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562V-MSM - Co-Cultivation Medium (+L-Methionine)

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Transformation of B104 m…

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We use this protocol and it's working

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Disclaimer

Abstract

This is part of the Leiboff Lab maize transformation protocol for somatic embryogenesis of B104 immature embryos. This protocol is a combination of Chen et al. 2022 and Kang et al. 2022 with some modifications based on material availability. This protocol is intended for the GRF-GIF/BBM somatic embryogenesis transformation strategy with the LBA4404 Met-auxotrophic *Agrobacterium* strain.

Freshly dissected immature embryos will be transferred from 700A liquid infection medium (containing induced *Agrobacterium*) to Co-cultivation Medium 562V-MSM. After draining excess infection medium and orienting embryos scutellum side up, plates are sealed with micropore tape and embryos and *Agrobacterium* will be co-cultured for 3 days in the dark at room temperature. Plan to promptly transfer from 562V-MSM to 605CefT to help suppress *Agrobacterium* contamination, which is extremely difficult to combat once it is noticeable. Co-Cultivation Medium contains added synthetic auxin (2,4-D) to encourage callus and embryogenic shoot growth. 562V-MSM is high in sucrose to encourage rapid plant growth. 562V-MSM contains no selective agents, and provides L-Methionine to permit LBA4404 Met-*Agrobacterium* growth. After 3 days of co-cultivation, all prior trials exhibited a minor, white film of bacterial growth on the plate surface, with little to no detectable growth on embryos themselves.

562V-MSM solid media should be prepared in 15x100 (standard) petri plates, planning for ~20 embryos per plate. Material grown on 562V-MSM will be sealed with micropore tape and be incubated at 20-25C (room temperature) in the dark. Embryos are ready to move off 562V-MSM after 3 days. There may be noticeable growth/swelling on the scutellum side of the embryo at this time, but do not be alarmed if this is not obvious.

Planning

1 Estimate the volume of 562V-MSM you will need based on the following:

$$Volume = 30mL * NumberPlates$$

Each unique construct will need a round number of plates; make sure to round up!
Check the table below to plan your media

Mixing Heat-Stable Ingredients

2 Retrieve the following heat-stable ingredients:

1. MS Basal Salts - Stored in Main Lab, Dry Powder, 4C Refrigerator, Top Shelf
2. 2,4-D (5 mg/mL) - Stored in Main Lab, 1.5mL Aliquots, -20C Freezer, Bottom drawer 'Tissue Culture 1'
3. Sucrose - Stored in Main Lab, Dry Powder, Chemical shelf 'S', use Fowler refillable container
4. Agar, Phyto - Stored in Main Lab, Chemical shelf 'A'

3 Retrieve a graduated cylinder for measuring your final solution
Place a stir bar at the bottom on a beaker that is ~1.5x the volume of your solution
Rinse stir bar+beaker and graduated cylinder with MQ H2O, discard rinse water in sink
NOTE: Any soap or detergent residue will interfere with the tissue culture process; if you see suds, rinse again or find different glassware

4 Add approximately 90% of your final media volume in MQ H2O to your beaker
Place beaker on a magnetic stir plate
Turn stir plate on to generate a vigorous stir

5 Using a fresh weigh paper and dry spatula/scoopula/pipette tip for each ingredient, add the following to your beaker:

	A	B	C	D	E
	Ingredient	100 mL	200 mL	300 mL	600 mL
	MS Salts	0.44 g	0.88 g	1.32 g	2.64 g
	2,4-D	40 uL	80 uL	120 uL	240 uL
	Sucrose	3.00 g	6.00 g	9.00 g	18.00 g

6 Thoroughly rinse all used tools with running water
Place clean tools in drying rack
Return chemical reagents to their original storage location

Adjust solution pH to 5.7 with 0.1 M KOH

7 Turn on the Hanna Instruments pH meter
Unscrew and remove the small green pH probe exchange cover and set cap aside
Gently remove the probe from the storage tube and set storage tube aside
Using squeeze bottle, rinse the glass probe with H2O, catch rinse water in a waste beaker
Gently blot probe with laboratory tissue paper to dry

8 Using adjustable arm, lower the pH probe into the beaker with stir plate on
Ensure that the stir bar does not strike the probe
Electrode at the base of the probe must be fully submerged

9 Using a plastic transfer pipette, add 0.1M KOH to your solution until you measure pH 5.7
NOTE: KOH can be added rapidly until pH 5.4, then add one drop at a time to reach pH 5.7
Solution pH between 5.6 - 5.8 is acceptable

10 Using the adjustable arm, remove the pH probe from the beaker
Using squeeze bottle, rinse the glass probe with H2O, catch rinse water in a waste beaker
Gently blot probe with laboratory tissue paper to dry
Return the probe to the storage tube -- Ensure the electrode bulb is fully submerged in storage solution
Return and secure the small probe exchange cover
Turn off the pH meter

Bring solution to target volume, add phytoagar, and autoclave

11 Turn off the stir plate and remove your beaker
Hold a large stir bar in your hand to stabilize the one in your beaker
Pour your solution into the graduated cylinder -- Do not include the stir bar
Add a small amount (50-100 mL) of water to your beaker
Carefully add water from the beaker to the graduated cylinder until your solution reaches the target volume -- Do not include the stir bar

12 Retrieve a clean dry bottle and matching cap
Using a fresh weigh paper and dry spatula/scoopula:

	A	B	C	D	E
	Ingredient	100 mL	200 mL	300 mL	600 mL
	Phytoagar	0.8 g	1.6 g	2.4 g	4.8 g

Add phytoagar to dry bottle
NOTE: Adding phytoagar to dry bottle avoids clumping which is undesirable for final media

13 Loosely place the cap over the bottle
Add a small piece of autoclave tape on the cap and bottle
Place the bottle in an autoclave-safe bin
Autoclave 20-25 min using the 'Liquid' setting
NOTE: Recommended autoclaves are in Cord 3112 and 4112. Complete cycle will take ~1 hr.

14 Rinse all used tools and glassware in running water
Place clean items on drying rack
Return chemical reagents to their original storage location

Adding Heat-sensitive Ingredients

15 Return to the autoclave to pick up your solution -- Be prompt, sucrose can degrade if left too long
Using autoclave gauntlets, gently seal the cap of the bottle
Swirl the autoclaved solution to evenly mix phytoagar

16 Carefully return to the lab with autoclave bin and sealed bottle
Place your sealed solution into the large 55C water bath in the main lab
Discard any liquid remaining in the autoclave bin and return to bin storage
NOTE: Your solution needs to reach ~55C before adding the heat-sensitive ingredients

17 Retrieve the following heat-sensitive ingredients:
1. Silver nitrate (1 mg/mL) - Stored in Main Lab, -20C Freezer, Bottom drawer 'Tissue Culture 2'
2. Acetosyringone (100 mM) 'AS' - Stored in Main Lab, -20C Freezer, Door shelf 5
3. L-Methionine (25 mg/mL) 'L-Meth' - Stored in Main Lab, 4C Refrigerator, Crisper drawer

Place reagents in a tube rack and move to laminar flow hood to thaw
Note: Acetosyringone (in DMSO) takes a LONG TIME to thaw

18 Turn on the laminar flow hood, airflow and lamp
Using 70% EtOH spray bottle and paper towels, sterilize the working area inside the laminar flow hood
Retrieve sterile petri plates
Using a fine-tipped sharpie, write '562V-MSM' and the date along the bottom rim of the plate

19 When your solution reads 55C with a digital thermometer gun,
transfer your sealed bottle to the laminar flow hood.
The bottle should be warm, but safe to handle.
Sterilize the outside of the bottle and your gloved hands with 70% ethanol spray.

20 Using a fresh filter tip for each ingredient, add the following to your bottle:

	A	B	C	D	E
	Ingredient	100 mL	200 mL	300 mL	600 mL
	Silver nitrate	100 uL	200 uL	300 uL	600 uL
	AS	10 uL	20 uL	30 uL	60 uL
	L-Meth	100 uL	200 uL	300 uL	600 uL

Used tips may be disposed of in regular lab waste -- no contact with rDNA or modified cells is anticipated.

21 Gently swirl media bottle to mix thoroughly, but avoid introducing bubbles.
Pour media into plates, ~30 mL per plate.
NOTE: Each plate should be more than half-full with media.

Close plates to solidify in laminar flow hood.

22 Using paper towels, clean any spilled media and discard in regular lab waste.
When plates are poured, rinse media bottle in lab sink and hang on bottle rack to dry.
Return reagents to their original storage location.
Using 70% EtOH spray bottle and paper towels, sterilize the working area inside the laminar flow hood for the next worker.

23 Leave closed plates to solidify in the laminar flow hood with the fan on, 3 hrs - overnight.
NOTE: Keep plates ~10 cm (4 in) away from the back of the flow hood to avoid drying out.
When plates are solid, wrap in a clean plate bag or individually seal with parafilm and store upside-down at 4C, up to 1 week.

Protocol references

Chen, Zongliang, Juan M. Debernardi, Jorge Dubcovsky, and Andrea Gallavotti. 2022. “The Combination of Morphogenic Regulators BABY BOOM and GRF-GIF Improves Maize Transformation Efficiency.” *bioRxiv*. https://doi.org/10.1101/2022.09.02.506370.

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