Intro to Plant Tissue Culture v1.1

Binomica Labs

Aim of the Experiment

In this experiment, we're going to learn how to regenerate an entire plant from leaf tissue through the process known formally as **somatic embryogenesis** or "babies from the body".

Background

All plant cells are **totipotent**, meaning they have all the necessary genetic machinery to divide into all the necessary and specialized cell types required to form a whole plant. This magical process is made possible by the ratio of two hormone families found in all plants: **auxins** and **cytokinins**. Auxins are produced at the apex or tip of the growing plant, otherwise known as the **Shoot Apical Meristem (SAM)**, and cytokinins are produced in the tips of growing roots, known as the **Root Apical Meristem (RAM)**. Since these two hormones are concentrated at the furthest locations away from each other, a gradient of concentration forms. Along this gradient, cells begin to differentiate into specific tissue types dictated by the ratio of the two hormones at that location. A common example of this is when gardeners take cuttings of a plant to propagate. Some plants do not produce roots by themselves and require a little chemical assistance, so there are commercially available products known as rooting powders which contain the auxin hormones. When gardeners apply auxin to the base of a cutting, it shifts the ratio of auxin to cytokinin such that auxins are in the overwhelming majority. When auxins are high and cytokinins are low, cells in contact with such a ratio begin to form roots. The reverse effect is also true, where high cytokinin and low auxin promotes shoot formation. This phenomenon is evident when gardeners trim their hedges. The sudden absence of auxin produced in the now-severed SAM triggers a cascade of signals which activate auxiliary buds located at the node of the stem (where leaf or branch meets stem) and begin to grow. Physically removing SAMs promotes bushiness and stout growth, which is the cornerstone of the Japanese art of Bonsai. We are going to exploit these chemical cues and reprogram leaf cells to build a plant from the ground up: Callus → Shoots → Roots → Done. Let's get started!

Materials

- Wild Type Tobacco Seeds
- 6-Benzylaminopurine 1 mg/mL
- Napthaleneacetic Acid 1 mg/mL
- MS Medium with Gamborg B5 Vitamins
- Sucrose
- Agar
- Distilled Water
- Potassium Hydroxide 1M (sodium bicarbonate option)
- Hydrochloric Acid (acetic acid option)
- Food Coloring (optional)
- Diamond 2 oz Condiment Cups
- 100 mm Sterile Petri Dishes
- Gloves
- Isopropyl Alcohol 70%
- Paper Towels
- Pipette tips for 10 µL, 200 µL, and 1000 µL
- Parafilm M Tape
- Potting soil or Perlite/Vermiculite Mix
- Jack's Classic 20-20-20 General Purpose Fertilizer

Equipment

- pH Meter
- Autoclave
- Pipettes (2 µL to 1000 µL ranges)
- Magnetic Stirrer
- Laminar Flow Hood / Dead-air Box
- 6"+ tweezers with a fine tip
- Light Rack outputting at least 2000 lux set to 16hr days
- Scalpel holders for #11 blades

Procedure

1. Gather all the necessary equipment and go through this entire document to understand what is required in terms of time and resources. Once you start there is no turning back!

2. Prepare MS media at your desired volume as follows:

   MS Basal Medium with Gamborg B5 Vitamins 4.43 g/L
   Sucrose 30 g/L
   MES Buffer 0.5 g/L
   Gelzan Agar 2 g/L
   pH 5.7

   See Appendix (below) for recipes and step by step protocol.

3. Melt and pour ~100 mL of MS media into four 100 mm sterile Petri dishes (25 mL per dish). No hormones shall be added in this batch. These plates will be your seed germination media for the next step.

4. Now we'll surface sterilize some seeds. Under laminar flow hood, place a clean paper towel onto your work surface. Ensure that the surface is wiped down and sanitized

thoroughly before starting. Start by spraying the paper towel entirely, using 70% isopropyl alcohol, such that the towel is soaked.

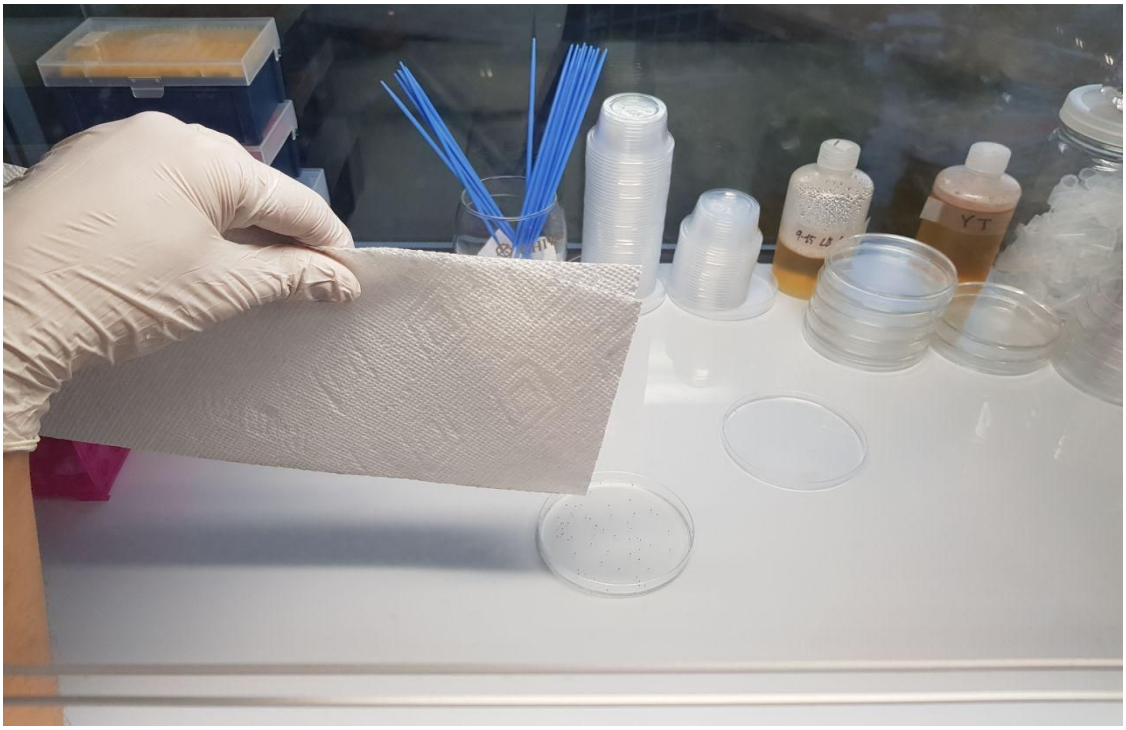
5. Sprinkle a small amount of tobacco seeds onto the center of the paper towel such that the inner parts of the towel looks peppered. Less is more in this case but any excess can be stored in a sterile container for later use.

6. Thoroughly spray the seeds with 70% isopropyl alcohol. Use the force of the spray to spread apart any clumps of seeds. Be sure to start the spraying softly, as to not blast the seeds away. They tend to stick to the towel once soaked, so you can be a bit more forceful with the spraying, but initially you want to start with a light mist.

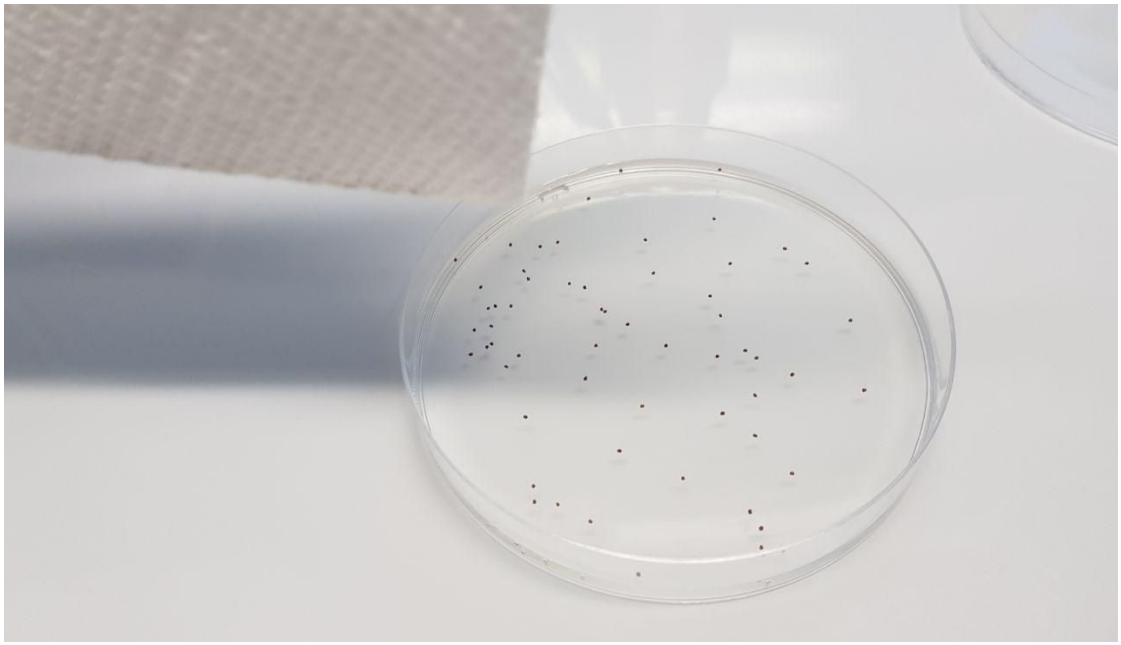
7. Allow the seeds and paper towel to completely dry under laminar flow. Depending on room temperature and humidity, this can be anywhere from 5 to 30 minutes. Determine if the towel and seeds are completely dry by trying to move a corner of the towel with a gloved hand. If you can easily move the entire paper towel with no resistance, then the towel is dry. If it looks damp or it resists movement when you tug on it, allow it to dry further.

8. Repeat step 6 and 7 while keeping in mind the seeds are now drier than before, so they may fly off the paper towel if the first blasts of alcohol spray is particularly forceful.

9. Once the seeds are dry for the second time, they are now surface sterilized and ready for sowing. With a gloved hand, fold the paper towel in half. Lift the folded towel into the air and tap it so the seeds from the walls collect in the crease of the fold in the center of the towel.



10. Remove the Petri dish lid to the seed starting MS media dishes. Carefully sprinkle some of the seeds from the folded towel by carefully tapping the sides of the towel at an angle. Be sure to just lightly pepper the plate. Not too dense. Remember, less is more. Do this to two of the four poured plates. We will keep the other two wrapped in seran wrap in the fridge as a backup in case the first two show signs of contamination. That way you'll have two chances to ensure sterile seed conditions.

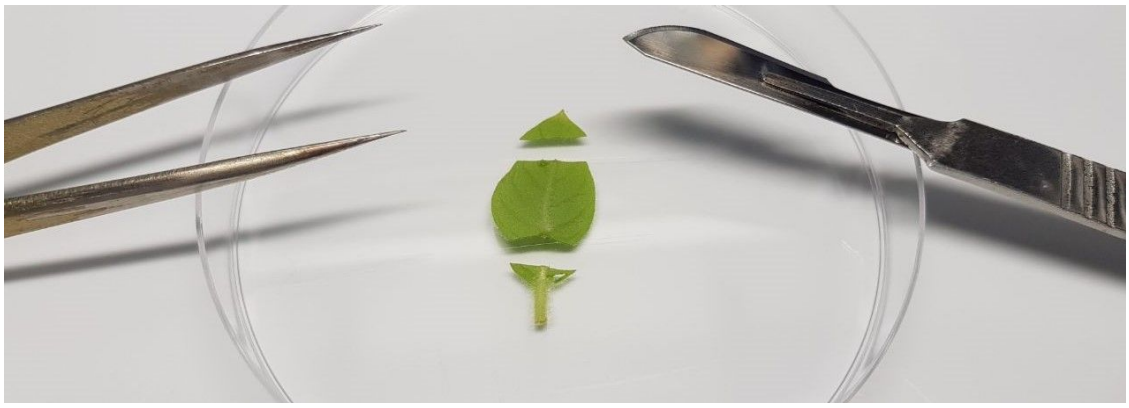


11. Seal the dishes with two strips of parafilm and place them under 16hr photoperiod at room temp. Allow seeds to germinate and grow until leaves of plants are at least 0.5 cm in diameter or the leaves have touched the top of the Petri dish lid.

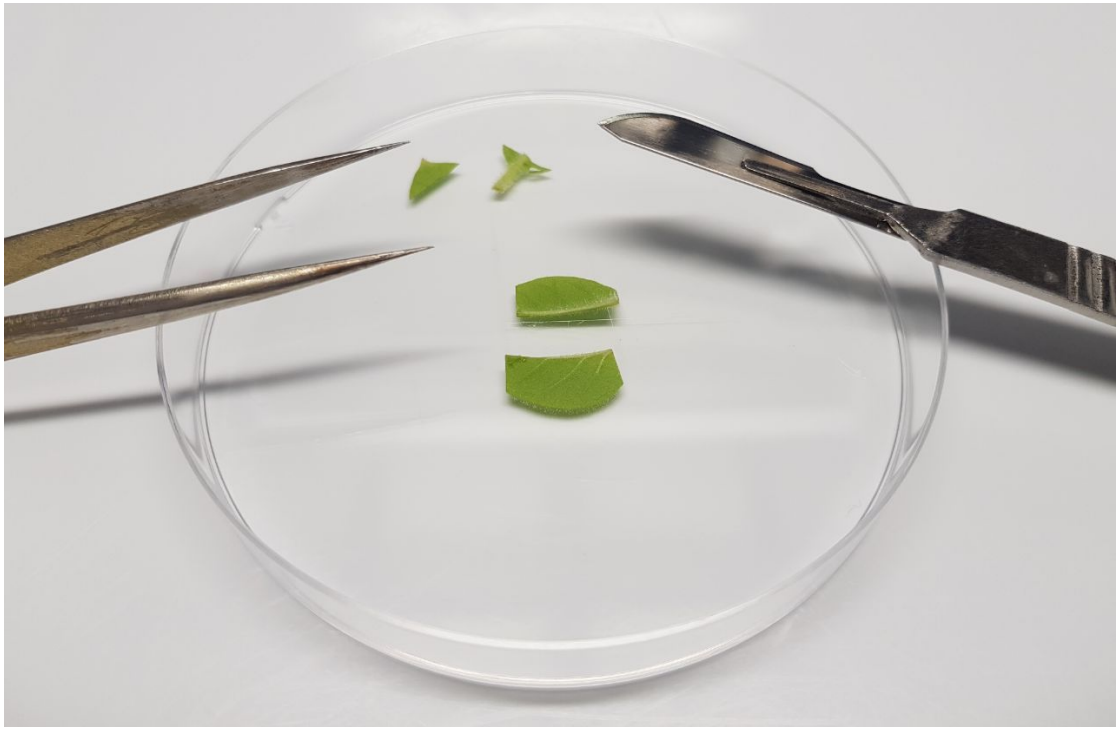
12. Now that the seedlings have grown to maturity, let's start culturing the tissue. First thing is to prepare the shoot inducing medium known as **MSBN**. This is just standard MS media but we add two hormones, **6-benzylaminopurine (BAP)** and **Naphthaleneacetic acid (NAA)**, just before we pour the plates. The final concentration of the two hormones is 1 mg/L for BAP and 0.1 mg/L for NAA. For every milliliter of MSBN you have, you'll want to add one microliter of BAP and 0.1 microliters of NAA. Remember to add the hormones once the media bottle has cooled down like the hottest cup of coffee that you are willing to drink. General rule of thumb is if you can hold the molten media bottle for 10 seconds in a gloved hand without pain, the media is ready for hormones. Dispense 25 mL per Petri dish and allow to cool and set under laminar flow.

13. Under laminar flow, remove the parafilm seal from each Petri dish containing your mature seedlings. Using a sterile tweezer and #11 scalpel, carefully excise a single large leaf from the seedlings and place it onto a sterile Petri dish or cutting mat.

14. Trim off the tail and tip of the leaf (petiole and apex respectively).

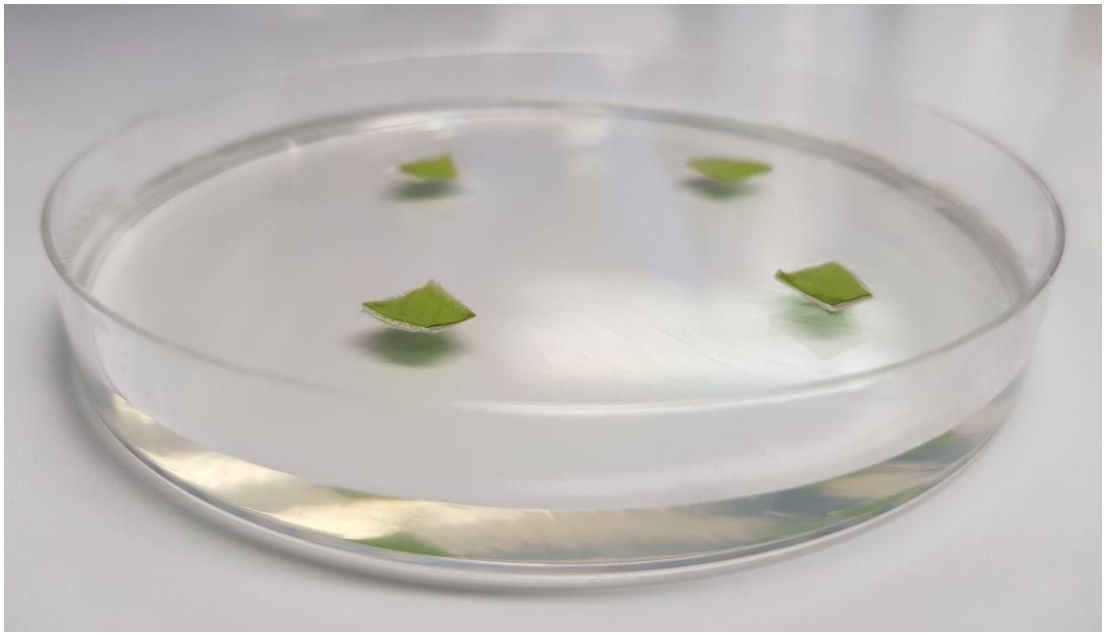


15. Carefully dissect the leaf down the midrib in half.

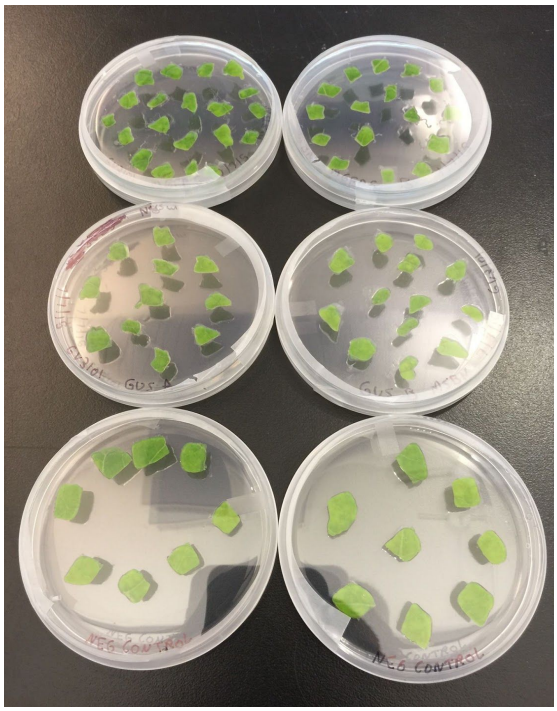


16. Carefully dissect the halves in half such that you are left with four squares of uniform tissue.

17. Now we are going to plate the leaf tissue. With tobacco leaves, as with many other species, there is a darker green side and a lighter green side. The lighter side tends to be the underneath part. When plating leaf tissue in-vitro, you always want to place the leaf tissue light side up. Just lay the leaf onto the media and the capillary action will suck the leaf right onto the surface of the media. Do not bury the leaf in the media.



18. Repeat steps 13 through 17 for as many dishes as you desire. Just a single Petri dish with 8 or so leaf sections is enough for the minimal experience. I recommend at least two plates of 8 to 10 squares of leaf tissue each, so that you can get a sense of the kind of variations the same tissue may have in later steps as well as more practice in the cutting technique. Everyone does things their own way, so feel free to experiment with what cutting method works best for you.

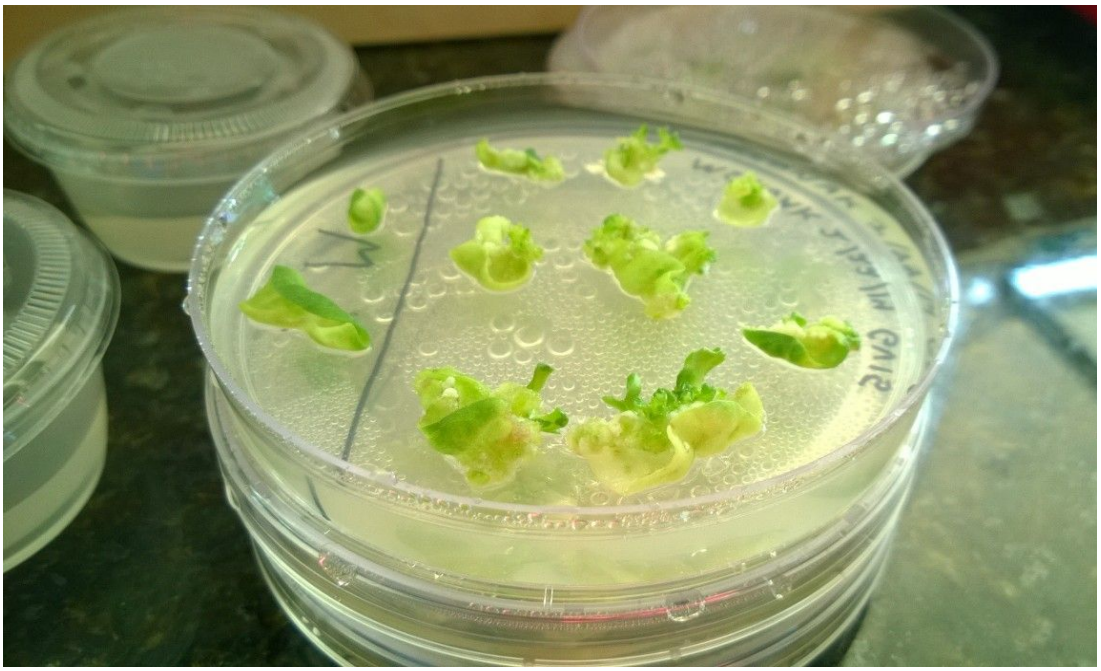


19. Seal the dishes with two strips of parafilm and place them under 16 hour photoperiod at room temperature.

20. The first few days are critical, so make sure you check once a day for any signs of contamination. This can manifest as a halo of bacterial smears along the perimeter of the leaf tissue or very thin wisps of fungal hyphae, which look like starbursts radiating from a central point. If you see fungal or bacterial grow originating from the edges of the Petri dish, this is most likely airborne contamination. If the bacteria or fungi originate from the tissue then it was either your utensils, your starter culture, or you.

21. After about one week, you'll start to see some significant swelling of the leaf tissue, as it takes in the auxins, which causes an increase in water uptake. Auxins are responsible for cell elongation, so the leaf tissue will expand. You may also notice the edges of the leaf are starting to thicken and small bumps may be visible. This is the beginning of callus tissue and the subsequent re-programming of the cells. This is a good sign. Allow the cultures to sit for another week.

22. At this point you should be seeing significant changes in the appearance of the leaf tissue. Small bumps are visible and even the beginnings of shoots emerge from the larger bumps. It's now time for your first replate! Prepare MSBN media, but this time we'll be pouring into the 2 oz condiment cups. You ideally want one cup for each explant in culture. This is where your choice of how many leaf tissue squares to start with will either be a blessing or a curse. Each ketchup cup requires about 25 mL of media, so prepare accordingly. Always make a little more than the desired amount of media to account for evaporation during meltdown. Add the hormones when cool enough to hold for 10 seconds without pain and allow to set under laminar flow. For every condiment cup of media poured, ensure you have a corresponding empty sterile cup which will act as a lid. Can't tell you how many times I poured media into these things and forgot to sterilize lids for them. The actual condiment snap-on lids themselves are made of polystyrene, thus are NOT AUTOCLAVABLE so please don't put them in the autoclave or else it will melt and make a literal hot mess. Use another cup as the lid instead.



23. Under laminar flow, transfer each explant to their new home with a sterile tweezer. Be sure to push the culture into the medium such that just the very top of the tissue is on the surface. This ensures a uniform contact point with the hormones in the media and establishes which way is up and out of the “soil”. A departure from total moisture gives the plants an idea of what is above ground and what is below, so it helps to bury the explant a little. Not too deep, just a bit below the surface.

24. Place a cup on top of each culture and seal the two brims with a strip of parafilm. This forms a nice culture vessel that is reusable and cheap as hell. Label the vessel with the media name and date along the waterline of the bottom cup: it's good to trace back and see how long cultures have been out for, in case you lose some to contamination. Place these back on the light rack for another week or until clear signs of shoots form.

25. Because there is a bit of randomness to the formation of shoots, some cultures can be along further than others. We'd like to respond to these cultures instinctively and on a case by case basis. If there is what looks to be a clear baby plant with stem and leaves growing out of your culture, it's time to transplant those into rooting media. Before we do this we must first prepare the media itself. This consists of MS media with the addition of just 0.1 mg/L NAA and we'll refer to this media as **MSN** from now on. Make enough media for each of the cultures you think are ready for rooting. You can make extra cups and store them in the fridge with their original lids (wiped down twice with alcohol and dried under laminar flow) if you have space. The best practice is to melt and pour fresh media as you need it. Hormones last about a month in the fridge in media.

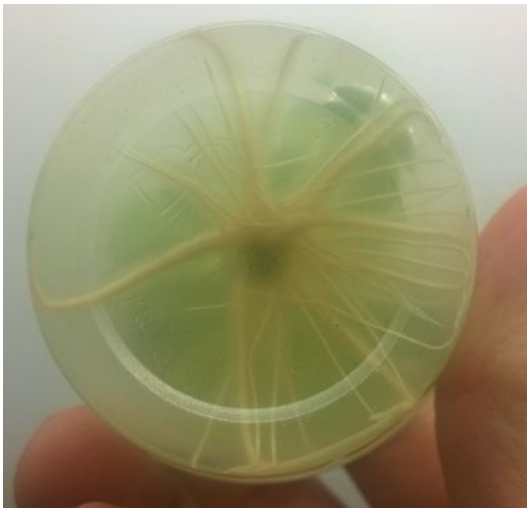
26. Open the culture vessels containing shoots taller than 0.5 cm with a visible stem. The stem is important since it imparts a distance between the SAM and crown tissue (where stem meets soil), which helps the differentiation process. No roots will form on tissue that is just leaves growing out of callus.

27. Carefully excise each shoot at the base of the stem and transfer it to the center of an MSN media plate. Bury the stem enough so the plant is self standing. Repeat with all remaining vessels and seal them with a cap and two strips of parafilm. Place on light rack with a 16 hour photoperiod.

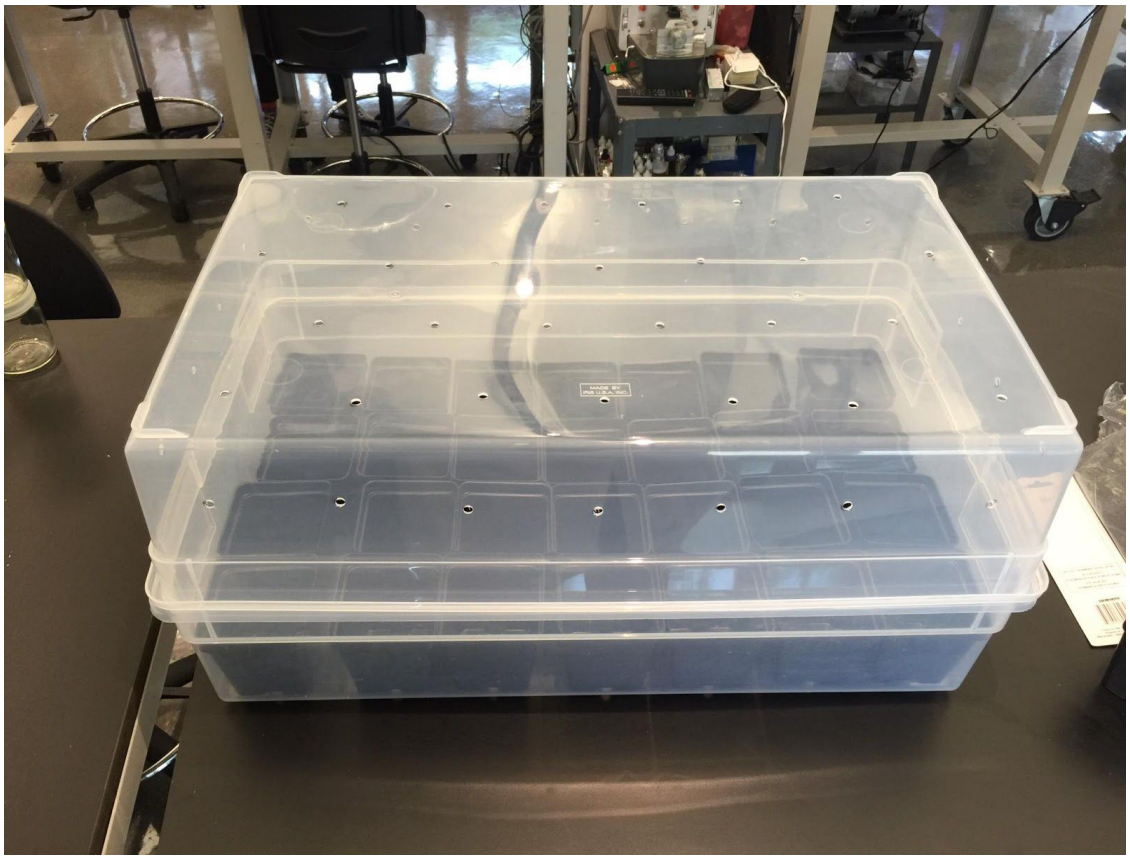
28. After about a week you'll start to see a swelling of the submerged region as well as the beginnings of little white bumps along said region. These are cells differentiating into root tissue and can vary in speed of

formation due to a number of factors including but not limited to: health of the plant, height of the stem, leaf dimension, humidity, etc. It's best to just let the plants do their thing.

29. After a little over another week, the roots of the plant should have taken over the vessel. The general rule is once the roots touch the walls of the culture vessel, they are ready to be transplanted into soil. If you do not see a decent amount of roots forming, just let your plant grow. At this point replating is not needed nor advised. Patience is a virtue. :)



30. Once the plants have produced a decent amount of roots, prepare some soil and a humidity chamber. This can be as simple as two tupperware bins placed on top of eachother with holes drilled evenly and taped over. The tape has a little bit of a tag that you can peel off. You want to divide your holes by 6 such that each day after moving the plants to soil you can take off the tape from holes labeled with the corresponding day.

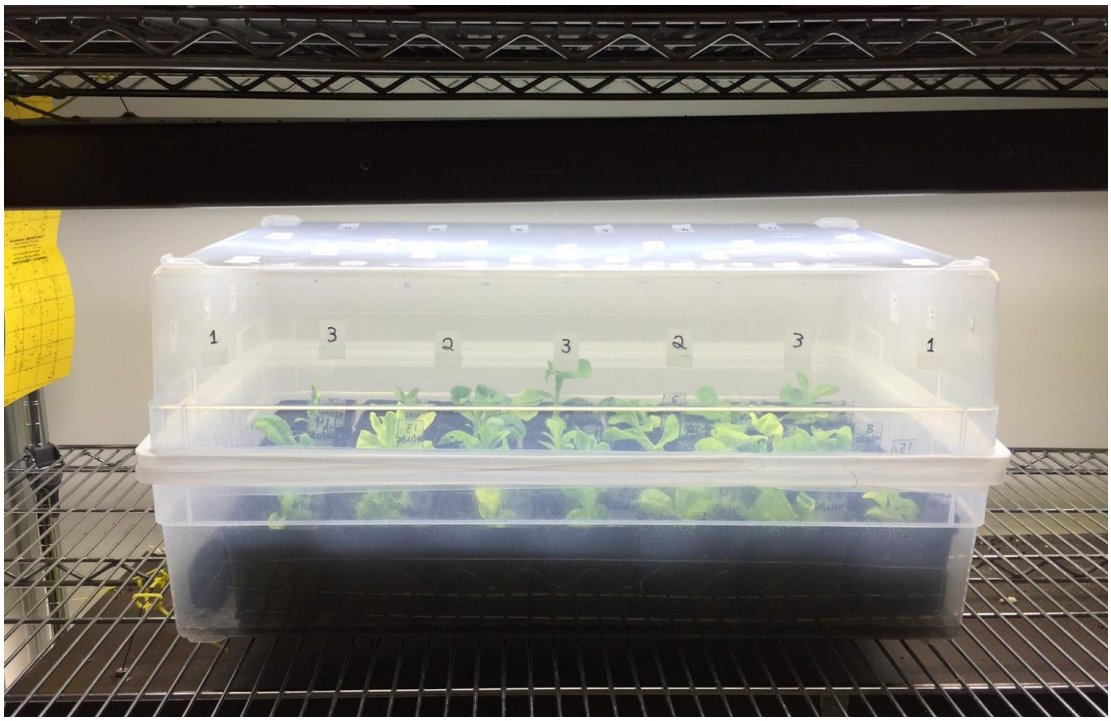


31. For a soil mix I prefer to use a 50/50 mixture of organic perlite and vermiculite. The reason for “organic” is not to be fashionable and hipster but rather to know the media is devoid of any nutrients. This is important for proper fertilization if things like media and feeding schedule R&D is your thing. Else you can go for general purpose Miracle Grow Potting Soil. Regardless of what medium you chose, fill enough pots of 4” pots to the top and then moisten until water drains out from below. This prepares soil to receive the fragile cultured plants without drying them out.

32. Once your soil and humidity chamber is set up, in another tupperware container, fill the vessel halfway with tepid or room temperature water. Carefully, with gloved hands, remove each of your rooted tobacco cultures and place them into the water. Gently remove the agar from the roots of each plant, paying close attention to the center of the root mass. The medium contains sugar, which microbes and fungi love; so, to avoid overgrowth of contaminants, you want to make sure all the media is removed. There is a slipperiness to roots still laden with agar, so tease out each root until a resistance is felt.

33. Sow each of your plants into their own pot and use a squirt bottle to moisten the soil such that the material around the plant makes contact with the stem. Pour enough standard strength Jack's Classic 20-20-20 fertilizer solution such that the pots are sitting in one inch of said solution. This can vary depending on the size of your humidity chamber, but the general rule is between one and two finger widths of liquid.

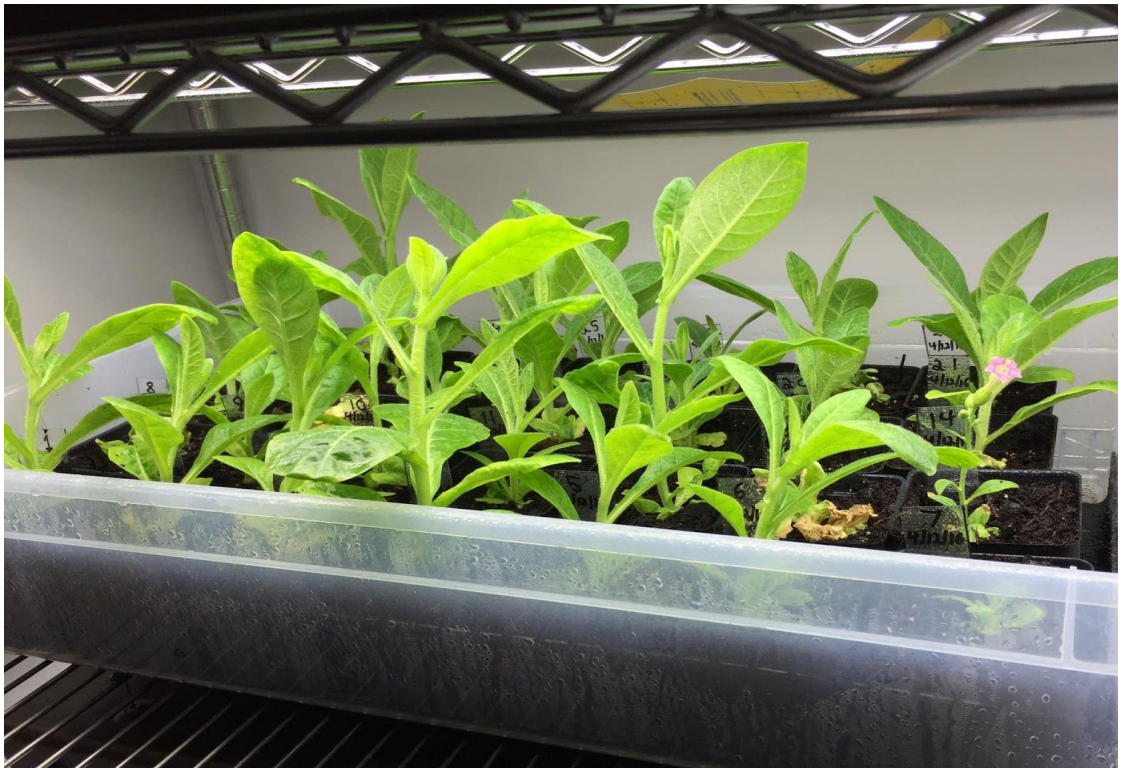
34. Place drilled upper tupperware container to cover the humidity chamber and seal the two containers with tape. Ensure all of the drilled holes are covered with a tape tag and they are evenly labeled such that there are equal amounts of taped holes for each dehumidification day. Place the humidity chamber under cool white fluorescent lights at 2000-3000 lux and a 16 hour day, 8 hour night photoperiod.



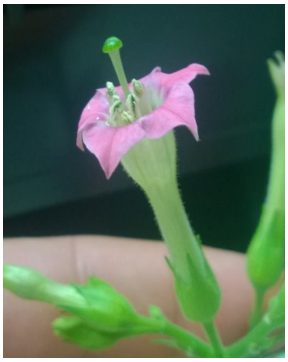
35. Every 24 hours, remove the labels with the corresponding day-count since the cultures were transplanted. This will gradually lower the humidity from the near 100% conditions in-vitro to the 20-40% humidity of the real world. The gradual decrease in humidity will trigger the closure of these tiny mouths on the underside of the leaves, called stomata. In nature, these special cells open to allow for gas exchange, which is an integral part of photosynthesis, but close during drier conditions to prevent dehydration. In tissue culture, these cells remain open because the humidity never changes, so the sudden shock of being moved from such a comfortable womb-like setup to the harsh reality of daily life would cause the plant to almost immediately wilt and die.

36. After the 7th day, remove the tape from along the bring of the humidity chamber and take off the lid entirely. Allow the plants to grow with the lid off for a week, ensuring there is a constant water level of a finger. There still could be some minor wilting, but this will subside with time.

Due to hormonal imbalances, flowering may occur during initial growth. Despite how the cultures look, they will all normalize to the standard tobacco phenotype as they mature.



37. Allow the plants to grow with a fertilization every two weeks, using Jack's Classic 20-20-20. Standard fertilization is done by simply watering the plants using the fertilizer. Watering schedule depends entirely on the ambient humidity and temperature, but as a general rule never allow the soil to dry entirely. Best is to add a finger's worth of water to a tray holding several pots and replenish the water when the level drops to just moisture along the tray. Because this is stagnant water, a biofilm of bacteria may form. A remedy for this is to dilute hydrogen peroxide 3% with water in a 4:1 ratio (water : H_2O_2) and water with this mixture once. This will burn the bacteria and fungi, but the roots of the plant are large enough to resist the oxidation. At this point these plants are essentially house plants, so feel free to experiment with lighting conditions.



38. Flowers will form within two months of growth. The plant self pollinates, meaning that seed pods will form shortly after.

39. A single plant can yield an entire 15 mL conical tube worth of seeds. Once the seed pods begin to brown, stop watering the plant and allow for it to dry naturally. Once the seed pod stalk is dry, carefully cut it off and place it onto a sheet of printer paper. Pinch the tip off of each seed pod and pour the seeds out of the pod onto the paper. Store these seeds in a 15 mL tube and label it with the date and the cultivar name "*Nicotiana tabacum* var. "Petite Havana" wild Type". Store the tube in a dark, cool place until future use.

You've reached the end of this experiment! I hope you've learned a lot and feel a bit more confident in your ability to conduct this basic but critical skillset of plant tissue culture. Here is a quick experiment to dive further into the world of plant cloning and tissue culture. Enjoy!

Open-Ended Experiment

Now that you've garnered the skills needed for basic tissue culture, let's see if you can try your hand at domesticating a wild plant from your surroundings. This can be a favorite ornamental in your home, a random weed on the street, or something that catches your eye. There are 8.7 million eukaryotic species of life on this planet and many of these are plants. Genetically, very little is known about plants outside of the model organisms. At the time of this writing, there are currently only ~140 fully sequenced species of plants and most are commercially incentivised. To quote the infamous Donald Rumsfeld "*There are things we know we don't know and things we don't know we don't know*". This line holds true for the rich biodiversity this planet has to offer. There is a high probability that the random plant that catches your eye while going for a walk has yet to be sequenced and, rarer, still to be reliably cultured in the lab. The definition of what a species is, and furthermore what a model organism is, seems nebulous at best. If you wish to work with a different plant, you must first identify the species visually, then scour the internet for leads as to the current state of knowledge regarding this particular species. Learn about where it grows, its distribution, prefered climate, life cycle, etc. Once all this background legwork is completed, what remains is the bleeding edge of our understanding and a solid place for you to contribute meaningfully to the compendium of human knowledge.

Any progress made in the furthering of our understanding of these organisms is important and valuable, for no research is wasted research and we honestly do not know what to expect or gain from this endeavor unless we look. And that is our challenge to you! Find an organism that you find interesting and behind the steps toward attempting to culture this lifeform. Here are some steps toward achieving this. Enjoy!

Procedure

1. Obtain a whole plant from the wild and bring it back to your lab. If a whole plant is not possible, legal, or worth the effort, obtain a tissue sample large enough for several Petri dishes worth of attempts. DO NOT take plants from private property or without the express permission of the landowner. Just ask or inquire. There are nice people on this planet that may be willing to help. :)

2. Once back at the lab, wash the tissue thoroughly with tap water and keep in a vessel of cold water until ready to culture. Note detached tissue pieces do not keep well and should be processed as soon as possible.

3. Prepare a bleach solution using chlorine tablets used to purify water. These are sold under many brands and ef-Chlor is one of them. The main ingredient of this is sodium dichloroisocyanurate (NaDCC for short). This is a lower pH bleach that is less harsh to plant tissue but strong enough to get rid of contaminants like fungal spores and bacterial cells. These tablets can be sourced easily and good keywords to look up on Amazon or eBay are “effervescent chlorine tablets” or “water purification tablets”. All of these suppliers also provide the amount of active chlorine present per pill on the product label. Conveniently enough, the 167 mg ef-Chlor tablets have 100 mg of active chlorine. This means a single pill added to 50 mL of distilled water will produce a solution of 2000 ppm.

4. For general purpose sterilization, make a 500 ppm solution, add 1g of your desired tissue to a 50 mL conical tube and 25 mL of said solution along with a drop (5 µL) of dish soap. Vortex or shake this vigorously to dislodge any debris that the initial washing may have left behind and allow to sit (ideally gently shake) overnight.

5. The following day, using sterile tweezers, scalpels, and aseptic technique, section the desired tissue into 5 mm square pieces and place them abaxially (belly side up) onto MSBN media (MS media with 1 mg/L BAP and 0.1mg/L NAA). Culture them as you would tobacco and record your results. Monitor for contamination and change your sterilization regimen accordingly. You can modify the concentration of NaDCC, time spent in solution, shaking vs no shaking, more or less soap, maybe a more thorough rinsing with soap initially. WRITE DOWN EVERYTHING YOU DO.

6. The next step is where the fun begins: COMBINATORIAL EXPERIMENTAL DESIGN! The hormone recipe I gave you in MSBN is a kind of one-size-fits-all and, much like the clothing style where not everyone looks good in a one-size-fits-all, so too do some plants. Each species of plant may have drastic differences in the way they respond to the ratio and kind of hormones they are given. The general rule when culturing an unknown is to use natural hormones. These hormones, namely Indole-3-Acetic Acid and Zeatin are a little more expensive than the synthetic NAA and BAP respectively, BUT the could have much more potent effects. By modulating the ratio and kind of hormone in the media, you can systematically deduce which pair and concentration works for each step in the tissue culture pipeline. Test out a table of values where you iterate through 0, 0.1, 0.5, 1, and 2 mg/L of either cytokinin or auxin. Prepare small batches (two or four Petri dishes worth) of each concentration and slowly but surely determine the needed ratios for the callus induction step.

7. Once callus is achieved, divide the callus and run it through the same table again to see where it starts to form shoots.

8. Transplant the shoots to a medium containing 0, 0.1, 0.5, 1, and 2 mg/L of only auxin to induce rooting. As always, record your results.

9. Once you finish all of these steps and have successfully regenerated your plant of interest, compile your notes and let us help you prepare for submission to a pre-print server for publication because you just developed brand new knowledge on how to domesticate a wild plant in the lab. A genuine contribution to our knowledge base. Congrats!

This entire process can take months and plant biotech is a patient person's game. We wish you clean cultures, vibrant growth, and much enjoyment in your botanical adventures. Have fun and a massive **THANK YOU** from all of us at Binomica Labs for supporting our cause of providing the tools and resources for anyone to conduct small thoughtful science!

References and Further Reading

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant Physiologia Plantarum, 15(3), 473-497. doi:10.1111/j.1399-3054.1962.tb08052.x

Svab, Z., P. Hajdukiewicz and P. Maliga. 1975. Transgenic tobacco plants by co- cultivation of leaf disks with pPZP Agrobacterium binary vectors. In "Methods in Plant Molecular Biology-A Laboratory Manual", P. Maliga, D. Klessig, A.. Cashmore, W. Gruissem and J. Varner, eds. Cold Spring Harbor Press: 55- 77.

Appendix

Making Murashige and Skooge Medium (MSA)

1. Calculate how much media you wish to make. This determines the concentration of the reagents which will always be states in grams per liter such that we keep a standard of measurements. Adjust the following recipe of reagents according to your desired volume:

    MS Basal Medium w/ Vitamins: 4.43 g/L
    MES Buffer: 0.5 g/L
    Sucrose: 30 g/L
    Gelrite (Gelzan): 2 g/L

2. Depending on the size of your autoclave, wash and prepare glass bottles with enough volume such that the media you are about to make can be divided into them. Never fill any glass bottle that is about to be autoclaved more than half way, so make sure you take this into account when dividing media for sterilization.

3. Due to the size restriction of my small upright autoclave, I can only prepare 2 volumes of 500 mL at a time. As a result, I prefer distributing the autoclaved media into 3 sterile 250 mL Pyrex bottles, each holding 150 mL of media. Your choice of smaller containers is limited by the size of your microwave oven, which tops out at the 500 mL Pyrex bottle on average, so media tends to be stored in one of those bottles with a volume of about 300

mL of media. Your mileage may vary. Ensure that whatever smaller bottles you choose, you sterilize them ahead of time.

4. To a beaker that is around the size of the final volume you wish to make (in this case I am making 500 mL of media), fill the beaker 80% of the way with distilled water. We do not have a MilliQ water purifier so we use store-bought Poland Spring brand water. Any store-bought distilled water works fine.

5. Add a magnetic stir bar and place the beaker onto a magnetic stirrer on medium speed. If you do not have one, manual stirring works too.

6. Begin by adding 30 g/L of Sucrose. This can be store bought granular sugar. Domino works fine here, no need for fancy stuff.

7. Allow the sugar to dissolve, then add 4.43 g/L of the Murashige and Skooge Basal Medium powder (stored in the fridge).

8. Add MES buffer powder to the media at 0.5 g/L.

9. Allow all the reagents to dissolve. While this is happening, calibrate your pH meter according to the user manual. Each pH meter is different, but ensure it is accurate to at least 0.1 pH units AND has automatic temperature compensation (known as ATC). Always calibrate your pH meter prior to use. Be sure to wash off the probe with distilled water prior to submersion into each of the calibration buffers and then once more once the calibration is complete.

10. Place the pH meter probe about one centimeter into the swirling vortex of the medium. Ensure the probe is deep enough into the liquid that no air gaps exist between probe bulb and media. These probes are submersible so do not be afraid to dunk a decent amount of the probe into the solution.

11. The reading on the probe should be somewhere in the ballpark of 3.8 to 4.2 pH, assuming you are using the basal medium with no additives. Regardless of the initial pH, adjust it while stirring the medium using 1 M potassium hydroxide solution (baking soda works as an alternative) in increments of 100 µL. Keep count of how many 100 µL volumes you've dispensed, for future records. Once the pH reaches around 5.5, add the KOH slowly and allow the pH meter value to settle before continuing. You do not want to overshoot. Keep adding dropwise when the pH is 5.68 and stop once the meter reads a stable value of 5.7.

A margin of error from 5.70 to 5.72 is acceptable. In case of overshooting, lower the pH with hydrochloric acid or vinegar.

12. Once pH is adjusted to 5.7, wash off the probe of the pH meter and store it in its storage buffer or proper cap.

13. Dispense the entire contents of the beaker into a volumetric flask whose volume is exactly that of the desired final media volume. Use a 1L flask for 1L of medium. Bring the volume of the medium in the flask to the fill line on the neck of the flask. Use the meniscus to ensure you don't overshoot.

14. To a bottle that will contain your media during autoclaving, add the appropriate amount of Gelrite or Gelzan agar powder, such that the final concentration is 2 g/L. Example: To a 1L Pyrex bottle I'll add 1 g of gelrite because it will eventually be filled with 500 mL of MS medium. Double check to be certain your math makes sense and the proper concentration of agar is achieved. Do so for all the media bottles that will be autoclaved. Adding agar to the main batch is unwise, since the agar will not dissolve and rather be a colloidal suspension that may settle, leading to uneven distribution of agar prior to autoclaving and ultimately non-uniform firmness of media batch-to-batch.

15. Using either a graduate cylinder or volumetric flask, dispense the appropriate amount of media that will be required for the bottles containing agar that will be autoclaved. Remember to make sure that whatever volume you calculated is no larger than half the capacity of the bottle about to be autoclaved. Example: do not fill a 1L Pyrex bottle beyond the 500 mL mark if you are autoclaving liquids, since it will boil over.

16. Cap the bottle and give it a swirl. Then loosen the cap such that it is still attached to the bottle but can be rattled without coming off. This allows for the superheated steam to penetrate the medium but not build up pressure and break the bottle. THIS IS VERY IMPORTANT. ALL CAPS OF BOTTLES TO BE AUTOCLAVED MUST BE LOOSELY CLOSED. NO SEALED BOTTLES ARE TO BE AUTOCLAVED. Also, sorry for the caps concerning caps.

17. Place the bottle in the autoclave and apply a strip of autoclave tape to the cap. This will change color from white to black if the proper temperature is achieved. A bit of peace of mind for the paranoid folks where a single bacterium cell can spoil all the fun.

18. Allow your autoclave to run as per its user manual. Typical run is 15-30 mins at 121 psi (1 atm). Once autoclaving is done, carefully… CAREFULLY remove the media from the

HOT autoclave AFTER venting properly. Move the media to the laminar flow hood and allow it to cool to a hot cup of coffee you are still willing to drink (55°C).

19. Add hormones if needed (MSBN media is just a modification of MS with the addition of BAP 1 mg/L and NAA 0.1 mg/L) at this time. DON'T ADD HORMONES TO PLATES USED FOR STARTING SEEDS!

20. Carefully pour 25 mL (pour slowly until the Petri dish bottom is covered and then stop) into each sterile Petri dish. 150 mL makes for 6 thick plates, so consider that as your ruler when pouring. If you are making media just for storage, pour the media into pre-sterilized smaller bottles to the halfway mark and close tightly.

21. Store at room temperature if no hormones are added. Store in fridge if antibiotics or hormones are used. Keep at room temperature for 6 months or until the agar begins to degrade.