contaminated barley.jpg contaminated flask.jpg

contaminated plates.jpg Dried barley inoculum.jpg flask and lid.jpg flask with PDB.jpg

flask with Rhizoc growing in PDB.jpg Flasks with Rhizoc growing in PDB.jpg ground inoculum.jpg Impulse polybag sealer.jpg

Partly colonized barley.jpg plate colonized with Rhizoc.jpg plate colonized with Rhizoc.jpg plates for long term storage.jpg

spawn bag.jpg wiley mill.jpg rhizoctonia solani inoculum production.docx

GUIDELINES

always use aseptic technique.

materials	manufacturer	Manufacturer #	specifications
Petri plates	Kord-Valmark	2910	100 x 15mm
Sterile blank concentration disks	Difco	1599-35	NA
Sterile barley	Montana milling	NA	hullless
Cryogenic tubes	Thermo scientific, nalgene	5000-0020	2ml
Incubator	NA	NA	temp: 25°C, photoperiod: 16h, humidity: 50-70%
Biosafety cabinet	NA	NA	NA
Core borer	Carolina	712202	size 3/16 in
Potato dextrose broth	MP biomedical	ICN 1008617	NA
Battled cell culture Erlenmeyer flask	Triforest enterprises	FBC05005	500ml
Filter cap	Triforest enterprises, vented duocap	DCPC-69	.22 micron
Shaker	NA	NA	temp: 25°C RPM: 190
Spawn bag	unicorn bags	UG-050418-B	10-B, 5 micron
Nanopure water	Thermo scientific, genpure pro UV	50131948	.2 micron, UV, cleaning solution
Impulse polybag sealer	ULINE	PFS-400	600w
Cloth bags	NA	NA	NA
Antibiotic	Coming	30002CL	penicillin, streptomycin: 10,000 µg/ml
Wiley mill	Thomas scientific	1188Y48	sieve size: 4mm

Preparation of culture for long term storage

- 1 Obtain desired isolate from either fungal isolation techniques or material transfer from a third party resource.
 - 1.1 For sugar beet, the Rhizoctonia solani strain R9 (AG2-2IIIB) has been used for identifying lines of sugar beet with strong resistance.
- 2 Prepare petri plates containing 25ml of potato dextrose agar and place sterilized medium to be colonized for long term storage atop of petri plate.
 - 2.1 Suspend 39g of Difco™ Potato Dextrose Agar in 1L of purified water. Mix and heat with frequent agitation until the powder is completely dissolved. Autoclave at 121°C for 15 minutes. Let cool and aseptically, in a biological safety cabinet, pipette out 25ml for each sterile petri plate. Let solidify and return to sleeve for future use.
 - 2.2 a. Sterile concentration disks or sterile barley kernels used as medium to be colonized for long term storage.

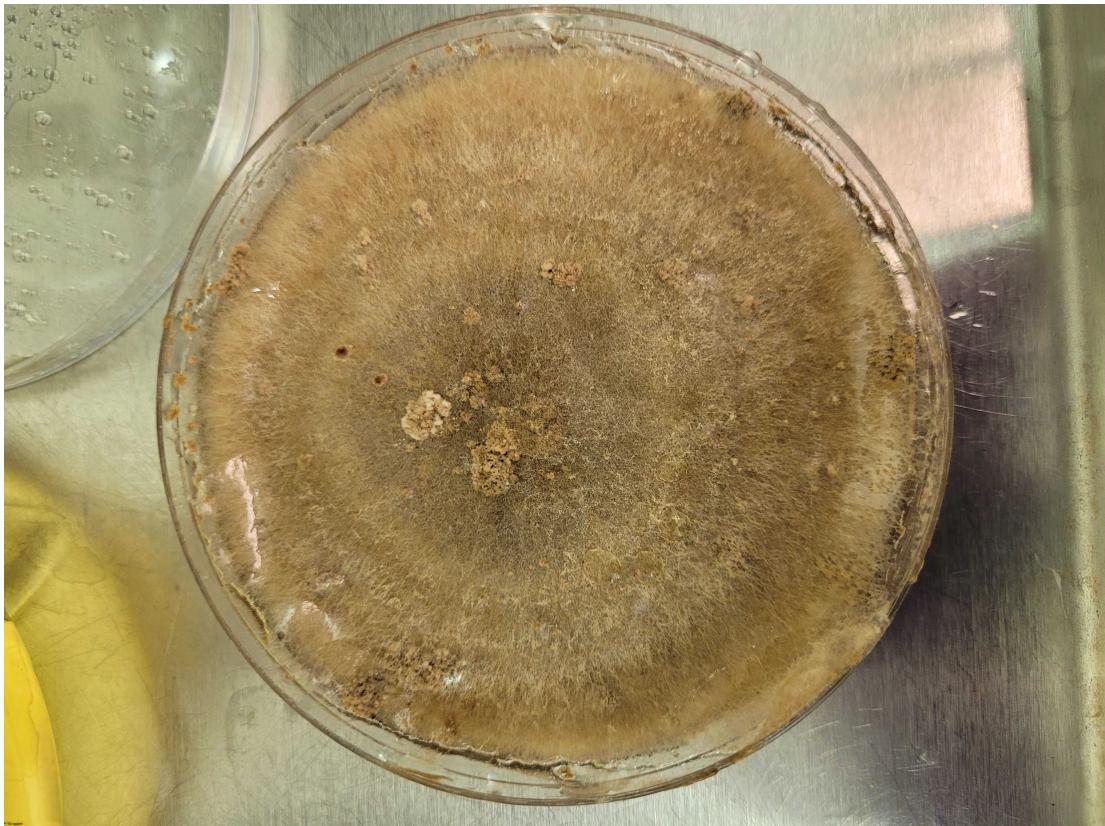
2.3

- b. Roughly 10 sterile kernels or Blank sterile concentration disks per petri dish.



colonization plates for long term storage

- 3 Using aseptic technique, in a biological safety cabinet, transfer desired fungal isolates onto petri plate described in step two of preparation of culture for long term storage.
- 4 Incubate petri plates at 25°C with a photoperiod of 16 hours for 4-5 days to fully colonize plate. (Webb et al., 2011, doi:10.1111/j.1744-7348.2011.00464.x)



colonized plate of *Rhizoctonia solani*

- 5 Once the petri plate is fully colonized, in a biological safety cabinet, using aseptic technique, transfer sterile medium colonized with isolate of interest into sterile cryogenic tubes and store at -80°C up to 21-26 months. (Sneh & Adams, 1996)

- 5.1 a. This will be your stock (generation 1, G1) for long term storage. Preparation of working cultures will be derived from this long term storage.

Preparation of working cultures from long term storage

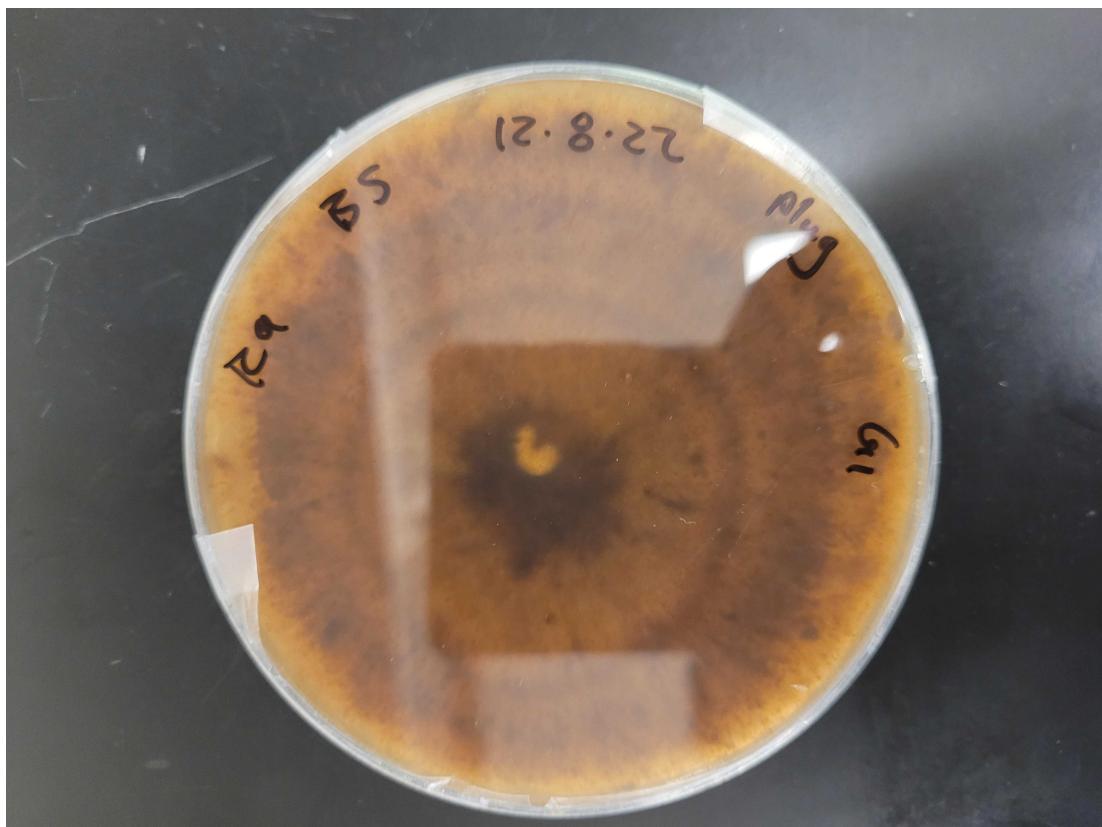
- 6 Obtain isolate of interest from long term storage.

Using aseptic technique, in a biological safety cabinet, transfer 5-10 pieces of colonized medium

7 from long term storage onto a petri plate containing 25ml potato dextrose agar and incubate at 25°C with a photoperiod of 16 hours for 4-5 days to fully colonize plate.

7.1 a. This will be the first generation colony. (G2)

7.2 b. These plates will be your working cultures for inoculum production.



colonized plate of *Rhizoctonia solani*

7.3 c. check plates daily for contamination.

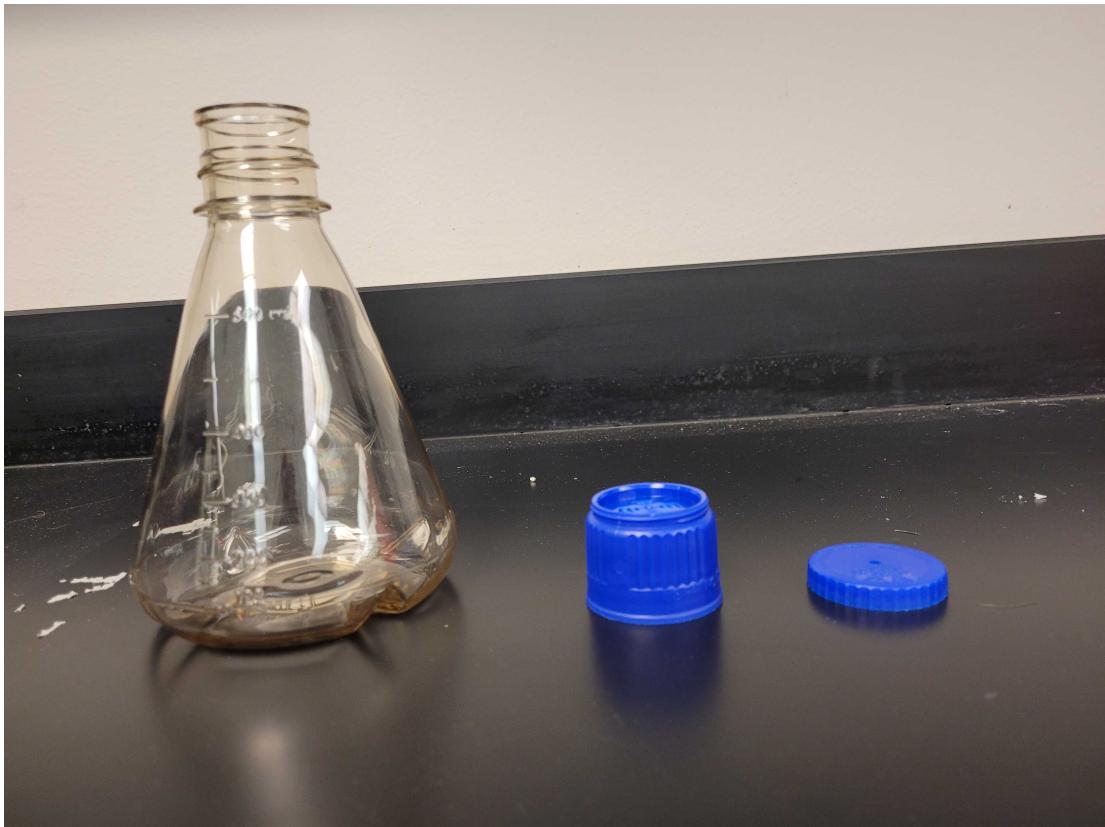


plates with fungal and bacterial contamination

- 7.4**
 - i. These plates should be kept in incubator at 25°C with a photoperiod of 16 hours for the duration of production.
- 8** If production requires more plates, using a sterile core borer, transfer G2 colony core onto a new PDA petri plate containing potato dextrose agar to form next generation (G3).
- 8.1**
 - a. On account of genetic drift, do not exceed past third generation (G3) (Lakshman et al., 2016)

Potato dextrose liquid broth inoculum production

- 9** Prepare 400ml potato dextrose broth (PDB) according to manufactures specifications in a 500ml baffled cell culture Erlenmeyer flask with a vented Duocap.



Erlenmeyer flask with a vented Duocap

9.1 a. Filter of the vented Duocap should be .22 microns.

10 Autoclave flask with PDB at 250°F (121.1°C) for 30 minutes on liquid cycle.

10.1 a. Make sure filter cap is loose on the flask with the secondary cap for the filter tightened.

11 Tighten the lid to the flask and allow flasks containing PDB to cool at room temperature.



Erlenmeyer flask with a vented Duocap containing PDB

- 12** Once cool, using aseptic technique in a biological safety cabinet, inoculate each flask with several (2-4) cores from a fully colonized working culture petri plate.
 - 12.1** a. Use a sterile core borer for cores.
- 13** Remove the secondary cap to expose filter, while the cap is still tight onto the flask, and incubate on a shaker for 7 days at 200 RPM at 25°C.



flasks containing *Rhizoctonia solani* incubating on a shaker

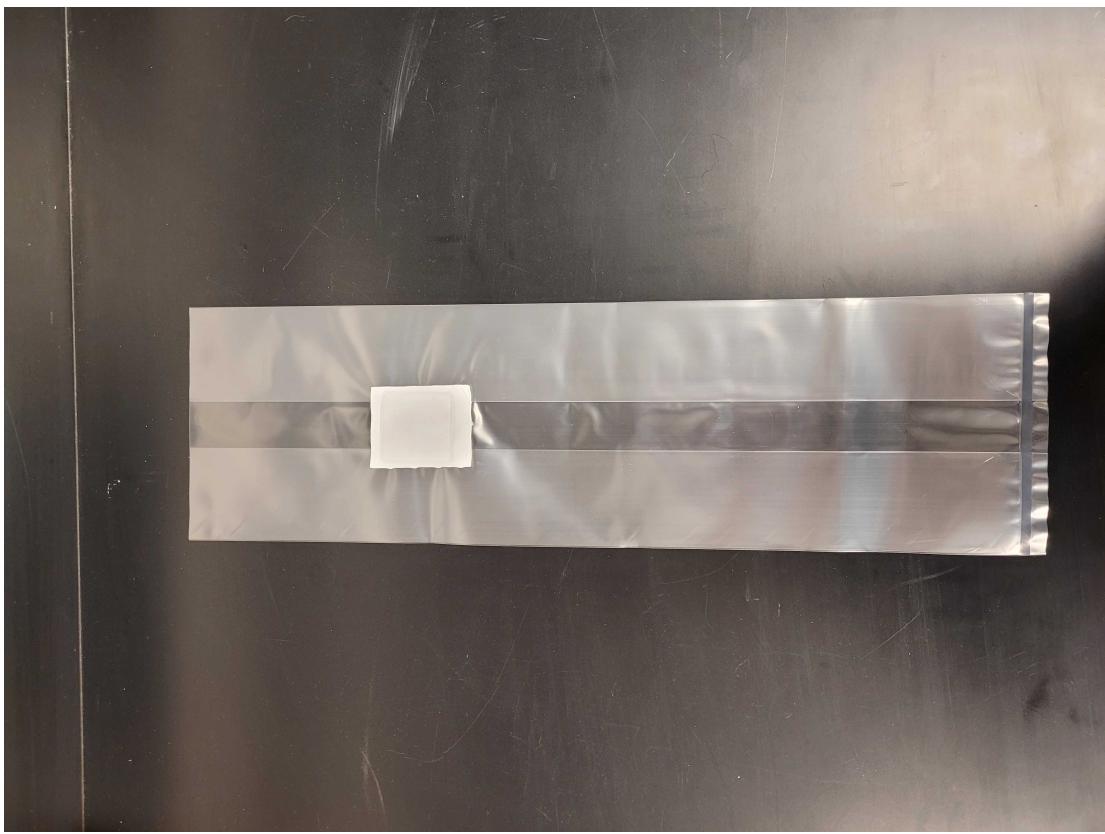
- 13.1** a. check flasks daily for contamination.



fungal contamination in a flask with PDB

Spawn bag production

- 14** Fill a type 10B polypropylene spawn bag with 2L of hull-less barley (~1.7kg) and add 550ml NANOpure filtered water.



type 10B polypropylene spawn bag

- 14.1** NANOpure water is water purified using Barnstead/Thermolyne Nanopure lab water system which produces NCCLS, CAP, or ATSM type 1 water with having 18.2 megohm purity with little to no biologicals present.
- 14.2** a. Seal bag with impulse poly bag sealer.



poly bag sealer

14.3 b. Mix barley and water in bag and let rest horizontally with filter facing up for 1 hour.

15 Place spawn bags into cotton cloth bags and place them into autoclave pans vertically.

15.1 a. Spawn bags are placed into cloth bags in order to not melt plastic spawn bags on metal autoclave pans.

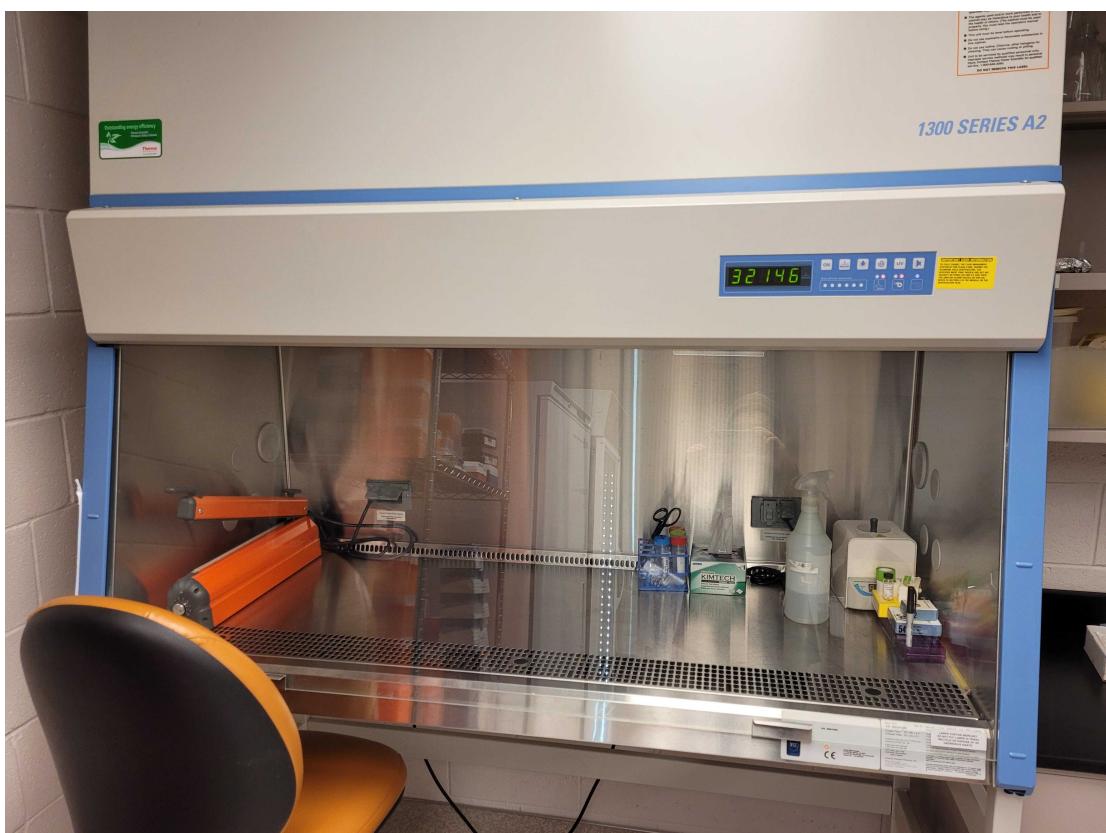
16 Autoclave spawn bags at 250°F (121.1°C) for 45min sterilization and 15min evacuation on liquid cycle.

16.1 a. Repeated two times in order to fully sterilize barley.

17 Remove spawn bags and set out to cool horizontally with filter facing up until room temperature.

17.1 a. Bags should not exceed 24hours of cooling.

18 Using aseptic technique, in a biological safety cabinet, add 4ml of antibiotic (10,000 ug/ml penicillin and 10,000 ug/ml streptomycin) to each colonized flask described in “potato dextrose broth inoculum production” step.



biological safety cabinet

- 18.1** a. Let sit for 15min.
- 19** Using aseptic technique, in a biological safety cabinet, open sterilized spawn bags and transfer one colonized flask of liquid inoculum into bag.
- 19.1** a. Seal spawn bag using impulse poly bag sealer.
- 20** Mix sealed spawn bag of barley with liquid inoculum and incubate horizontally with filter facing up at 25°C with a photoperiod of 16 hours and humidity at 50-70%.



partially colonized spawn bag

- 21** After 24hours, mix spawn bag containing barley and liquid inoculum by hand and return to incubator vertically for 5-10 days or until fully colonized.



fully colonized spawn bag

- 21.1** a. Check spawn bags daily for contamination.



contaminated spawn bag

- 21.2** Bacterial contamination of spawn bags show a "soupy" non cohesive barley with an unpleasant "sour" smell.
Fungal contamination of spawn bags show differing colors and possible hyphae that form spores.
R9 should appear only white and cohesive throughout the barley without any fruiting or spore structures.
- 21.3** Contaminated spawn bags should be autoclaved at 121°C for 30 minuets and disposed.

Inoculum

- 22** Once barley is fully colonized with desired isolate, open bags and let air dry at room temperature (20°C) with circulating air and a relative humidity of ~15% until completely dry.

- 22.1** Once Barley is completely dry using the proper PPE (mask, safety glasses/goggles, long shirt/pants, and hearing protection) grind the dry inoculum with a Wiley mill fitted with a 4mm sieve to reduce particulate size for application.
- 22.2** This gives final colonized inoculum ready for application.