Rhizoctonia solani inoculum production



Preparation of culture for long term storage

1. Obtain desired isolate from either fungal isolation techniques or material transfer from a third party resource.
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**.**
	1. Blank sterile concentration disks or sterile barley kernels used as medium to be colonized for long term storage.
	2. Roughly 10 sterile kernels or Blank sterile concentration disks per petri dish.
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)
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)
	1. This will be your stock (generation 1, G1) for long term storage.

Preparation of working cultures from long term storage

1. Obtain isolate of interest from long term storage.
2. Using aseptic technique, in a biological safety cabinet, transfer isolate 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.
	1. This will be the first generation colony (G2 )
	2. These plates will be your working cultures for production.
		1. Cultures should be kept in incubator at 25°C with a photoperiod of 16 hours for the duration of production.
		2. Check daily for contamination.
3. If production requires more plates, using a sterile core borer, transfer G2 colony onto a petri plate containing potato dextrose agar to form next generation (G3).
	1. On account of genetic drift, do not exceed past third generation (G3) (Lakshman et al., 2016)

Potato dextrose broth inoculum production

1. Prepare 400ml potato dextrose broth (PDB) according to manufactures specifications in a 500ml baffled cell culture Erlenmeyer flask with a vented Duocap.
	1. Filter should be .22 microns.
2. Autoclave flask with PDB at 250°F (121.1°C) for 30 minutes on liquid cycle.
	1. Make sure filter cap is loose on the flask with the secondary cap for the filter tightened.
3. Tighten the lid to the flask and allow flasks containing PDB to cool at room temperature.
4. 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.
	1. Use a sterile core borer for cores.
5. 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.
	1. Check daily for contamination.

Spawn bag production

1. Fill a type 10B polypropylene spawn with 2L of hull-less barley (~1.7kg) and add 550ml NANOpure filter water.
	1. Seal bag with impulse poly bag sealer.
	2. Mix barley and water in bag and let rest horizontally with filter facing up for 1 hour.
2. Place spawn bags into cloth bags and place into autoclave pans vertically.
	1. Spawn bags are placed into cloth bags in order to not melt plastic spawn bags on metal autoclave pans.
3. Autoclave spawn bags at 250°F (121.1°C) for 45min sterilization and 15min evacuation on liquid cycle.
	1. Repeated two times in order to fully sterilize barley.
4. Remove spawn bags and set out to cool horizontally with filter facing up until room temperature
	1. Bags should not exceed 24hours of cooling.
5. 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.
	1. Let sit for 15min
6. Using aseptic technique, in a biological safety cabinet, open sterilized spawn bags and transfer one colonized flask of liquid inoculum into bag.
	1. Seal spawn bag using impulse poly bag sealer.
7. 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%.
8. 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.
	1. Check spawn bags daily for contamination.
9. Once barley is fully colonized with desired isolate, open bags and let air dry on trays until completely dry.
	1. This gives final colonized inoculum for application.
	2. Further grinding of barley using a Wiley mill with a 4mm sieve to reduce particulate size for application may be performed after drying of colonized barley kernels.

**Citations**

Lakshman, Dilip k, et al. “Molecular Identification, Genetic Diversity, Population Genetics and Genomics of Rhizoctonia Solani.” *Perspectives of Plant Pathology in Genomic Era*, 2016, pp. 55–89.

Sneh, Baruch, and Gerard C. Adams. “Culture Preservation Methods for Maintaning the Genetic Integrity of Rhizoctonia Spp.. Isolates.” *Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*, 1996, pp. 139–145., https://doi.org/10.1007/978-94-017-2901-7\_12.

Webb, K.M., et al. “Long-Term Preservation of a Collection of Rhizoctonia Solani Using Cryogenic Storage.” *Annals of Applied Biology*, vol. 158, no. 3, 2011, pp. 297–304., https://doi.org/10.1111/j.1744-7348.2011.00464.x.