Node Propagation of Switchgrass

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This protocol can be used to clonally propagate switchgrass under sterile conditions in six weeks. It describes how to prepare and propagate the nodes on an MS-based media. These shoots are ideal for experiments that would require pest and pathogen-free switchgrass clones, such as microbiome studies.

SWITCHGRASS NODE PROPAGATION PROCEDURE.docx

Switchgrass Node Shoot Medium with Benomyl.docx

Switchgrass Rooting Medium with IBA & Ben.docx

SWITCHGRASS FIRST PLANTING PROCEDURE.docx

Image Guide to Switchgrass Propagation.pptx

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A key aspect of these micropropagation techniques is maintaining sterile conditions throughout the entire process. Sterile gloves and the use of 70% ethanol should help keep the hood area clean. After each use, flame sterilize the scalpel and forceps used for cutting or the transferring nodes/shoots before.
Keep a close eye on any areas that may have been brushed by gloves/tools or accidentally contaminated. If any contamination should appear, promptly transfer any uncontaminated shoots to fresh media and discard the rest. If nodes from particular plants appear to be heavily contaminated with endophytes, 33 mg/L of Rifampicin (in DMSO) can also be added to the initial propagation media to eliminate the endophytic pathogens.

Media Preparation:

MS Basal Medium with Gamborg's Vitamins (Sigma)
D-(+) Maltose monohydrate powder, suitable for plant culture (Sigma)
dd H_2O
Phytoblend plant agar (Caisson Labs)
6 Benzylaminopurine (stock 15mM) (Caisson Labs)
Indole-3-Butyric Acid (IBA) Sigma
Benomyl (Sigma) 5mg/ml aliquots (dissolved in DMSO)
DMSO
1 liter beaker
1 liter graduated cylinder
1 liter bottles with screw caps
15ml sterile centrifuge tube (Corning)
microfuge tubes (~1.5ml)
pH meter and calibration standards
1 M KOH, 0.1M KOH
Stirring platform and stir bars
Heated 57 deg.C water bath
Autoclave/autoclave tape
Sterile Petri Plates 100x25mm
PhytaTray II, Sigma
100ml Filter Flask, Sigma
Aluminum Foil

Node Propagation:

Scalpel and #10 blades
Forceps
Petri dishes 100x25mm and 100x15mm
Clorox Bleach (6% sodium hypochlorite)
TritonX-100
Sterile ddH_2O
Sterile 50ml centrifuge tubes
Graduated cylinder - 100ml
Timer
Sharpie marker
Nitrile gloves
10ml individually wrapped sterile pipettes

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Pipette bulb/pipettor
Laminar flow hood, EtOH, & flame source for sterilizing instruments
70% ethanol in spray bottle
Kimwipes, large
Variable speed rocking platform
Switchgrass strain of choice
Micropore tape, 1" wide
Time Tape

Planting Procedure:
Rooted Switchgrass shoots
3 inch square plastic pots or similar
2-ply cheesecloth
Clean (autoclaved or microwaved) soil mix, such as Redi-Eart
Mosquito Bits (Summit Chemical) to control for Fungus Gnats
Scissors: medium and fine point
Sharpie marker
Nitrile gloves
Time Tape
Plastic flat trays, inserts, ~3 inch pots, and clear plastic domes: short and tall
diH_2O
Nutrient Water

Prepare node propagation MS-based media:
- For 1 liter medium: 24 100x25mm petri plates = 2 sleeves
8-10 PhytaTrays with 100-125ml media

Prepare 6-BAP, Benomyl, and IBA stock solutions (see below)

Prepare Propagation Media (Plates)

1 MS Basal Medium: weigh amount on bottle per liter (4.4g for Sigma Aldrich product MS0404-10L), add to 800 ml ddH2O with stir bar

2 Add 30 g D+Maltose, and dissolve.

3 Adjust pH to 5.5 with 1M KOH.

4 Add MS medium to 1000ml cylinder and bring to 1000ml.

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5 Add 4 g PhytoBlend agar to each of two 1L bottles. Add 500ml MS medium to each bottle.

6 Loosely cap, add autoclave tape and autoclave 45 minutes at 121 deg C. Place in a water bath set to 57 deg C. until cool enough to handle.

7 In hood, add 333ul filter-sterilized 15mM 6-benzylaminopurine (6-BAP) stock to each 500ml of medium and swirl bottle
- **Final concentration = 10uM B-BAP**

7.1 **6-BAP Stock solution:** (15 mM, 100mls) Add 337.88 mg to a small tube, and add a several drops of 1M NaOH to dissolve.
Add ddH_2O to tube and transfer all to 100 ml graduated cylinder. Continue adding ddH_2O to small tube and then pouring it into cylinder until certain all 6-benzylaminopurine has been transferred to 100 ml cylinder.
Fill to mark with ddH_2O, and then filter sterilize.
Wrap bottle with aluminum foil - light sensitive. Store at 4 deg C.

8 In hood, add 500ul 5mg/ml stock Benomyl (well vortexed after freezing), to each 500ml cooled medium bottle.
- **Final concentration = 5 mg/L Benomyl**

8.1 **Benomyl stock (5mg/ml):** Add 50mg Benomyl to 10ml DMSO in a 15ml sterile plastic centrifuge tube. Tighten cap and vortex until dissolved. This is a 1000x preparation. In hood, aliquot into 1.5ml microfuge tubes. Do not add more than 1 ml per tube. Store at -20deg. C.

9 Pour medium into 100 x 25mm petri plates ~1/2 full. Let cool completely before packing. Store at 4 deg. C

PhytaTray Propagation Media

10 **Repeat steps 1-8** to prepare MS media with 10uM 6-BAP and 5 mg/L Benomyl

11 In hood, assemble PhytaTrays carefully ahead of time. The larger piece is the "bottom" into which the medium is poured. Crack lids, and pour ~100ml medium into PhytaTray and cover with shorter lid piece, snapping in place.

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Let cool completely before packing and storing at 4 deg C.

Rooting Media (in PhytaTrays)

12 **Repeat steps 1-6** to prepare MS media (but do not add the 6-BAP or Benomyl to this media)

13 In hood, add 5.0 ml IBA to each bottle containing 500ml of medium (1.0mg/L of IBA).

- For lowland genotypes, add 10.0 ml IBA to each bottle with 500ml of medium (or 2 mg/L) to increase the chance of root formation

13.1 **100mg/L IBA Stock:** dissolve 10 mg IBA powder in a few drops of 1 N NaOH in a microfuge tube. Add ~0.75 ml dd H_2O to tube and then pipette that solution into a 100 ml graduated cylinder. Continue adding dd H_2O to microfuge tube several times and pour liquid into cylinder until certain that all IBA has been rinsed from microfuge tube. Bring volume to 100ml with dd H_2O. Filter sterilize using filter flask assembly. Wrap bottle with aluminum foil and store at 4 deg. C.

14 If using Benomyl, thaw Benomyl (5mg/ml) aliquots and add 250ul to medium (final concentration: 2.5mg/L). Swirl to mix, and pour/divide into PhytaTray II containers. 1 liter medium will be sufficient for 7-8 PhytaTrays. Once solid, store at 4 deg. C.

14.1 *Note on Benomyl: If using nodes from greenhouse or field plants, Benomyl should be used in all media to control for the endophytic fungus Sarocladium strictum. If nodes are from plants grown in growth chambers and previously determined to be Sarocladium-free, I suggest as a safeguard, to add Benomyl at 5mg/L initially only in petri plates containing Propagation Medium. In the future, it may not be necessary to use Benomyl.

Node Propagation

15 Spray 70% EtOH on all metal surfaces of laminar flow hood, and wipe down well with Kimwipe. Place MS-M culture medium plates in hood and tilt lids slightly to dry moisture on medium

16 If switchgrass has visible dirt, rinse with ddH2O using a pipette or whatever is effective. Using a scalpel or sharp scissors, cut switchgrass internode tissue ~1 cm above and below the culm node. Do not take the node closest to the root

16.1 If unable to start work immediately, place node+tissue in a petri dish with a bit of sterile ddH2O to keep them moist. Cover dish.

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17 In hood, place node tissue in 50ml centrifuge tube(s), 10-15 per tube, depending on size

18 Add 25 ml 75% bleach/Triton X-100 solution to each tube. Screw on cap TIGHTLY. Place on rocking platform set to speed 4.5, and rock for 15 minutes. (Place tubes in the bottom half of a 150mm x 15mm petri plate to prevent them from rolling off platform.

18.1 Prepare 75% bleach/1% Triton X-100 solution **fresh each time**. Use deionized H2O

19 At the end of the bleaching time, remove tubes from rocker, and work in the laminar flow hood. Using a sterile 10ml pipette, remove bleach solution and add to waste bottle. Add ~40ml sterile ddH2O to each tube. Cap tightly and place on rocker for 5 minutes. Remove wash water with a pipette. Repeat this wash step for a total of 6 sterile ddH2O washes using a fresh pipette each time, and remove all

20 Dump nodes carefully from one tube into a 100 or 150 mm diameter sterile petri dish. Transfer one node to the lid of the petri dish and, using a sterile scalpel and forceps, cut node in half lengthwise (If nodes are large enough to easily cut). Place node halves, cut side down, on propagation medium, 4-6 nodes per plate, depending on size

21 Mark identifier/date, etc., on top of plates. Seal plate with 1 round of Micropore tape. Place plates in Growth Room (16/8 photoperiod, ~80-90 uE/M2/sec, ~78 deg. F room temperature.

22 Monitor often for mold contamination and shoot formation and record. If a node becomes contaminated, carefully remove uncontaminated nodes and plate on fresh medium.

23 Shoots should begin to form within 1 week. Check often, and note the condition of medium. When it begins to crack and recede, nodes must be transferred to fresh propagation medium prepared in PhytaTrays (~100-125 ml medium per PhytaTray).

Transfer to MS-M PhytaTray

24 Label PhytaTray using a piece of Time Tape (Sharpie markers do not write well on the PhytaTray plastic)

25 In hood, in a sterile petri dish, aseptically trim any necrotic/brown edges from the tissue, and transfers nodes with shoots to the PhytaTrays. Do not transfer any nodes that have not

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produced shoots - discard these.

Transfer to Rooting Medium PhytaTray

26 After 2-3 weeks, aseptically transfer shoots (repeat step 25) to Rooting Medium prepared in PhytaTrays. Return PhytaTrays to Growth Room. Monitor often for root formation and contamination.

27 When >=1 cm long roots have formed, rooted shoots may be planted in pots containing "clean" or sterile soil mix (Redi-Earth Propagation Mix or other suitable mixture).

Planting Procedure

28 Cut cheesecloth into 2-ply squares to fit in the pots and place in the bottom of each

29 Place tray insert into flat tray, and fit in the 32 pots. Add autoclaved soil to each pot and tap tray on the counter several times to settle soil but without compacting it

30 Cover with a dome and store in dark at 4 deg. C until ready to use

31 To a fresh tray and tray insert, insert sufficient pots for the number of rooted shoots to be planted

32 Add Mosquito Bits to each of these pots (~15-20 Bits per pot) and mix them into the soil just prior to planting

33 Label each pot appropriately using Time Tape or a small plastic plant marker stake

34 Remove the lid of the PhytaTray and fill it 2/3 with diH2O

35 Carefully remove a rooted shoot from PhytaTray containing Rooting Medium. Using a fine point scissors cut off any excess dead tissue

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36 Dip the rooted end of the shoot into the water and gently massage away any MS-M medium that has adhered to the plant

37 Place the cleaned plant into a pot to which a small depression has been made. Gently pat the soil mix up around the base of the plant

38 Add ~1/2 diH2O & 1/2 Nutrient Water to each pot by top-watering. Add enough to thoroughly moisten the soil mix and so a small amount drips through the cheesecloth. Place a tall clear dome on the flat. Place under lights in the Growth Room.

39 Monitor flat daily. Remove dome after ~3 days. Discard any dead/dying shoots. Feed with Nutrient water once a week. Let the soil dry between waterings. Monitor for fungus gnats.

40 Once plants appear to be developing new shoots and are large enough, they can be transplanted into larger pots as necessary

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