Use of Plant Tissue Cultures for the Demonstration of Plant Mineral Nutrient Deficiency Symptoms

Michael J. Bosela[1], Nicholas Becker[1], and Kirk Bradtmiller[2]

[1]Department of Biology
Indiana University-Purdue University, Fort Wayne
2101 E. Coliseum Boulevard
Fort Wayne, IN 46805-1499
boselam@ipfw.edu (author for correspondence)
becknd01@ipfw.edu

[2]Department of Biology
Indiana University
1001 E. Third Street
Bloomington, IN 47405-3700
kabradtm@indiana.edu

Biography

Michael Bosela is an assistant professor of Biology at Indiana University-Purdue University at Fort Wayne (IPFW). He has a B.A. in Biology from Oberlin College (1991), a M.S. in Botany and Plant Pathology from Michigan State University (1995), and Ph.D. in Plant Science from North Dakota State University (2002). Dr. Bosela's research specialty is in woody plant tissue culture and biotechnology. He is especially interested in research on the development and optimization of basic tissue culture and biotechnology techniques**.** At IPFW, Dr. Bosela teaches a wide variety of plant biology courses, including Introductory Horticulture, Plant Physiology, General Botany, and Field Botany.

Introduction

In hydroponics, plants are grown in nutrient solutions instead of soil. Hydroponics experiments are widely used in plant science instruction, but can be time-consuming to set up and maintain, especially for derivative techniques such as sand culture that require regular and repeated treatment application (watering). In this lab an alternative technique, plant tissue culture, is used for the demonstration of mineral nutrient deficiencies. Tissue cultures are sterile cultures of cells, tissues, organs, or whole plants, grown on nutrient media. These media typically contain both inorganic (mineral) and organic (carbohydrate) nutrition, as well as hormones and gelling agents to 'set' the media. Since tissue cultures are sterile and are kept in closed containers that restrict the degree of growth they require little maintenance. Tissue cultures also offer viewing advantages, particularly for the evaluation of nutrient deficiency effects on root architecture, and may be superior to conventional hydroponics for the demonstration of deficiency symptoms for immobile nutrients.

In this lab, students compare seedling tissue cultures of tomato (cv. Micro-Tom) grown on eight different media; positive and negative controls and six experimental media each lacking one of the following essential nutrients (calcium, nitrogen, phosphorous, magnesium, potassium, or iron). To assist instructors with little or no prior plant tissue culture experience, I have provided detailed protocols for several of the more specialist components of the experimentation (e.g., media preparation, seed sterilization, etc.). References to plant tissue culture training opportunities and web-based plant tissue culture resources are also included.

Student Outline

Background

Plants make their own carbohydrates via photosynthesis, but are dependent upon the external environment for their inorganic (mineral) nutrition. In fact, in both agricultural and natural ecosystems, plant growth is typically limited by the mineral nutrient that is present in the lowest quantity relative to the plant's needs as stated in Leibig's 'Law of the Minimum' (Taiz and Zeiger, 2006). In addition, the importance of mineral nutrient management in agriculture is expected to increase in the future as the rate of plant growth is hastened by increases in the atmospheric carbon dioxide concentration, both via direct effects on the photosynthesis and via indirect effects on the climate (global warming), exacerbating the need for effective fertilization programs

Hydroponics is an important tool for plant mineral nutrition research. In hydroponics plants are grown in mineral solutions instead of soil or potting media. Thus, the experimenter is able to directly control the types and concentration of mineral nutrients. By excluding specific mineral nutrients, researchers can assess which are essential for plants and document deficiency symptoms for the mineral(s) being evaluated. The evaluation of nutrient toxicity effects and nutrient interactions (e.g., competition for uptake, etc.) is also possible. In this exercise a modified hydroponics technique is used to demonstrate deficiency symptoms for six mineral nutrients; calcium [Ca], nitrogen [N], potassium [K], phosphorous [P], magnesium [Mg], and iron [Fe] in tomato.

For any essential mineral nutrient, prolonged deficiency will ultimately result in adverse effects on plant growth. However, for some mineral nutrients, especially those required at relatively low concentrations (<0.1% plant dry weight, termed **micronutrients**), 'visual deficiency symptoms' (**necrosis**, **chlorosis**, etc.) may precede growth inhibition. Changes in resource allocation are also a possible plant response. For example, deficiencies of nitrogen, phosphorous, and iron, may cause shifts in carbohydrate allocation from shoot to root growth, an adaptation that would be expected to facilitate mineral nutrient acquisition, at least for plants grown in the soil where mineral nutrient movement is restricted both physically and chemically (via adsorption).

In this lab, you will evaluate **tissue cultures** of tomato (cv. Micro-Tom) for nutrient deficiency symptoms. Tissue cultures are cultures of cells, tissues, organs, or whole organisms grown under sterile conditions on nutrient 'media'. Compared with hydroponic media, tissue culture media are generally more complex, containing carbohydrates, vitamins, hormones, and a gelling agent, in addition to the mineral nutrients. In this experiment a standard hydroponic medium (Hoagland #2) is employed but it also contains sucrose (10 g/l) and agar (8 g/l). Because agar is extracted from living organisms (red algae), it contains mineral nutrient impurities that could delay the onset of deficiency symptoms. Sucrose also has the potential to confound the experiment by reducing the dependency of the plants, in particular the roots, on photosynthesis for their growth. However, prior research has established that seedlings of 'MicroTom' exhibit typical nutrient deficiency symptoms in tissue culture (Bosela, 2008). In addition, tissue cultures are easier to maintain than conventional hydroponic experiments and may be superior to conventional hydroponics for the demonstration of deficiency effects for immobile nutrients.

There are several components to this exercise. Initially, you will evaluate the seedlings for visual symptoms of nutrient deficiency and also for differences in the 'degree' of shoot growth. The quantitative data (shoot heights, weights, etc.) will be combined across student groups, to produce a class data set, and analyzed to determine if differences in shoot growth (weight) between the treatments are 'statistically significant'. You will also prepare a key for the identification of nutrient deficiencies in tomato. Overall, the lab design includes a strong emphasis on data analysis and interpretation.

Part I: Visual Evaluation of (Shoot) Nutrient Deficiency Symptoms

A data collection table and instructions are provided in **Appendices A** and **B**. For each of the major types of nutrient deficiency symptom, a series of questions is provided to guide your data collection, using the positive control seedlings as a reference to verify the treatment effects. For symptoms affecting the leaves the pattern, or sequence, of symptom development both within and between leaves, is often of diagnostic importance. For mineral nutrients that are **mobile** in the phloem, as the nutrient concentration within the plant becomes limiting the nutrient is moved (recycled) from older to younger leaves, near the growing shoot tip, and nutrient deficiency symptoms will appear first in the older leaves. In contrast, '**immobile**' nutrients are generally only transported in the xylem and cannot move between leaves. Thus, leaves will only have access to

these nutrients if they are being absorbed and transported to the shoot tip at the time of leaf initiation. If the supply of the nutrient is finite and decreasing relative to the plant's needs younger leaves will show more severe deficiency symptoms than older leaves.

Part II: Shoot Growth Data

After your visual evaluation of the seedlings has been completed, use the data table and instructions in **Appendix C** to collect quantitative data on the degree of shoot growth. The shoots will need to be cut off, at the root-shoot junction, and removed from the culture vessels for weighing. However, since plants that have been cultured in non-ventilated culture vessels, as employed in this study, are highly susceptible to wilting when exposed to ambient humidity conditions, the culture vessels should not be opened until right before the weighing and the shoots should be returned to their vessels, with the caps reapplied, after weighing to keep them hydrated, and healthy, for the remainder of the class period. Before leaving turn in your data to the instructor to be included in the class data set.

Part III: Using Statistics to Evaluate Nutrient Deficiency Effects on Shoot Growth

The instructor has calculated average (mean) values for the quantitative data (shoot heights, weights, etc.) These are estimates of treatment effects on plant growth. Based on the amount of variability in the data set; i.e., between the height or weight values of the different seedlings, we can calculate a range of values that would be expected to contain the 'true value', for each variable, at a given probability (confidence level). In statistic courses the true value is generally described as the value that would be obtained if the entire 'population' was sampled. For experimental research, such as here, it is the value that would be obtained if the sample size was infinite.

A 95% confidence interval range is the range of values that is 95% likely to contain the true value. Thus, it is a measure of the precision of an estimate. The larger the confidence interval, the lower the precision. In general, precision and sample size are directly (positively) related, meaning that as the sample size (level of replication) increases the precision of an estimate also increases.

Confidence intervals and margins of errors are related statistics. Numerically, the margin of error is half the confidence interval. It is the amount you would add and subtract from the mean to calculate the upper and lower limits of the 95% confidence interval range. For example, if 45% of the voters in a survey favored Candidate A and 51% favored Candidate B, with a margin of error of 6%, we can be 95% confident that the true value for Candidate A would fall between 39 and 51% and the true value for Candidate B, between 45 and 57%. Since the outer values of the confidence interval range overlap, the race is 'too close to call'. Statistically, it is a tie. In contrast, if the percentage support values were 41 and 59%, the confidence interval ranges would not overlap and the differences would be deemed 'statistically significant'. What this means in practical terms is that if the survey was repeated 100 times, the percentage support values for each candidate would fall

within these ranges for 95 of the 100 surveys. The percentage support would be higher for Candidate A less than 5% of the time.

In the natural sciences confidence interval ranges can be used to assess whether treatment effects are statistically significant. In this case if the confidence interval ranges of the averages being compared overlapped, the treatments would be considered statistically equivalent. If the ranges did not overlap the differences would be declared 'statistically significant'. In general 95% probability levels are used to assess statistical significance, as in the voting example, but other values (90%, 99%, etc.) can be used depending on the level of certainty desired for the experimental conclusions.

Assignment: Use the class averages and 95% confidence intervals to answer each of the questions. Your answers should be typed and turned in on a separate sheet of paper.

1. For what nutrient deficiency treatments was the mean shoot weight significantly lower than for the positive control at a 95% level of confidence?

2. Was the mean shoot weight higher for any of the deficiency treatments than for the positive control?

3. If the answer to Question 2 is yes, were the differences in mean weight between the positive control and the deficiency treatment(s) statistically significant at the 95% level of probability?

4. Did the mean heights or weights differ significantly between the P and K deficiency treatments (95% level of confidence)? Explain?

5. Was the mean shoot weight value higher for the negative control or the N deficiency treatment? Were these differences statistically significant at the 95% confidence interval?

Part IV: Preparation of a Nutrient Deficiency Key

Keys are tools used for the classification, and identification, of objects or conditions. Keys are arranged as a series of choices, or questions, about the specimen being evaluated that ultimately result in its positive identification. Keys are hierarchical in organization and most are also dichotomous, meaning that two choices are presented at each step, or couplet. Positive identification

is achieved via the evaluation of successive traits, as illustrated in the key provided for MARS brand candies (See the next page).

Key for the identification of candy types produced by the MARS company:

1. Candy is chocolate-based ……..……..……..……..…… 2
1. Candy lacks chocolate, but is fruity ……..……..…..……... 3
2. Candy has a hard, colored coating ..... M&Ms
2. Candy has a chocolate surface ……..……...……. 4
4. Candy has caramel ……..... 5
4. Candy without caramel ……..……..……..…... 6
5. Candy has peanuts ……..……..……..……... Snickers
5. Candy without peanuts ……..... 7
7. Candy with nougat filling ……..……..…... Milky Way
7. Candy with cookie bar ……..……..……..……..……... Twix
6. Candy solid chocolate ……..……..... Dove bar
6. Candy with nougat filling ……..……..…… Three Musketeers
3. Candy with hard surface ….……..……..……..……...…. Skittles
3. Candy soft, taffy-like …...……...……...…….. Starburst

You will prepare a key for the identification of nutrient deficiency symptoms in tomato (cv. Micro-Tom) for the six mineral nutrients evaluated (N, P, K, Mg, Ca, and Fe). The key should be typed and formatted as in the MARS candy example. Both choices (leads) for each couplet should be placed on adjacent lines and the couplets should be nested (Couplets 4-7 are nested within Couplet 2) and indented to show the hierarchy.

General Advice and Tips: The process of designing and construction your key can be facilitated using a heuristic device, termed a branching diagram. **Branching diagrams** are outlines of ways the samples being evaluated (nutrient deficient seedlings) can be organized into groups of progressively smaller size, one trait (branch) at a time, until each sample is separated from all of the rest ('keyed out'), based on a unique combination of traits. In general the most major (easily observed) traits are used for earlier branches in the diagram and more minor traits for later branches; with growth inhibition being an example of a major trait and the pattern of chlorosis within a leaf being a more minor trait. A branching diagram corresponding to the MARS candy key is provided in the **Appendix D**. Branching diagrams can be used as templates for the construction of keys by assigning couplet numbers to each node, or branch point, from left to right and top to bottom for branch points falling within the same tier (column). More detailed guidance is available in **Appendix E.**

Student Appendix A: Nutrient Deficiency Data Collection Table

Table 1. Compilation of Shoot Nutrient Deficiency Symptoms

Nutrient Deficiency	Symptoms
Negative Control (Distilled Water)	
Nitrogen	
Phosphorous	
Potassium	
Calcium	
Magnesium	
Iron	

Student Appendix B: Nutrient Deficiency Data Collection Instructions

1. Growth Inhibition (Visual Assessment)

- What organs are affected (shoots, leaves, or both)?
- How severe is the inhibition (or promotion)?

COMMENTS: The heights and weights of the shoots are directly measured in Part II of this lab (Shoot Growth Data). At this point you are charged with making visual assessments of the degree of growth inhibition (or promotion) for the nutrient deficiency treatments, expressed as a percentage of the degree of growth for the positive controls. If sufficiently broad percentage growth category ranges are used (0-25%, 25-50%, 50-75%, 75-100%, etc.) the differences in growth can be scored quickly and accurately by sight. However, since the degree of growth can be variable between seedlings within treatments you should evaluate multiple seedlings (3-4) from each treatment, as well as from the positive control, before assigning percentage growth ratings.

2. Leaf Abscission

- What leaves are affected, distal (younger) or proximal (older)? If the proximal leaves are affected is the abscission limited to the cotyledons or are the foliage leaves also affected? NOTE: In some cases the leaves may be dead but remain loosely attached to the stems. These leaves should still be scored as positive for abscission.
- How many leaves have been abscised as expressed in either absolute terms or percentage terms, for treatments where the degree of abscission is more extensive?

3. Tissue Death (Necrosis)

- Which organs are affected (stem or leaf)?
- Is the necrosis localized to specific regions of the affected organs?

COMMENTS: Necrosis is being evaluated here as a **primary symptom** of nutrient deficiency, not as a secondary symptom associated with leaf abscission. This means that the necrosis should be apparent at an early stage in leaf development; prior to the onset of chlorosis or other nutrient deficiency symptoms, and should be localized to specific regions of the affected leaves (tips, margins, or as individual spots of necrosis). When spots of necrosis are present on the leaves, they can be small and numerous, termed **stippling**, or larger with irregular margins, termed **splotching** or **mottling**. For some nutrient deficiencies the shoot and root tips may be directly affected. In such cases, branching is generally induced from lower (proximal) regions on the

affected root or shoot and the branches may partly obscure the root or shoot tip and thus may interfere with the diagnosis.

4. Chlorosis: Chlorosis is a condition in which the leaves (and stems) of a plant do not have a normal green color. Chlorotic tissues may be light green, yellow, or white. For mobile nutrients, the affected leaves will generally have a normal green color when they are young but will become chlorotic with age, as mineral nutrients are exported from the older, lower leaves to the growing regions of the shoot. An opposite pattern is observed for immobile nutrients. Since nutrient recycling between leaves is not possible, the chlorosis appear first in the youngest, more distal, leaves and may be apparent from even the earliest stages of leaf ontogeny. In such cases, the degree and severity of chlorosis would be expected to increase for each successive leaf from the stem base towards the growing tip.

- Are all leaves equally affected, or is the chlorosis specific, or more severe, for the younger (more distal) or older (more proximal) leaves?
- What color are the affected regions (light green, yellow, light yellow, or white)?
- What is the pattern of chlorosis within the leaves? Is the chlorosis localized to the leaf margins, termed **marginal chlorosis**, the leaf veins (**veinal chlorosis**), the regions between the veins (**interveinal chlorosis**) or is the entire leaf affected (**diffuse chlorosis**). For any particular leaf the percentage of the leaf area impacted and severity (degree of green color loss) would be expected to increase as the leaf ages. Thus, it is not uncommon for leaves to start out with partial chlorosis (marginal, veinal, etc.) and for the chlorosis to spread across the entire leaf with time. In such cases, the chlorosis would be classified based on the initial pattern. Thus if any leaves are detected with definite marginal or veinal/interveinal chlorosis, this would be the designation assigned.

COMMENTS: For some nutrient deficiencies the leaves may be darker green than is typical for control plants.

5. Excessive Red-Purple (Anthocyanin) Coloration: Anthocyanins are water-soluble plant pigments that can vary from red to purple or blue in color depending on their specific structure and the pH. Several types of stress (nutrient, temperature, etc.) can induce higher than normal levels of anthocyanin synthesis and pigmentation.

- What organs, and regions of these organs are affected? Is the degree of purple coloration more severe for the distal or proximal leaves or stem regions?
- For leaves are all of the leaf regions equally affected or is the pigmentation limited to, or clearly more severe, for specific regions; i.e., veins vs. interveinal tissues, upper vs. lower surfaces, etc.?

6. Other (Morphology): Changes in plant morphology (leaf shape, root branching frequency, etc.) are also possible and should be noted if present.

Student Appendix C: Shoot Growth Data Table

Shoot Weight and Height Data Table

Nutrient Deficiency	Number of Nodes[1]	Shoot Height (cm)[2]	Shoot weight (g)[3]
Positive Control			
Negative Control (Distilled Water)			
Nitrogen			
Phosphorous			
Potassium			
Calcium			
Magnesium			
Iron			

[1] Nodes are points of leaf attachment. Count the number of nodes with leaves ≥ 1 cm long. The cotyledons of tomato are produced in pairs, as is typical for dicots, but the rest of the leaves are produced individually (one leaf per node). For treatments affecting plant height, the node counts can be used to pinpoint the cause of the height differences; i.e., differences in the degree of stem elongation, per see, vs. the rate of shoot apical meristem activity (number of nodes)

[2] Measure the height of the seedling from the base to the tip of the main stem, excluding leaves or axillary shoots that project above the shoot tip from the measurements.

[3] Cut off the shoots at the shoot-root junction and weigh them together with any abscised leaves.

Student Appendix D: Branching Diagram for MARS Candy Key

Student Appendix E: Additional Key Construction Tips

1) The characteristics used to distinguish between the taxa being compared at any particular couplet should be unambiguous and mutually exclusive. Distinctions based on degree (larger/smaller, etc.) should be avoided unless the differences are consistent and can be quantified, i.e., leaves 6-8 cm long vs. 3-4 cm, plants less than 50% as tall as the positive controls, etc.

2) Both statements, or 'leads', in a couplet should be parallel, meaning that they should evaluate the same trait or set of traits and should be worded similarly; i.e., 'leaf chlorosis appearing first or most severe in the lower leaves' vs. 'leaf chlorosis appearing first or most severe in the upper leaves'.

3) For couplets that discriminate based between taxa on the presence/absence of a trait, the first lead should be used for the positive alternative and the second for the negative, but without using wording such as 'not as above', or 'plant otherwise' for the second lead. Each lead should be a complete and independent statement that is able to stand on its own.

4) The initial couplets should distinguish between the taxa based on the most visually dramatic or easily scored differences; e.g., growth significantly inhibited vs. growth not inhibited, etc; with subsequent couplets focusing on 'finer' differences. This approach is logical and reflects the natural sequence in which the traits would be evaluated and differences between the specimens detected.

5) If the taxa being compared at a couplet differ consistently across multiple traits, all these traits can be incorporated into the leads, separated by semicolons. This way it is still possible to key out specimens exhibiting on a subset of the diagnostic traits. The evaluation of multiple traits also gives more certainty to the decisions made at each couplet.

Materials

Equipment

- Steam autoclave (See Appendix 5, 'Aseptic Technique')
- Laminar flow hood (See Appendix 5, 'Aseptic Technique')
- Heat sterilizer, Bunsen burner, etc. (See Appendix 5, 'Aseptic Technique')
- Fluorescent lighting unit or environmental chamber[1]
- Heated water bath (at least 10-12 inches tank size)
- Heated stirring pad
- pH meter and buffers (pH 4, pH 7)
- Balances (0.01 g accuracy)
- Micropipetters (200-1000 uL capacity)
- Beakers (0.5-4 liter capacity, glass or plastic)
- Graduated cylinders (50-1000 mL capacity range)
- Autoclavable media containers, 0.1-1 liter capacity (See Appendix 5, 'Aseptic Technique')
- Culture vessels (See Appendix 4, 'Culture Vessel Selection Guide')
- Measuring tools (spatulas, measuring spoons, etc.)
- Stainless steel forceps (6-8 inches length)
- Oven mitts

Plant Materials:

- Tomato (*Lycopersicon esculentum* cv. Micro-Tom) seeds[2]

Disposables:

- Micropipetter tips (200-1000 uL capacity)
- Petri plates, polystyrene, 100 x 15 cm diameter
- Filter paper circles (9 cm diameter)
- 1.7 mL microfuge tubes
- Labeling tape
- Sharpie® ink markers
- Nitrile or latex gloves

Reagents:

- Purified water (distilled or deionized)[3]
- Bleach (NaOCl)[4]
- Ethanol (70% ethanol:30% water)
- Tween-20 detergent (polyoxyethylene sorbitan monolaurate)
- Mineral salts (KNO_3, $Ca(NO_3)_2 \cdot 4H_2O$, $NH_4H_2PO_4$, $MgSO_4 \cdot 7H_2O$, Fe-EDTA, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $H_2MoO_4 \cdot H_2O$, $NaNO_3$, $MgCl_2 \cdot 6H_2O$, Na_2SO_4, NH_4Cl, $NaH_2PO_4 \cdot H_2O$, $CaCl_2 \cdot 2H_2O$, KCl)[5]
- Sucrose[6]

- 1M NaOH and 1 M HCl (in dropper bottlers)
- Agar[7]

Materials (Footnotes)

[1] Fluorescent lights are the preferred type of lighting for plant tissue culture. They generate less heat than incandescent or halogen bulbs and can be placed close to the cultures, generally within 6-18 inches, without raising the temperature of the culture environment to a significant degree. Tissue cultures should never be placed in the direct sunlight since the amount of radiational heating of the media and plants is liable to exceed the rate of heat transfer away from the vessels, particularly for traditional culture vessels with non-ventilated lids (Appendix 4), and the air temperatures inside the culture vessels may reach levels high enough to injure or kill the plants.

Since plant tissue cultures are supplied with carbohydrates (sucrose) via the culture medium they are not entirely dependent on photosynthesis for their growth and do not require as much light as greenhouse or field-grown plants. Light intensities ranging from 40-80 µM m^{-2} $second^{-1}$ are sufficient, and typical, for plant tissue cultures, compared with light intensities of up to 1000-2000 µM m^{-2} $second^{-1}$ for full sun at midday. Continuous (24 hr) lighting is possible but generally is not beneficial and can be injurious for some plant species, such as tomato (Globig et al., 1997; Hillman, 1956; Tibbets et al., 1990). Instead, plant tissue cultures are typically grown under long day photoperiods, such as the 16-hour photoperiod employed here. Appliance timers can be used for photoperiodic control and instructions for preparing a simple fluorescent lighting unit from are available in a previous ABLE publication (Stone, 1996).

[2] 'Micro-Tom' is a dwarf cultivar of tomato that grows just 6-8 inches tall (Scott and Harbaugh, 1989; Meissner et al. 1997). Because of its small stature it is well adapted for use in plant tissue culture. In this exercise, Micro-Tom seedlings grow to heights of 8-10 cm (3-4 inches) after about 10 weeks of culture on positive control medium (Appendix 11) and thus fit within traditional plant tissue culture vessels (Appendix 4, 10-15 cm tall). In contrast, in preliminary tests the seedlings of three determinate, non-dwarf cultivars ('Summerpink', 'Scotia' and 'Rutgers') grew to heights of 12-15 cm after just one to two weeks. In fact, these cultivars grew faster in tissue culture than in the greenhouse, perhaps as a result of the lack of air movement/mechanical perturbation. Seeds of Micro-Tom can be purchased from the Totally Tomatoes Company (Randolph, WI, http://www.totallytomato.com, 1-800-345-5977, Product 00468), the Tomato Growers Supply Company (Fort Myers, FL, http://www.tomatogrowers.com, 1-888-478-7333, Product 6536), and the Ball Seed Company (Chicago, IL, http://www.ballhort.com). A 1/32 oz package (Totally Tomatoes, $13.50) provides about 200-300 seeds.

[3] Plant tissue culture media are prepared using purified water to ensure a high degree of control over the mineral nutrient content of the medium. Traditionally, (double) distilled water has been employed. Deionized water is also acceptable but the reverse osmosis membranes and/or ion exchange resins used for deionization may introduce trace levels of organic contamination. In contrast, distillation removes both ionic and organic contaminants.

[4] Household bleach (Chlorox®, etc.) has traditionally been prepared with 5.25% NaOCl but the concentration may vary between brands and products (3-6%). Bleaches with additives (fabric softeners, perfumes, etc.) should be avoided since the effects of these ingredients on plant tissue cultures is unknown and may contribute to the experimental error. Since NaOCl decomposes when exposed to the air, the bleach containers should be tightly sealed between uses and working solutions (0.5-1.5% NaOCl) should always be prepared fresh on the day of use.

[5] Mineral salts may be purchased with different levels of hydration than indicated. The degree of hydration affects the formula weight and thus the quantity (weight) required to prepared solutions of a given molarity, but has no effect on the biological response. The ions supplied are the same irrespective of the degree of hydration.

[6] Sucrose (table sugar) may be purchased at the grocery store or from a scientific supplier. In theory, the purity should be greater for reagent grade sucrose, but many tissue culture labs, both commercial and academic, have reported using table sugar without any obvious adverse effects.

[7] Since agars are prepared from living organisms (red algae), they invariably contain mineral nutrient impurities. However, the levels of mineral nutrient 'contamination' can vary widely between different brands and purities of agar (Appendix 3). Agars can also vary in the ratio of gelling polysaccharide (agarose) to non-gelling polysaccharide (agaropectin and/or alginate), and thus may produce gels of different hardness at the same concentration (George, 1993). This variability is present both between and within brands since the algae used for agar preparation are harvested from natural populations and the specific taxa harvested and growth state of the algae may vary between harvest dates (batches). The agar used in my research lab (Phytotechnology Labs, Micropropagation Agar, Product A111), is prepared from *Gracilaria* species of algae. Although I have not tested other agar brands, for most of the mineral nutrients evaluated the concentration ranges present in agars (Appendix 3) are presumably not high enough to preclude symptom development over the course of 8-10 weeks of plant growth. However, the effects of differences in agarose:agaropectin ratio on the experimental outcome are less certain. Since water availability and gel hardness are inversely related parameters, differences in agarose content could affect the degree of seedling growth, and thus the rate of symptom development, across all nutrient treatments. In addition, indirect effects on mineral nutrition are possible since agaropectin is negatively charged and thus is capable of binding (adsorbing) a fraction of the cationic nutrients (Ca^{2+}, K^{+}, etc.). The bound nutrients may be less readily available to plants, adversely affecting plant mineral nutrition.

Methods

Media Preparation: A traditional hydroponics medium (Hoagland's #2 nutrient solution, Hoagland and Arnon, 1938) is employed. Hoagland's medium provides all of the essential plant mineral nutrients, except nickel. However, nickel has only recently been recognized as a required plant nutrient and is needed at such low concentrations that the amounts present in seeds are generally sufficient for the entire life cycle of herbaceous plants (Hopkins and Huner, 2004). In addition, for plant tissue cultures, the gelling agent brings in additional nickel.

In contrast with Hoagland's #1 solution, which contains nitrate (NO_3^-) as the only source of nitrogen, Hoagland's #2 contains both nitrate and ammonium (NH_4^+). Since nitrate uptake is coupled with hydroxyl, or bicarbonate, secretion from plant roots; raising the external pH, and ammonium uptake is coupled with proton exudation, lowering the pH, the inclusion of both forms of nitrogen tends to stabilize the pH of the medium (Brady and Weil, 2002; George, 1993; Hershey, 1992).

Solutions of Hoagland's medium are prepared by dilution from concentrated mineral nutrient stocks (Appendix 2). The positive control solution contains all of the mineral nutrients as specified by Hoagland and Arnon (1938) and distilled water is used as the negative control. The nutrient deficiency solutions are prepared by ion substitution. In this technique, the counterions of the excluded mineral nutrients are added as sodium or chlorine salts. For example, the magnesium-deficiency medium is prepared by using sodium sulfate in place of magnesium sulfate (Appendix 2). The sulfate concentration and total ionic strength of the medium are unchanged, but magnesium is excluded. The nutrient deficiency media contain higher concentrations of sodium and/or chloride than the positive control, but neither of these minerals is essential for plant growth and they are generally not thought to have adverse effects on plant growth at the low to moderate concentrations.

The stock solutions can be stored in a refrigerator (4 ºC) or freezer (-20 ºC) and should be replaced whenever precipitation or microbial growth are apparent, with the exception of the sodium sulfate stock (1 M) which naturally precipitates at 4 °C and needs to be redissolved by heating prior to each use.

After the nutrient solutions have been prepared they are adjusted to pH 5.7-5.8 using NaOH (0.1-1 M). For conventional hydroponic experiments the media would be complete at this point, but for plant tissue culture additional components (sucrose, gelling agent) still need to be added and the media are sterilized by autoclaving. I have generally used sucrose at 10 g/liter and agar (8 g/liter) as the gelling agent. Since agar requires heating to 45-50 ºC to dissolve (George, 1993), it is not added until the media has been divided into containers for autoclaving and dissolves while the medium is being sterilized.

Autoclave times of 20 minutes (121 ºC, 15 psi), using a 'liquid cycle' setting, are sufficient for the sterilization of media volumes of up to 1 liter (See Appendix 5, 'Aseptic Technique'). If the media are being sterilized in Pyrex bottles the lids should be loosened slightly (¼ to ½ turn) to prevent pressures from building up in the headspace of the bottle. Erlenmeyer flasks are generally sealed with foil closures (two layers, crimped around the lid) and thus are naturally better-ventilated and less likely to break during autoclaving. The volume of the flask should be twice as great as the media volume both to prevent boiling over, as the pressure is released at the end of the autoclave cycle, and to facilitate mixing and pouring after the media has cooled. In contrast, Pyrex bottles can be used at 80-100% full volume.

After the autoclave cycle is complete the media should handled gently since spurts of boiling can be induced as residual positive pressures in the bottles are released by shaking or knocking. They should be removed from the autoclave promptly, their lids, or closures, should be tightened, and they should placed in a 65 °C water bath (2-3 inches water depth) for 30-40 minutes for temperature equilibration. After cooling, the media are mixed by swirling to ensure that the agar is equally dispersed and poured. If reusable culture vessels are employed (glass, polycarbonate, etc.) they will also need to be steam sterilized prior to use and can generally be autoclaved at the same time as the media. The vessels are capped during autoclaving and should only be opened again at the time of media pouring in a sterile transfer hood.

For a traditional laminar flow hood, with an 18-24 inch deep working compartment, the vessels should be poured in rows of four to five at a time, and opened from the back to front of the hood, to ensure that your arms and hands, a potential source of contaminants (bacterial spores, etc.), do not pass over or behind an open vessel (Appendix 5, 'Aseptic Technique'). After the media has been poured (~25 mL per baby food jar, ~50 mL per GA-7 vessel, Appendix 4), the culture vessels are closed, from front to back, and can be removed from the transfer hood. The media will generally start to harden within 10-15 minutes of pouring and should not be handled at this point since they will not form uniform gels if they are disturbed during setting. The media can be used immediately after they have cooled to room temperature, but it is generally beneficial to wait three to four days to confirm their sterility.

Media Planning and Volumes: The media volumes required are affected by many variables including the class size, the student group size and number of seedlings evaluated per group, and the type of culture vessel. For example, for a class of 25 students working in groups of five with four replicates (seedlings) per group I would need a minimum of 20 seedlings per treatment (5 groups x four seedlings per group). These calculations assume that each seed germinates and produces a healthy seedling, that there are no losses to contamination, and that each seedling develops typical deficiency symptoms and thus is suitable for student use. However, since one or more of these assumptions is typically not meet, I generally prepare about 25% more replicates (seedlings).

Although some types of plant tissue culture vessels are designed to accommodate multiple explants (Appendix 4), I would recommend using smaller culture vessels (baby food jars, GA-7 vessels, etc.) and sowing a single seedling per vessel to ensure that the mineral nutrients in the media are not depleted prematurely, especially for the positive controls. Since the individual culture vessel is the basic unit of replication in tissue culture experiments, this approach ensures that each seedling is a separate replicate and thus is desirable from an experimental design perspective. Thus, for the example from the previous paragraph (5 student groups with four seedlings each) I would prepare 25 culture vessels of each media type, each with a single seedling. Using baby food jars, I would need 500 mL of each medium type (25 jars x 25 mL of medium per culture vessel, Appendix 4).

Seed Sterilization: The seeds are surface-sterilized (disinfested) with ethanol and bleach, in a laminar flow hood (Appendix 5). A wide variety of containers can be used for the sterilization, but I have generally found standard (1.7 mL) microfuge tubes to be the most economical and easiest to use. However, to ensure a high enough ratio of sterilant volume (1000 uL) to seed volume the tubes should be filled only one-third to one-half way full of seeds (50-100 seeds per tube on average) and for large experiments multiple tubes of seeds may need to be sterilized.

The sterilization is started with the addition of the ethanol (70%, with mixing). After one to two minutes the ethanol is removed and replaced with bleach (1.5% NaOCl) for 10 minutes with mixing by inversion every few minutes. The bleach is prepared with a few drops of Tween-20 detergent to lower the surface tension and ensure good contact with the seeds. After the bleach has been removed the seeds are rinsed four to five times with sterile (autoclaved) distilled water. After each aliquot of water (1000 uL) has been added the tubes are mixed to ensure that any residual films of bleach on the seeds are diluted into the water. As soon as the seeds have settled the rinses can be removed.

A micropipetter with sterile (autoclaved) tips is used to measure the samples of ethanol, bleach, and rinse water used for the sterilization. New tips should be used for each successive aliquot, including the water rinses. The final aliquot of rinse water is left in each tube and used to pour the seeds into sterile plastic petri plates with two sterile filter paper circles (9 cm) per dish. The water serves as a carrier for the seeds and also moistens the filter paper sheets.

Direct sowing is recommended, but petri dishes of seeds can be sealed with deformable plastic film (Parafilm® or Nescofilm) and stored at 4 °C for a matter of days to a week or so before sowing if necessary. Storage at room temperature is possible but the seeds may start to germinate prior to transfer to the culture media and the seedlings are liable to be damage or killed during handling and placement in the culture medium. The amount of seeds sterilized should be about 25%

greater than the number needed for the experiment to mitigate against losses during the sterilization. The extra seeds can also be used as replacements for any seeds that fail to germinate (See below).

Seed Sowing and Culture: Since tomato seeds do not require germination pre-treatments (cold exposure, seed abrasion, etc.) they can be directly sown onto the experimental media. Germination generally begins within four to six days of sowing and is completed within 10-12 days. Using current or one year old seed stocks (Totally Tomatoes, Randolph WI) I have generally obtained germination frequencies of 80-90% or higher. Seeds that fail to germinate can be replaced using leftover seeds from the seed sterilization (see above). These seeds can be stored at 4 °C but new seedlings will be one to two weeks behind the others in the experiment. Alternatively the extra seeds can be sown into a separate culture vessels (5-10 seeds/vessel) and transplanted into the experiment at the 10-12 day point.

The forceps used to handle the seeds should be at least one or two inches longer than the height (depth) of the culture vessel to ensure that the 'handling end' of the forceps is never inserted into the vessel (Appendix 5, 'Aseptic Technique'). Using traditional culture vessels (glass baby food jars, GA-7 vessels, etc.) a single seed should be allotted to each vessel. The seeds are placed on the medium surface in the middle of each culture vessel. As soon as the vessels have been closed they can be removed from the sterile transfer hood.

The seedlings are cultured under a 16 hr photoperiod using fluorescent lighting (See Materials, Footnote 1). The culture vessel lids can be sealed with Parafilm® or Nescofilm® but this is not generally necessary, provided that the culture environment (light shelf, environmental chamber, etc.) is reasonable clean and sanitary.

Notes for the Instructor

Pros and Cons of using Plant Tissue Cultures for Hydroponics Demonstration: Prior to developing the lab exercises described here, I used sand cultures to demonstrate plant mineral nutrient deficiency symptoms in my teaching. The sand provides a physical support for the seedlings but it does not contribute significantly to their mineral nutrition so stereotypical nutrient deficiency symptoms are generally observed. However, frequent watering is required; daily or every other day at later time points in the experiment, increasing the reagent costs and the likelihood of application errors. In comparison, plant tissue cultures require no maintenance after the seeds are sown.

The same ease of maintenance applies for conventional hydroponics (solution culture), provided that the containers housing the plants have close-fitting closures, but more space is required since the containers (Mason jars, etc.) are generally larger than used for plant tissue culture (Appendix 4). Tissue cultures may also be superior for the demonstration of nutrient deficiency symptoms for immobile mineral nutrients, such as calcium and iron, as well as for the evaluation of treatment effects on root growth. Since immobile nutrients are not transported in the phloem they are dependent upon transpiration, or root pressure generated, xylem flow for their long distant movement (root to shoot) in plants. However, for experiments employing traditional (non-ventilated)

culture vessels, such as this experiment, the rate of transpiration is limited by water vapor accumulation inside the air space of the culture vessel exacerbating the nutrient stress and hastening symptom expression.

Tissue culture media, like soil, includes a stationary phase, formed by the crossing linked gelling agent molecules, that limits nutrient movement both physically, via the hydraulic restriction of water flow, and chemically, via cation adsorption for gelling agents that include negatively charged components (agar, gellan gum, carageenen, etc.). Thus, nutrient acquisition strategies based on changes in root:shoot ratio, root architecture, or substrate modification (media acidification, chelating agent secretion, etc.) are more likely to be effectual, and expressed, in gelled media than in liquid media. Root phenotypes (branching, root hair density, etc.) are also more easily evaluated in tissue culture than solution culture since the media is stationary and the culture vessels are transparent. In contrast, the containers used for solution culture are typically wrapped in foil, or are opaque, to prevent algal growth and root hair development appears to be inhibited in solution culture (Epstein and Bloom, 2004)

The primary disadvantages associated with using tissue cultures for hydroponics demonstration are the more substantial infrastructural requirements and the greater level of technical expertise required. Traditionally, plant tissue culture has required several larger pieces of equipment, such as autoclaves and laminar flow hoods that may not readily available to faculty at colleges or smaller universities. However, a wide range of alternative protocols that do not require such expensive equipment have been developed in recent years. For example, plant tissue culture media can be sterilized using microwaves or pressure cookers instead of autoclaves (Phytotechnology Labs, 2008; Stiff, 2004; Taji, 1996) and 'transfer boxes' constructed from plexiglass or acrylic (Stone, 2006), or even from plastic-lined cardboard or PVC frames (Stiff, 2004) can be used in place of laminar flow hoods to create a clean working environment. Biocides (antibiotics, fungicides, chlorine, isothalozones, etc.) can be added to the culture medium at low concentrations, both as an alternative to heat sterilization and as a safeguard against contaminant introduction during culturing (Niedez, 1998; Herman, 2006; Paul et al., 2002).

Tissue Culture Training and Resources: For readers interested in learning basic plant tissue culture techniques, a wide variety of plant training opportunities are available including hands-on workshops offered by the non-profit organization, Kitchen Culture Education Technologies (KCET, http://www.hometissueculture.org/htcgworkshops.htm) as well as by the Environmental Horticulture Department at the University of Florida (see http://hort.ifas.ufl.edu/2006PlantTCWorkshop.pdf for an example). Videos demonstrating basic plant tissue culture techniques are also available at YouTube (see http://www.hometissueculture.org/ for links) and training CD/DVDs are available from Dr. de Fossard ('Plant Tissue Culture Propagation' available at http://users.bigpond.net.au/rdefossard/home.htm) and Dr. Carol Stiff ('Basic Plant Tissue Culture' and 'Plant Tissue Culture for the Classroom and Home', available at http://www.hometissueculture.org, see the online catalog). Plant tissue culture kits, emphasizing skills development are available from the Home Tissue Culture Group (http://www.home-tissueculture.org) as well as from Carolina Biological (Career Skills in Plant Tissue Culture).

Plant Species and Cultivar: Historically, tomato has been one of the most widely used plants for hydroponics demonstration. In conventional hydroponic experiments, tomato exhibits stereotypical deficiency symptoms for most major mineral nutrients and symptom interpretation is facilitated by its cauline (non-rosette) growth habit and relatively long juvenile period. Tomatoes are also generally easy to grow and exhibit relatively high levels of disease and pest resistance. Similar advantages apply in tissue culture, but when traditional tissue culture vessels (10-15 cm tall) are employed, the use of extreme dwarf cultivars, such as Micro-Tom, is necessary to ensure that the seedlings will not outgrow the culture vessels prior to the onset of symptom development (See 'Materials', Footnote 2).

Appendix 9 summarizes the major types of nutrient deficiency symptoms exhibited by tissue cultures of Micro-Tom in this experiment. In general, the phenotypes are similar to those that have been reported for studies employing conventional hydroponics techniques (Berry, 2006; Epstein and Bloom, 2004; Hoagland and Arnon, 1938; Woolley and Broyer, 1957); but a few of the symptoms are somewhat unusual. For example, we are not aware of any prior reports of calcium deficiency effects on stem diameter in tomato, or more generally, but stem hypertrophy was observed at 100% frequency for the calcium deficient medium (Appendix 10). Since similar phenotypes were not apparent, for the other tomato cultivars evaluated in our preliminary research the response appears unique to the Micro-Tom cultivar but we are not able to offer a satisfactory explanation the phenotype at this point.

The leaf epinasty exhibited by the magnesium deficiency seedlings was also somewhat unusual, but is not entirely without precedent. In fact, leaf epinasty was apparently also engendered by magnesium deficiency in the tomato cultivars employed by Hoagland and Arnon (1938) in their pioneering research on tomato hydroponics, as gleaned from an evaluation of their photomicrographs, but they do not state this directly. In contrast, alternative types of leaf distortion (e.g., hyponasty, leaf 'puckering') have been reported in other studies (Berry, 2005; Woolley and Broyer, 1957).

Leaf necrosis is a standard potassium deficiency symptom, but the location of the necrosis (marginal, spotty, or both) is variable between species and perhaps also between culture environments. Although necrotic stippling has been observed for tomato in prior studies (Epstein and Bloom, 2004; Woolley and Broyer, 1957) in both cases the leaves also exhibited marginal necrosis and the stippling was either slower to develop or was only apparent for a subset of the experimental plants. In contrast, stippling was the only type of necrosis observed in this study; a preferable phenotype for demonstration (teaching) purposes since necrosis is visually distinctive and is less likely to be confused with necrosis developing secondarily as a consequence of leaf abscission.

The primary drawback associated with using tomato (Micro-Tom) as the model species for this lab exercise is the lack of clear nutrient deficiency effects on the roots. In contrast, for each of the other three species that we evaluated in our preliminary experimentation (aspen, tobacco, and carnation) the roots were responsive to nutrient deficiency treatments (data not shown).

Timing - Plant Growth: Since the rate of symptom development is variable between the different nutrient deficiency treatments, the allotment of 8-10 weeks for plant growth, prior to data collection, is a compromise. For most of the nutrient treatments evaluated (-N, -P, -K, Ca) the visual deficiency symptoms develop rapidly and are best observed at an earlier time point, perhaps after 4-6 weeks. Beyond this time point, the leaves may start to die and abscise, especially for the –N, -P, and –K treatments. Long culture periods (8-10 weeks) may also confound the interpretation of the calcium deficiency symptoms. Organ (leaf) abscission is not a problem in this case, but axillary branching can interfere with the diagnosis of the primary symptoms, stunting and death or the growing shoot tip (Appendix 10). In contrast, for the –Fe and –Mg treatments, deficiency symptoms develop more slowly and may be apparent for only a subset of the seedlings after 8-10 weeks.

In his Plant Physiology Lab Manual, Ross (1974) recommends sowing the seeds for the nutrient deficiency treatments at different time points, to ensure a synchronous rate of symptom development, but this strategy would not be suitable here because it would confound the plant growth comparisons (Student Handout, Part II). A better option would involve modifying the lab so have the students evaluate the seedlings at multiple time periods, perhaps after 4-5, 8-10, and possibly even 12-14 weeks. (See also 'Treatment Evaluation and Data Collection'). Alternatively, photographs of the seedlings could be taken at earlier time points and shared with the students at the point of final data collection.

Timing – Treatment Evaluation and Data Collection – The data collection components of this lab (Parts I and II) can be completed in a standard three hour lab period if the students work in teams and clear directions are provided during the lab period. In general about 1.5-2 hours are required for the evaluation of visual deficiency symptoms including 15-20 minutes for general instructions and group practice (see 'Evaluation of Visual Deficiency Symptoms'). To expedite data collection I sometimes tell students how many of the treatments will exhibit each type of deficiency symptom so they can check their results along the way and hopefully will keep more focused in general. I also engage the student groups on a regular basis during the lab period instead of waiting for them to ask questions. This way difficulties that they may be having can be resolved promptly and if the same types of problems apply across multiple student groups class announcements can be made to resolve the confusion. \

Another option worth considering, from a time management perspective, would involve modifying the exercise to have students collect visual deficiency data at multiple time points (see 'Timing – Plant Growth', above). This way the students would be familiar with the data collection protocol and the basic types of deficiency symptoms for each of the nutrient treatments by the time of the final data period leaving more time for shoot growth data collection (Part II, Student handout) and discussion.

Since the shoot growth data (heights, weights, etc.) is quantitative the data collection process tends to go quickly. As long as the number of replicates per student group is not excessive, and students don't have to wait in line to weigh their samples one hour should be sufficient for this part of the data collection process. In addition, if necessary the node count data could be excluded without affecting the learning opportunities offered by the lab to a significant degree. I use this data to discriminate between growth inhibition at the level of shoot apical meristem activity (number of

leaves produced) vs. at the level of the internodes (mean internode length), but this data is not used for the subsequent portions of the lab exercise (Student Handout, Parts III and IV).

Evaluation of Visual Deficiency Symptoms: Because of the large number of deficiency symptoms that are possible and the wide variety of traits the students are asked to evaluate for each type of symptom (Appendix B, Student Handout), the data collection process is challenging and requires some training. Towards this end, I usually prepare an extra nutrient deficiency treatment (sulfur) that I evaluate together with the students at the start of the lab. This treatment is not a formal part of the experiment; i.e., it is not included in the statistical testing or nutrient deficiency key, but it works well for demonstration purposes since the seedlings exhibit three of the five major classes of nutrient deficiency symptom (growth inhibition, chlorosis, and excessive purple coloration) and the phenotypes are strong and easily scored.

Necrosis is generally the most difficult symptom for students to score. Primary necrosis, occurring prior to leaf abscission, is observed for two of the nutrient deficiency treatments (-Ca and –K). For potassium (-K), the necrosis develops early in leaf ontogeny, prior to the onset of leaf chlorosis, and is visually distinctive because of its stippling pattern. However, since the potassium deficient seedlings exhibit precocious leaf abscission, most of the affected leaves may be abscised and 100% necrotic, or nearly so, before the point of final data collection (8-10 wks). Precocious leaf abscission is not a problem for the calcium deficient treatment; but necrosis is often only observed for a subset of the seedlings and is so highly localized to the shoot or leaf tips that it is easily overlooked. In contrast, because the abscised leaves often fail to completely separate from the seedlings, presumably as a result of the lack of sufficient air (and leaf) movement to break residual connections between the leaves and the stem, students routinely to misinterpret secondary necrosis as primary necrosis (-P, -K, -Mg).

Another difficulty that students sometimes have relates to the transitory nature of some of the chlorosis distribution patterns. Since the percentage of chlorotic tissue tends to increase over time for any individual leaf, leaves tend to become diffusely chlorotic as they age even for treatments, such as the magnesium and iron deficiency, that induce interveinal chlorosis.. In fact, if the students are only evaluating the seedlings at the 8-10 week time point it is possible that most of the leaves for the seedlings assigned to these treatments may be diffusely chlorotic. In this case, the students should be encouraged to examine all of the leaves particularly those at the transition between affected and unaffected regions of the shoots, for leaves in an early stage of symptom development. If there are any examples of leaves with definite, non-diffuse chlorosis (inteveinal, marginal, etc.) they would assigned this designation/classification to the treatment.

Interpretation of Nutrient Deficiency Symptoms:

For treatments exhibiting strong and fast-developing chlorosis (-Mg, -N, -S, -Fe) the excluded mineral nutrients play a direct role in chloroplast biogenesis and/or maintenance. Nitrogen and magnesium, for example, are components of the chlorophyll molecule. Magnesium plays major

roles in the regulation of Calvin cycle enzymes and contributes to the maintenance of charge balance across the thylakoid membranes during electron transport. Iron and sulfur are essential functional components of many of the electron carrier proteins involved in photosynthetic electron transport (cytochrome b_6f, ferridoxin, Photosystem I). Phosphorous deficiency is unusual in that it is associated with increases, not decreases, in the degree of green coloration. However, the effect is presumably indirect, and is mediated by reductions in cell (leaf) size resulting in higher chloroplast densities per unit tissue volume.

The induction of higher than normal levels of anthocyanin pigmentation is a common deficiency symptom for mineral nutrients that are structural components of one or more major classes of biological molecule. Nitrogen for example, is used for the synthesis of both proteins and nucleic acids, as well as for the hormones indole acetic acid, an auxin, and all cytokinins. Sulfur is also used for the synthesis of proteins, since the amino acids cysteine and methionine both contain sulfur atoms, and phosphorous is used for the synthesis of nucleic acids and nucleotides (ATP, etc.). Any stress that adversely affects the synthesis of primary metabolites, such as proteins, is liable induce higher rates of anthocyanin synthesis. Anthocyanin synthesis provides an outlet (shunt) for the carbohydrates produced via photosynthesis. If the carbohydrates were not consumed via secondary metabolism the entire photosynthetic process would be liable to feedback inhibition depriving plant of the ATP and NADPH generated via photosynthetic electron transport.

Necrosis, observed for the calcium and potassium deficiency treatments, is the most difficult nutrient deficiency symptom to explain at the cell and molecular level but several possible mechanisms have been identified. Perhaps most importantly, both nutrients are known to play major roles in cell membrane function. Potassium is a major osmoregulator and charge carrier. Potassium import mediates stomatal opening and most turgor movements (sleep movements, etc.). Potassium also contributes to the maintenance of charge homeostasis across plant cell membranes via the activity of voltage gated potassium channels (rectifying channels). Calcium, in contrast, physically stabilizes cell membranes, presumably via the cross linking of phosphate and carboxylate residues on the membrane surface. Calcium also contributes to the regulation of microtubulin turnover during cell division and to cell adhesion by cross-linking pectin molecules in the middle lamella. Thus, the preferential susceptibility of the growing tips of shoots and leaves to calcium deficiency is presumably partly a consequence of their high rates of cell division and cell wall synthesis at these locations.

Plant (Shoot) Growth Quantification: The balances used to weigh the shoots should be accurate to the nearest 0.01 g to discriminate between the average weight gains for the treatments, particularly when comparing the negative controls and nitrogen deficiency treatment. MS Excel is used to calculate average and 95% confidence intervals for each of the variables (shoot height, weight, and node number).

Preparation of Nutrient Deficiency Identification Keys: Detailed instructions and guidance are provided in the student handout. However, since most students do not have prior experience using identification keys it is sometimes helpful to give an additional example. An example that most students can relate would involve developing a key for identifying students in the class. For small

classes, with 10-20 students, a complete branching diagram could be constructed within a matter of minutes. Since most classes have roughly equal numbers of male and female students, the most straightforward approach would involve using gender (M/F) for the first branch point, and traits such as height (range), eye color, hair color, etc. for subsequent branch points within each gender. As long as the names of the students matching the different categories (phenotypes) for each trait are written above the branches in the diagram, the process is orderly and manageable and can be used as a model for students when they prepare their own keys. It may also be possible to demonstrate the use of multiple traits at some of the branch points as encouraged in the Student Handout (Appendix E) and demonstrated in the sample key (Appendix 12)

Literature Cited

Barbas, E., C. Jay-Allemand, P. Doumas, S. Chaillous, and D. Cornu. 1993. Effects of gelling agents on growth, mineral composition, and naphthoquinone content of in vitro explants of hybrid walnut tree (*Juglans regia* x *Juglans nigra*). Annales des Sciences Forestieres 50: 177-186.

Berry, W. 2006. Symptoms of Deficiency in Essential Mineral Nutrients. In: Online Companion to Plant Physiology, Fourth Edition. Eds (Taiz, L and Zeiger, E). Sinauer Associates, Inc. Sunderland, MA. WebTopic 5.1. Available online at http://4e.plantphys.net

Brady, N.C. and R.R. Weil. 2002. The Nature and Property of Soils, Thirteenth Edition. Prentice Hall Publishers, Upper Saddle River, NJ, 960 pp. Chapter 9, Soil Acidity pp. 363-411

Epstein, E. and A.J. Bloom. 2004. Mineral Nutrition of Plants: Principles and Perspectives, Second Edition. Sinauer Associates, Sunderland, MA, 400 pages.

George, E.F. 1993. Plant Propagation by Tissue Culture, Vol. 1 (2nd Edition). Exegetics Ltd, Edington, England, 574 pages, Chapter 9, Components of the Culture Medium, pp. 273-342.

Globig, S., I. Rosen, and W.H. Janes. 1997. Continuous light effects on photosynthesis and carbon metabolism in tomato. Acta Horticulturae 418: 141-152.

Herman, E.B. 2006. Protocols for micropropagation without autoclaving. Agricell Report 47:17-18. Available online at http://agritechpublications.com/article.htm

Hershey, D.R. 1992. Plant nutrient solution pH changes. Journal of Biological Education 26:107-111.

Hershey, D.R. 1995. Plant Biology Science Projects. John Wiley & Sons, Inc. New York, NY, 176 p. Part V: Hydroponics, pp. 107-119.

Hillman, W.S. 1956. Injury of tomato plants by continuous light and unfavorable photoperiodic cycles. American Journal of Botany 43: 89-96.

Hoagland, D.R. and D.I. Arnon. 1938. The water-culture method for growing plants without soil. University of California, College of Agriculture, Agricultural Experiment Station, Berkeley, CA Circular 347: December 1938, 39 pages.

Hopkins, W.G. and N.P.A. Huner. 2004. Plant Physiology, Third Edition. John Wiley & Sons, New York, NY, 560 pages. Chapter 12, Plants and Inorganic Nutrition, pages 241-258.

Meissner, R., Y. Jacobson, S. Melame, S. Levyatuv, G. Shalev, A. Ashri, Y. Elkind, and A. Levy.. 1997. A new model system for tomato genetics. Plant Journal 12: 1465-1472.

Neidez, R. 1998. Using isothiazolane biocides to control microbial and fungal contaminants in plant tissue cultures. HortTechnology 8:598-603.

Paul, A.-L., C. Semer, T. Kucharek, and R.J. Ferl. The fungicidal and phytotoxic properties of benomyl and PPM in supplemented agar media supporting transgenic Arabidopsis plants for a Space Shuttle flight experiment. Applied Microbiology and Biotechnology 55:480-485.

Phytotechnology Labs 2008. Lily Multiplication Kit (Product L577), Product Literature, 8 pages Available as a pdf file at http://www.phytotechlab.com, Downloaded 10/08

Puchooa, D., P.N. Purseramen, and B.R. Rujbally. 1999. Effect of medium support and gelling agent in the tissue culture of tobacco (*Nicotiana tabacum*). University of Mauritius Journal of Science and Technology Research 3: 129-144.

Rafaill, W.S. 1982. Plant tissue culture systems as instructional tools in the biological sciences. Pages 39-51, in Tested Studies for Laboratory Teaching, Volume 3 (C.L. Harris, Editor). Proceedings of the Third Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 216 pages.

Ross, C.W. 1974. Plant Physiology Laboratory Manual. Wadsworth Publishing Company, Belmont, CA, 200 pages.

Scherer, P.A. and E. Muller. 1988. Multielement analysis of agar and Gelrite impurities investigated by inductively coupled plasma emission spectrometry as well s physical properties of tissue culture media prepared with agar or the gellan gum Gelrite. Acta Hortitculturae 226: 655-658.

Scholten, H.J. and R.L.M. Pierik. 1998. Agar as a gelling agent: chemical and physical analysis. Plant Cell Reports 17: 230-235.

Scott, J.W. and B.K. Harbaugh. 1989. MicoTom: A miniature dwarf tomato. Florida Agriculture Experiment Station Circular 370: 1-6.

Selby, C, R. Lee, and B.M.R. Harvey. 1989. The effects of culture medium rigidity on adventitious bud production and tissue vitrification in needle cultures of Sitka spruce [*Picea sitchensis* (Bong. Carr.]. New Phytologist 113: 203-210.

Singha, S., E.D. Townsend, and G.H. Oberly. 1985. Mineral nutrient status of crabapple and pear shoots cultured in vitro on varying concentration of three commercial agars. Journal of the American Society for Horticultural Science, 110:407-411.

Stiff, C.M. 2004. Affordable plant tissue culture for the hobbyist. Tropical Vision Rare Fruit Review (Online Magazine) Volume1(3). Available online at http://www.quisquails.com/tu03content.html.

Stone, M. 2006. Propagation of miniature roses by plant tissue culture. Pages 229-263 *in* Tested Studies for Laboratory Teaching, Volume 27 (M.A. O'Donnell, Editor). Proceedings of the 27th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 383 pages.

Taji, A. 2007. Plant Tissue Culture for Home Gardeners (Online publication, 3 pages). Available online at http://www.une.edu/.au/agss/hort/horticutlural-sceience.hph,

Taiz, L. and E. Zeiger. 2006. Plant Physiology. Sinauer Associates, Sunderland, MA, 764 pages.

Tibbetts, T.W., S.M. Bennett, and C. Weixing. 1990. Control of continuous irradiation injury on potato with daily temperature cycling. Plant Physiology 93: 409-411.

Woolley, J.T. and T.C. Broyer. 1957. Foliar symptoms of deficiencies of inorganic elements in tomato. Plant Physiology 32:148-151.

Acknowledgements

Several IPFW undergraduates contributed to the research that was necessary for the development of this lab including Agna Win, Kirk Bradtmiller, Patrice Harris, and especially Nicholas Becker. Nick assisted with many of the earliest experiments and identified the Micro-Tom cultivar as a suitable model for the use in the experiments. Kirk helped with experiments evaluating the bioavailability of the mineral nutrients contributed by gelling agents. Drs. Carol Stiff (KCET), Ken Torres (Phytotechnology Labs), and Gary Seckinger (Phytotechnology Lab) helped me to collect and organize the data used to prepare the plant tissue culture vessel comparison table (Appendix 4).

Appendices

1. Composition of Hoagland #2 Nutrient Solution
2. Preparation of Nutrient Deficiency Solutions (and Controls)
3. Agar Contributions to Plant Mineral Nutrition
4. Plant Tissue Culture Vessel Selection Guide
5. Aseptic Technique (Guidelines)
6. Typical Plant Mineral Nutrient Deficiency Symptoms
7. Key for the Identification of Nutrient Deficiency Symptoms in Tomato (Woolley and Broyer, 1957)
8. Tomato Seedling Shoot Morphology and Growth Quantification - Photoplate
9. Sample Visual Deficiency Data - Tabular
10. Sample Visual Deficiency Data - Photoplate
11. Sample Shoot Growth Data
12. Sample Nutrient Deficiency Key
13. Online Plant Tissue Culture Resources

Appendix 1: Composition of Hoagland #2 Solution

Table 1. Composition of Hoagland's #2 nutrient solution. This solution differs from the original recipe in supplying the iron as EDTA chelate, rather than iron-tartrate. EDTA is a stronger chelating agent than tartaric acid and thus is more effective at limiting iron precipitation in the medium (George, 1993).

		Concentration	
Compound	Formula Weight	mg/L	μM
KNO_3	101.1	606.6	6000
$Ca(NO_3)_2.4H_2O$	236.2	944.8	4000
$NH_4H_2PO_4$	115.1	115.1	1000
$MgSO_4.7H_2O$	246.5	493.0	2000
H_3BO_3	61.8	2.86	46
$MnCl_2.4H_2O$	200.0	1.81	9
$ZnSO_4.7H_2O$	287.5	0.22	0.8
$CuSO_4.5H_2O$	249.5	0.08	0.3
$H_2MoO_4.H_2O$	162.0	0.02	0.1
FeEDTA	376	9.4	25

Appendix 2: Preparation of Nutrient Deficiency Solutions (and Controls)

Table 2. Preparation of Hoagland #2 nutrient solutions, complete (All) and deficiency solutions. The types and amounts (mL) of stock solution required per liter of working nutrient solution are indicated. Distilled water, without any minerals, is used as the negative control. (-N = nitrogen deficient, -P = phosphorous deficient, -K = potassium deficient, -Ca = calcium deficient, -Mg = magnesium deficient, -S = sulfur deficient, and -Fe = iron deficient)

		Stock Solution Preparation		**Preparation of Nutrient Solutions**							
Mineral Salt	**FW**	**Conc.**	**g/L**	**Volume of Stock Added (mL/liter)**							
				All	**-N**	**-P**	**-K**	**-Ca**	**-Mg**	**-S**	**-Fe**
KNO_3	101.1	1 M	101.1	6	-	6	-	6	6	6	6
$Ca(NO_3)_2.4H_2O$	236.2	1 M	236.2	4	-	4	4	-	4	4	4
$NH_4H_2PO_4$	115.1	1 M	115.1	1	-	-	1	1	1	1	1
$MgSO_4.7H_2O$	246.5	1 M	246.5	2	2	2	2	2	-	-	2
Fe-EDTA	376	25 mM	9.4	1	1	1	1	1	1	1	-
Micronutrient Stock: (Combine all five)[1]				1	1	1	1	1	1	1	1
H_3BO_3	61.8		2.86								
$MnCl_2.4H_2O$	200.0		1.81								
$ZnSO_4.7H_2O$	287.5		0.22								
$CuSO_4.5H_2O$	249.5		0.08								
$H_2MoO_4.H_2O$	162.0		0.02								
$NaNO_3$	85	1 M	85	-	-	-	6	8	-	-	-
$MgCl_2.6H_2O$	203	1 M	203	-	-	-	-	-	-	2	-
Na_2SO_4	142	1 M	142	-	-	-	-	-	2	-	0.025
NH_4Cl	54	1 M	54	-	-	1	-	-	-	-	-
$NaH_2PO_4.H_2O$	138	1 M	138	-	1	-	-	-	-	-	-
$CaCl_2.2H_2O$	147	1 M	147	-	4	-	-	-	-	-	-
KCl	74.5	1 M	74.5	-	6	-	-	-	-	-	-

[1] The micronutrient stock solution combines five mineral salts (indicated with yellow shading). The rest of the stock solutions single salts, or chelate (Fe-EDTA)

Appendix 3: Agar Contributions to Plant Mineral Nutrition

Table 3. Mineral nutrient concentrations in Hoagland's #2 nutrient solution, agar, and typical critical concentrations for plant tissues. The critical concentration is the concentration within a plant when the supply of the nutrient is slightly less than that needed for maximal growth (e.g., 90-95% maximal growth) and no other nutrients are limiting. At lower concentrations growth is progressively inhibited and visual nutrient deficiency symptoms (chlorosis, necrosis, etc.) may be apparent.

	Hoagland #2 solution		Agar (ppm, dry powder)1		Plants (ppm, dry wt)[2]
Mineral	ppm	µM	Range	Typical	ppm
Nitrogen (N)	210	15,000	15-2,500	750-1,500	15,000
Potassium (K)	235	6,000	40-3,900	500-1,500	10,000
Calcium (Ca)	160	4,000	40-4,500	1,000-2,500	5,000
Phosphorous (P)	31	1,000	30-2,400	250-1,000	2,000
Sulfur (S)	64	2,000	2,100-8,600	6,000-8,000	1,000
Magnesium (Mg)	49	2,000	75-2,400	500-1,000	2,000
Iron (Fe)	1.4	25	3-165	30-100	100
Chlorine (Cl)	0.6	18	--		100
Boron (B)	0.5	46	--		20
Manganese (Mn)	0.5	9	--		50
Zinc (Zn)	0.05	0.8	--		20
Copper (Cu)	0.02	0.3	--		6
Molybdenum (Mb)	0.01	0.1	--		0.1
Sodium (Na)	0	0	1,300-14,000	8,000-12,000	0

[1] Concentration ranges as reported in the primary literature (Barbas et al., 1993, Puchooa et al., 1999; Scherer et al., 1988; Scholten and Pierik, 1998; Selby et al., 1989; Singha et al., 1985). Collectively, these studies have employed a wide range of agar brands and purity grades. The second column lists the concentration ranges for agars of medium purity, as are typically used for plant tissue culture. Since agars are diluted into the media (8-12 g agar/liter), the concentration of mineral nutrients contributed to the media would be about 100-fold lower than those listed in the table. In addition, some of the mineral nutrients (N, P, S), may be present in both inorganic and organic form, and thus may be only partly available for plant absorption. This is especially true for sulfur, which is a component of the non-gelling fraction of agar (agaropectin).

[2] Taiz and Zeiger (2006)

Appendix 4: Culture Vessel Selection Guide

Table 4. Plant tissue culture vessel comparison table: The vessel dimensions are given in length by width by height order (L x W x H) for rectangular vessels and in diameter by height order (D x H) for round vessels. Bottom and top diameters, separated by a dash, are provided for the Erlenmeyer flasks. Single (maximal) diameter values are provided for the Sundae cups, but the top and bottom pieces are both tapered 3-4 cm. The height and volume values for the flasks exclude the neck region since this is not a useable growth space. The media volumes are estimates of the amount of media (mL) that would provide 1 cm of depth (4 cm for the culture tubes). Price ranges and vendors are listed for all vessel types except for Mason jars and plastic Sundae cups, which are available from national retailers and restaurant supply companies, but not from scientific suppliers (See 'Table Notes'). For each product, the vendors are listed from least to most expensive using superscript abbreviations. A plus sign (C + S, etc.) denotes vendors with the same pricing. C = Caisson Labs, F = Fisher Scientific, H = Home Tissue Culture Group, P = Phytotechnology Labs, S = Sigma-Aldrich, V = VWR International. Positive and negative aspects of the different types of culture vessels are discussed individually in the 'Table Notes.'

Vessel Type (Material)[1]	Dimensions, cm [~Volume]	Media Volume	Closures	Pricing and Vendors[2]
REUSABLE CULTURE VESSELS				
Magenta® boxes (Polycarbonate)	6.3 x 6.3 x 9.9 [400 mL]	40 mL	Polypropylene	1. Vessels + Lids: \$170-300/100$^{C+ P, S}$ 2. Lids: \$50-100/100^{P,C,S}
'Baby Food' jars (Borosilicate glass)	5.6 x 9.4 [225 ml]	25 mL	Polypropylene (B-caps)	1.Jars: \$57/100^{S} 2. B-caps: \$30-56/100$^{H+C,S}$
Mason jars (Soda lime glass) ***See the 'Table Notes' for safety considerations**	**a. 1/2 pint (8 oz)** 7 x 9.5 [240 mL] **b. Pint (16 oz)** 8 x 13 [480 mL] **c. Quart (32 oz)** 10 x 17 [960 mL]	a. 40 mL b. 50 mL c. 80 mL	Polypropylene (threaded)	1. Jars (8 oz): \$9/12 2. Jars (16 oz): \$10/12 3. Jars (32 oz): \$11/12 4. White polypropylene lids: \$4/12 5. Natural polypropylene lids: \$6/12^{P}
Erlenmeyer flasks (Borosilicate glass)	**a. 125 mL** [6.5-2.7 x 11.25] **b. 250 mL** [8-3 x 13]	a. 35 mL b. 50 mL	a. One hole rubber stoppers (cotton plug) b. Foam stoppers c. Silicone closures d. Bug-stopper closures	1. Flasks: \$40-65/12$^{F,S+V}$ 2. Stoppers: \$27-56/20-30$^{P+V, S}$ 3. Foam stoppers: \$62/200^{F} 4. Silicone lids: \$77/10^{P} 5. Bug-stoppers: \$110/10^{V+F}
Culture tubes (Borosilicate glass)	2.5 x 15 [75 mL]	15 mL	Polypropylene a. Kim-Kap™ closures b. Magenta® 2-way closures c. Bellco® closures	1. Tubes: \$56/125^{P}, \$179/500^{F} 2. Kim-Kap® lids: \$82-\$120/500^{F,P} 3. Magenta® lids: \$10-\$20/100^{C,P} 4. Bellco® lids: \$63/144^{P}
Combiness™ trays (Clarified	**a. 7.5 x 13 x 8** [800 mL]	a. 100 mL b. 80 mL	Natural polypropylene (Included), both non-	1. \$125/100^{C}

Polypropylene)	**b. 10 x 8** [640 mL]		ventilated and ventilated	
Culture trays (Polypropylene)	**a. 8 oz [240 mL]** 11.5 x 3.5 **b. 16 oz [480 mL]** 11.5 x 7 **c. 32 oz [960 mL]** 11.5 x 13.5 14.5 x 8	a. 100 mL b. 100 mL c. 100 mL	Polypropylene (Included)	1. 8 oz: $10/10^P$ 2. 16 oz: $13/10^P$ 3. 32 oz: $16/10^P$
PhytoCon™ trays (Polypropylene)	**a. 8 oz [240 mL]** 11.5 x 3.5 **b. 16 oz [480 mL]** 11.5 x 7 **c. 32 oz [960 mL]** 11.5 x 14	a. 100 mL b. 100 mL c. 100 mL	Polypropylene (Included)	1. 8 oz: $7/10^P$ 2. 16 oz: $8/10^P$ 3. 32 oz: $10/10^P$
DISPOSABLE CULTURE VESSELS				
Combiness™ trays (Polystyrene)	7.5 x 13 x 7-14 [700-1,400 mL]	100 mL	Polystyrene (Included)	1. $100/100 *The heights (depths) of the lids and bases both variable (multiple choices)
Phytatrays™ (Polystyrene - PT I) (PETG –-PT II)	**a. Phytatray I** 11.25 x 8.5 x 6.25 [600 mL] **b. Phytatray II** 11.25 x 8.5 x 10 [960 mL]	a. 100 mL b. 100 mL	Polystyrene or poly-ethylene terephthalate glycol (PETG) (Included)	1. Phytatray I: $84/100^S$ 2. Phytatray II: $144/100^S$
Clear-Con™ (Polystyrene)	**a. 6.8 x 6.8** [250 mL] **b. 6.8 x 11** [400 mL]	a. 35 mL b. 35 mL	Polystyrene (Included)	1.(6.8 x 6.8 cm): $215/320^P$ 2.(6.8 x 11 cm): $152/192^P$
Sundae cups (Polystyrene)	**a. 5 oz [150 mL]** 9.2 x 11.3 **b. 8 oz [240 mL]** 9.2 x 13 **c. 12 oz [360 mL]** 9.8 x 15.3	a. 30 mL b. 30 mL c. 30 mL	Polystyrene (Included)	1. 5 oz: $173/1,000 2. 8 oz: $186/1,000 3. 12 oz: $203/1,000

[1]Polypropylene transmits light but is not as clear as glass or polycarbonate. Polypropylene is also less heat resistant and are susceptible to cracking when sterilized in non-validated autoclaves that are prone to running over temperature (> 121 °C).

[2]Vendor Contact Information: **Caisson Labs** (North Logan, UT; 1-877-840-0500; http://www.caissonlabs.com), **Fisher Scientific** (Fairlawn, NJ; 1-800-766-7000; http://www.fishersci.com), **Home Tissue Culture Group** (Milton, WI; 1-608-302-2750; http://www.homtissueculture.org), **Phytotechnology Labs** (Lenexa, KS; 1-888-749-8682; http://www.phytotec-hlab.com), **Sigma-Aldrich** (St. Louis, MO; 1-800-325-3010; http://www.sigmaldrich.com), **VWR International** (West Chester, PA; 1-800-932-5000; http://www.vwrsci.com)

Culture Vessel Comparison Table - Notes

Magenta® Vessels: Magenta® vessels have traditionally been one of the most popular types of culture vessels. They come in two heights; 9.7 cm (GA 7 vessels) and 7.7 cm (GA-4), with interlocking polypropylene lids. Each vessel holds one to four plants, or shoot cultures, depending on their growth stage and size. Magenta vessels are available from a wide variety of vendors or from the manufacturer directly (Magenta Corporation, Chicago, IL; 773-777-5050, $118/100). Magenta vessels can be linked end-to-end using plastic 'coupling rings', doubling their height. Compared with glass culture vessels Magenta vessels are more light-weight and are less likely to break but they are more chemically 'reactive' and are susceptible to mineral or detergent spotting if they are not rinsed well with distilled water prior to re-autoclaving. Magenta vessels are also easily scratched by scouring pads (e.g., Scotch pads, etc.) and should only be cleaned with soft brushes or sponges. The square shape of Magenta vessels is beneficial since it reduces glare and facilitates the photographic documentation of cultures. Combiness culture vessels also employ a square design but since they are made from natural polypropylene they are not as clear and thus would not work as well for photographic documentation.

Baby Food Jars: At the current time Sigma Aldrich is apparently the only commercial supplier of baby food jars in U.S. They sell two sizes of jar, 100 mL (5.9 mm x 6.8 mm) and 175 mL (5.9 cm x 9.9 cm), with the larger jars being preferred for most tissue culture applications because they can accommodate taller cultures. These are the same jars baby foods are sold in, but the screw on metal lids that are used for the packaging of baby foods are not suitable for plant tissue culture since they are opaque and easily corroded by autoclaving. Snap on polypropylene closures, termed B-caps, manufactured by the Magenta Corporation (Chicago, IL), are used instead. Money can be saved by arranging with daycare centers/ nursery schools to recycle their baby food jars for tissue culture use, but with the trend towards using plastic instead of glass packaging for baby food jars this option will become less feasible. *Borosilicate apparently used for baby food jars cause less leaching and sometimes heated (greater chemical and temperature resistance)

Glass Culture Tubes: Aside from the vendors listed, glass culture tubes and closures can also be purchased from general scientific distributors such as VWR and Fisher Scientific. Since test tubes are not freestanding they must be used with a culture rack. The most economical option is the Magenta '7-way' tray. These trays, which hold 36 tubes each, are sold by both Caisson Labs ($3.23)

and Phytotechnology Labs ($3.54). Alternatively, flat-bottomed culture tubes are available from Phytotechnology Labs (2.5 cm x 9 cm, $89/144). Several brands of closure are available. The Magenta® closures are the thinnest and thus the least expensive. Since the lids are made from natural (non-clarified) polypropylene the Magenta closures are also have superior light transmission characteristics.

Mason Jars: Aside from their traditional use for home canning and food preservation, Mason jars can be used for plant tissue culture and are especially popular among hobbyists because of their relatively low cost and wide availability. Ball and Kerr brands of Mason jars, manufactured by Jarden Home Brands (Muncie, IN), can be purchased from a wide variety of online vendors including the Canning Pantry (http://www.canningpantry.com), KitchenKrafts (http://www.kitchendrafts,com", and Home and Beyond (Http://www.homeandbeyond.com), as well as from many national retailers (Krogers, Meijer, Menards, WalMart, Home Depot, Kmart, and Target). Both brands of jars come in same sizes and with both narrow (70 mm) and wide (86 mm) designs. The metal closures provided with the jars are not suitable for tissue culture use because they are opaque, are susceptible to corrosion during autoclaving, and have a modular in design, with separate lids and screw-on bands, that makes it difficult to add and remove the lids when working in a sterile culture environment such as a laminar flow hood. One-piece polypropylene 'storage' lids, are available but they are also opaque (white) and are not specifically rated for autoclaving (121 °C). Autoclavable lids prepared from natural (clear) polypropylene, are available from Phytechnology Labs (Product C566, 70 mm diameter). Phytotechnology Labs also sells the generic equivalent of Ball and Kerr brand Mason jars. These jars cost about twice as much as standard mason jars, but are purportedly prepared from borosilicate glass. Compared with soda lime glass, borosilicate glass exhibits higher levels of heat and chemical resistance. However, soda lime glass that has been de-alkalinized (USP Type II glass) is apparently nearly as durable as borosilicate glass and may also be autoclave safe. The technical support department at Jarden Home Brands was unable to tell me if the glass used for their jars meet USP Type II specifications but indicated that the jars had a 90 °F thermal shock guarantee and suggested they could withstand heating to 121 °C, if the rate of temperature change was gradual (15-20 minutes). However, since I have no direct experience using Ball or Kerr brand jars for plant tissue culture I cannot vouch for their autoclave resistance. In addition, susceptibility to thermal-stress induced breakage may vary between autoclaves based on differences in the rate of temperature change and degree of temperature control.

Erlenmeyer Flasks: Erlenmeyer flasks come in narrow and wide mouth forms. They are widely used for cell suspension cultures where their tapered shape is beneficial since it minimizes friction during mixing and ensures that the media does not contact the top, or lid, of the culture vessel.

However, for cultures of shoots or whole plants this shape is disadvantageous since it limits the space available for height growth. In addition, the neck's diameter may be too small to accommodate larger explants of tissue samples, especially when narrow (standard) mouth flasks are employed. The 125 mL flasks take #5 and #6 rubber stoppers (narrow vs. wide mouth). The 250 mL flasks take #6 and #8 stoppers. The prices ranges provided in the table are for narrow mouth flasks and closures. Wide mouth flasks (titration flasks) generally cost about 20-50% more. Closures for wide mouth flasks may cost double (100% increase).

Combiness™ Vessels: One of the major advantages of these vessels, especially for commercial labs and large research labs, is the option of buying vented lids. The vents, constructed from a hydrophobic membrane, facilitate gas exchange with the external environment while excluding contaminants (bacterial cells and spores, etc.). For many types of plant tissue cultures, particularly shoot cultures, the added ventilation improves growth and plant quality. Combiness vessels are available with three different sizes of vent. If the vent is too small the degree of aeration may be insufficient for the desired effects on the plant cultures. If it is too large could the rates of water loss and media drying may be excessive.

Sundae Cups: Like Baby Food jars, plastic Sundae cups are designed for use in food packaging, but they also work well for plant tissue culture. Although they are not marketed as sterile products they are generally sterile from the manufacturing process. The dimensions shown are for the WNA Comet brand (Sundae Cups/Dessert Specialty Containers) available online from the WEBstaurant store (http://www.webstaurantstore.com). Solo brand Sundae cups are available from the Reliable Paper Company (http://www.reliablepaper.com). In both cases, the cups (bottoms) and lids (tops) are separately purchased.

Appendix 5: Aseptic Technique

Aseptic (sterile) technique is a set of procedures and work habits that minimize the risk of contaminant introduction into a specimen or patient. Aseptic technique is especially important in plant tissue culture since the culture media generally contain both mineral nutrition and carbohydrates (sucrose) and thus can support the growth of a wide variety of bacteria and fungi. Traditionally, aseptic technique has required a wide range of specialized equipment including an autoclave, a laminar flow hood, and portable heating elements (glass bead sterilizers, Bunsen burners, etc.) to sterilize the tools used to handle the cultures (forceps, scissors, etc.).

Autoclaves use a combination of steam (wet heat) and pressure (121°C, 15 psi) for sterilization, with exposure times of 20-30 minutes being sufficient for the sterilization of most solid objects and solutions, up to 1-liter volume. The containers used for autoclaving should be made from stainless steel, low expansion borosilicate glass, such as Pyrex®, or heat-resistant plastic (polycarbonate, polypropylene, etc.). For liquids, the closures (lids) on the containers should be loosened slightly prior to starting the autoclave cycle to allow for pressure equilibration between the autoclave chamber and the 'headspace' above the liquids, minimizing the risk of breakage. In addition, most autoclaves have 'liquid' settings that limit the rate of pressure change during the autoclave cycle. When liquids are being sterilized in containers lacking screw-on lids (Erlenmeyer flasks, etc), the containers should be filled only half way, at most, to prevent boiling over, as the pressure is released at the end of the autoclave cycle. Solid items (e.g., pipette tips, forceps, filter papers, etc.) can be autoclaved in closable containers or wrapped in foil. After the autoclave cycle has completed, all containers should be checked to make sure their closures have not become loosened and should be subsequently opened only in a sterile transfer hood to maintain their sterility.

Perhaps the single most important item of equipment for the aseptic culture of plants is the laminar flow hood. At its essence, a laminar flow hood consists of a blower and a high efficiency particulate (HEPA) filter in sequence. The room air is pulled in through the blower and moved across the HEPA filter which retains all aerosols (suspended particles) of 0.3 μM diameter or greater, including microbial cells and spores. The filtered air is directed across the work compartment and towards the user at a constant velocity, as a laminar (non-turbulent) flow, in a horizontal or vertical orientation, depending on the hood design, to create a sterile working environment. Laminar flow hoods should not be confused with biological safety cabinets where the airflow is directed away from the operator and is HEPA-filtered for a second time prior to exhausting, but both can be used for plant tissue culture.

Laminar flow hoods should be turned on 15 minutes prior to use to flush out particles that have collected on the downwind side of the HEPA filter. Objects that do not need to be in the hood should be removed and those objects that are retained (sterilizers, media bottles, etc.) should be kept to the side to maintain a clean 'working' area in the center of the hood. In addition, for horizontal laminar flow hoods, where the air flow is from the back to the front, a clear path should be maintained between the back of the hood and the tissue cultures or other sterile objects in the working area. The inner surfaces of hood, especially the back wall/grill and bench top, should be wiped down with paper towels wetted with 70% ethanol prior to starting working, and again at

regular intervals during the work period. It is also customary to spray, or wipe down, with 70% ethanol and subsequently brought into the hood objects (pipette tip boxes, media bottles, etc.).

Since human skin contains an extensive microbial flora, the limbs and head of the experimenter are potential sources of contamination. To mitigate against these risks, you should wear disposable latex or nitrile gloves and should spray down your arms and hands with 70% ethanol on a regular basis while working. The gloves also offer some heat insulation and thus facilitate the pouring of media (65-80 ºC) and handling of heat sterilized culture tools. They also protect your skin against the drying action of the ethanol spray, an especially important concern during the winter and early spring in the northern hemisphere when the air is dry and skin desiccation is naturally a problem. However, your gloved hands are not necessarily sterile and thus should not be used to directly handle any of the genuinely sterile objects used in your work (plant samples, sterile pipette tips, etc.).

Each time your arms or head project into the hood, they create areas of turbulence, or backwash, that can carry particulates from the unfiltered room air into the hood. To minimize this risk you should sit straight up, instead of leaning into the hood, and should keep the sterile objects (plant cultures, etc.) being manipulated in the back half of the hood. Since watches and wrist jewelry can trap pockets of contaminated air around your arms, they should be removed prior to starting your work. Long-sleeve shirts should be avoided, or rolled up past the elbow, for the same reason.

The tools used to handle the tissue cultures (forceps, scalpels, etc.) should be heat sterilized prior to their first use and again at regular intervals using either a glass bead sterilizer, a Bunsen burner, or a ceramic-based heating element. Glass bead sterilizers are the safest and most popular option. They consist of a well of glass beads (1.5-2.5 mm diameter) heated to 250 °C. Tools are sterilized by immersion into the beads for 10-20 seconds. Ceramic sterilizers, such as a 'Bacti-Cinerator', can also be used but since these are designed for use with inoculating loops, the well is generally narrower and is heated to a much higher temperature (800 °C). Scissors and large forceps may not fit, or may only partly fit in the well, and the high temperatures generate shrink-swell stresses and can cause premature breakage of the tools. Bunsen burners use an open flame for sterilization (10-15 seconds exposure). Although, Bunsen burners have historically been widely used for the heat sterilization of plant tissue culture tools, since they are a fire hazard their use is discouraged.

Irrespective of the specific sterilization apparatus employed, the tools should be clean and dry before sterilization and scissors should be sterilized in a partly open position so the inner surfaces of the blades are exposed to the heat. If bits of plant tissue or culture media are left on the tools, this debris can form a black, charcoal-like residue on the tools, as well as on the glass beads. This residue can interfere with the efficacy of the tools, especially when the residue is present on the cutting surfaces of scissors or scalpels. The tools can be cleaned using Scotch or Brillo pads but this takes time and effort.

Functionally, all tools consist of two ends, a 'working end' that contacts the plant samples, accounting for 75-80% of the length, and a 'handling end', at the base. Although sterilization by heat conduction through the body of the tool is possible, since handling ends are not directly sterilized they should be kept to the outside of the culture vessels, reducing the effective length by

about 25%. In general, tools will air-cool to temperatures safe for the handling plant samples within one to two minutes of being removed from a sterilizer (or flame), but if faster cooling is desired, the tips of the tools can be immersed into a plate or vessel of sterile culture medium. The tools will generally melt the media at the point of contact, but will cool within seconds because of the high heat capacity of water.

Between uses, the tools should be set on a rack wrapped in foil or in appropriately-sized glass containers (petri dishes, 50 ml beakers, etc) with their working ends cantilevered into the open (sterile) air. The racks/glass containers should be placed in the rear of the hood, where there is little or no air turbulence, and wiped clean with 70% ethanol on a regular basis. The same handling principles apply to micropipetters, which can be set on top of the box of pipette tips between uses with the pipette barrel and plastic tip projecting into the sterile air. If there is any indication that the working ends of the tools, or micropipetter, have contacted a potentially non-sterile surface, such as the bench top surface of the hood, they should be re-sterilized to ensure that the sterility of the tissue cultures is maintained.

Appendix 6: Typical Nutrient Deficiency Symptoms (Across Plant Species)

Table 5. Plant mineral deficiency symptoms. Diagnostic symptoms are shown in bold italicized font.

Deficiency	Symptoms
Nitrogen	- ***strong inhibition of plant growth*** - ***diffuse chlorosis***, developing first in the lower leaves - ***strong purple coloration*** of the leaves (lower surface) and stems - stems may be tough and woody, with an increased lignin content - possible promotion of flowering (nitrogen favors vegetative growth)
Phosphorous	- ***moderate to strong growth inhibition*** - leaves ***unusually dark green***, sometimes also thick (leathery) in texture - leaves and stems often with a ***strong purple coloration,*** most severe for at the stem base; for the leaves the lower surfaces and veins especially affected - leaves may shed precociously from the base of the stem upwards as phosphorous is remobilized to the growing regions of the shoots - positive effects on root growth (branching) may be observed at initially, but the rate of photosynthesis eventually becomes limiting and root growth is inhibited
Potassium	- ***moderate to strong growth inhibition,*** stems may be slender with closely spaced leaves (short internodes) - precocious ***leaf necrosis,*** appearing as ***discrete spots, marginal necrosis,*** or both; the necrosis developing first on lower, older leaves - chlorosis of the older leaves, the ***chlorosis interveinal*** to variable; the rate of chlorosis (i.e., developing before or after the necrosis) variable between species - leaves may curl and desiccate, termed '***scorching***', particularly at their margins
Calcium	- deficiency symptoms appearing first in the shoot tip and young leaves since calcium is immobile - ***shoot tip stunted, chlorotic, or necrotic, followed by axillary branching*** - young 'growing' leaves stunted, chlorotic, or necrotic at their tips or margins. The faster the rate of plant (leaf) growth the greater the differential between the amount of calcium delivered in the xylem and the plant's needs, and the more severe the deficiency symptoms. Changes in leaf shape, resulting from growth inhibition at the leaf margins, are frequently observed. The leaves may be cupped down at their margins, termed epinasty, or unusually narrow to lanceolate in shape in cases of more severe growth inhibition - root growth poor, roots short and thick; sometimes also with tip necrosis and excessive branching
Magnesium	- ***interveinal chlorosis***, ***developing first in the oldest leaves*** - leaves may also develop 'overlapping' red or orange coloration with time

Sulfur	- ***moderate to strong growth inhibition*** - ***diffuse chlorosis*** appearing first in young leaves or at more or less the same time across leaves of all ages (sulfur is weakly mobile to immobile) - leaves and stems sometimes with a ***strong purple coloration***, especially along leaf veins
Iron	- ***interveinal chlorosis, developing first in the youngest leaves***, with time the affected leaves may become entirely chlorotic and ultimately bleached (white)

Appendix 7: Key for the Identification of Nutrient Deficiency Symptoms in Tomato

Appendix 7: (Woolley and Broyer, 1957)

LE 5.6. Key to nutrient deficiency symptoms on tomato plants

A_1 Symptoms appearing first or most severely on youngest leaves	B
B_1 Interveinal chlorosis present on young leaves	C
C_1 Black spots appear adjacent to veins. Smallest veins remain green. In older leaves the necrosis may appear as interveinal brown necrotic spots 2 to 4 mm in diameter located near the main veins	Manganese
C_2 Black spots do not appear. Smallest veins do not remain green. Necrotic areas, if present, are not associated with any particular part of the lamina	Iron
B_2 Young leaves do not show *interveinal* chlorosis, but young leaflets may show chlorosis toward the central basal portion of the leaflets	D
D_1 Dorsal sides of young leaflets show marked purpling. This purpling includes both veins and interveinal areas. The ventral surfaces of young leaflets are very dark green. Leaflets are small and curled downward. Oldest leaves may show slight interveinal chlorosis and necrosis	Phosphorus
D_2 Dorsal sides of young leaflets are not purple. Ventral surfaces of young leaflets often show central basal chlorosis. Growth may be distorted. Interveinal necrosis often appears at bases of young leaflets. Very young growing tissue shows necrosis	E
E_1 Plant tissues are very brittle, especially under conditions of low stress for water. Growth accompanying recovery from this deficiency is usually twisted, asymmetrical, and otherwise distorted	Boron
E_2 Plant tissues are soft and often flaccid even under conditions of low stress for water. Leaflets developing after onset of the deficiency are narrow and cupped downward, but there is not usually much twisting either under deficiency conditions or upon recovery	Calcium
A_2 Symptons neither appearing first nor being most severe on youngest leaves. Symptoms about equal over entire plant, or most severe on oldest or on recently matured leaves	F
F_1 Interveinal chlorosis present, possibly only as a mild mottling	G
G_1 Oldest leaves most chlorotic	H
H_1 Chlorosis definitely interveinal, so that at least the main veins remain green. Plants are not usually spindly	I
I_1 Chlorosis is the first visible symptom. Leaf edges curl upward in severe deficiencies	J
J_1 Necrosis appears as sunken necrotic spots which appear shiny from the back of the leaf. These spots have no particular location with respect to veins. Bright yellow and orange colors of the chlorotic leaves are common	Magnesium
J_2 Necrosis occurs as gradual drying of interveinal areas followed by drying of the remaining tissues. Bright coloration is not common in chlorotic leaves	Molybdenum
I_2 Chlorosis is not the first visible symptom. Leaf edges do not usually curl upward	K
K_1 Tip and marginal necrosis present on older leaves of mildly affected plants, appearing as a "scorch". Old or recently matured leaves may show interveinal chlorosis. Leaflets sometimes show small black interveinal necrotic dots. Neither excessive guttation nor "water soaking" is present	Potassium
K_2 Tip and marginal necrosis is absent. Only the oldest leaves of severely damaged plants show chlorosis. Necrosis appears as irregular sunken necrotic spots which may be veinal, interveinal, or adjacent to veins. Young leaflets sometimes show small black interveinal necrotic spots. At times of low stress for water, excessive guttation and "water soaking" of the leaf tissues may be observed	Zinc
H_2 Chlorosis general, so that veins do not remain green. Plants are often spindly	L
L_1 Veins become bright red. Petioles and petiolules tend to be twisted and/or vertically disposed	Sulfur
L_2 Veins are yellow or possibly somewhat pink. Petioles and petiolules do not show twisting or vertical disposition	Nitrogen
G_2 Oldest leaves not most chlorotic. Chlorosis, when present, appears as interveinal mottling on recently matured leaves. Small black necrotic dots appear on young or recently matured leaves	M
M_1 Tip and marginal necrosis is present on older leaves of mildly affected plants, appearing as a "scorch". Old or recently matured leaves may show interveinal chlorosis. Leaflets sometimes show small black interveinal necrotic dots	Potassium
M_2 Neither tip nor marginal necrosis is present on older leaves until the entire plant is severely affected. Some tip necrosis is often present on the youngest leaves. Younger leaflets often show small black interveinal necrotic dots, especially adjacent to the main veins. Necrosis is usually confined to interveinal tissues adjacent to main veins	Manganese
F_2 Interveinal chlorosis is not present	N
N_1 Leaf margins and tips wilt. Leaves do not show excessive guttation	O
O_1 Necrotic spots, when present, are sharply delimited and sunken. Old leaflet margins roll upward stiffly. Veinal necrosis is often present. Petioles and petiolules often bend abruptly and stiffly downward	Copper
O_2 Necrosis appears as bronze-colored areas of cellular necrosis. These areas are neither sunken nor sharply delimited. *Old* leaflet margins are not usually rolled upward. Veinal necrosis is not present. Petioles and petiolules do not show abrupt downward bending	Chlorine
N_2 Leaves show no excessive wilting except in cases of petiole necrosis. Wilting, if present, is not confined to leaflet tips and margins. Excessive guttation occurs under conditions of low stress for water. This is often accompanied by the appearance of water-soaked areas on the backs of the leaves. Necrosis usually appears as irregular spots which may or may not have any particular relationship to the veins	Zinc

From Woolley and Broyer, 1957.

Appendix 8: Tomato Seedling Shoot Morphology and Growth Quantification

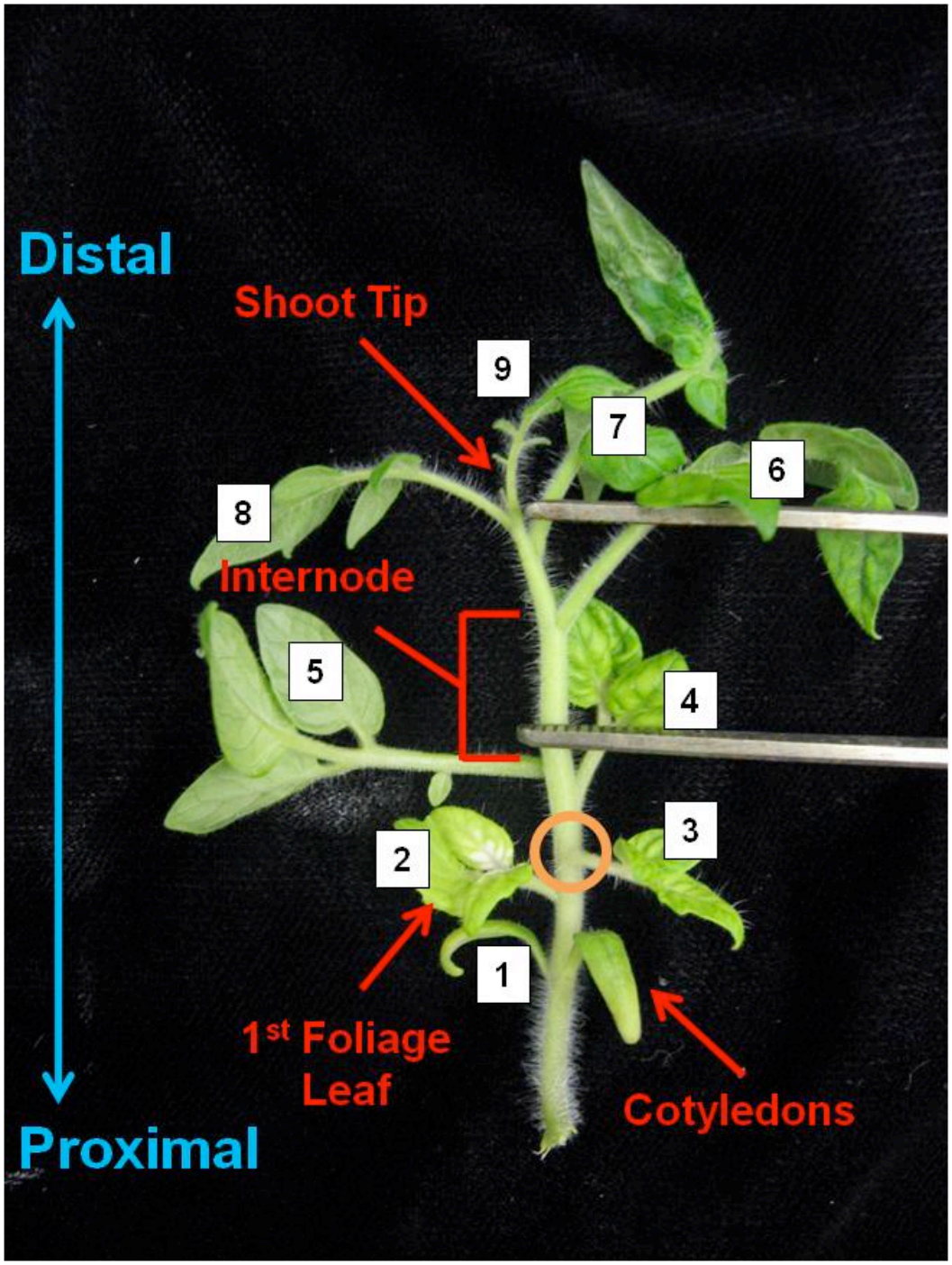

Figure 1. Tomato (*Lycopersicon esculentum* cv. Micro-Tom) seedling after 8-10 weeks of growth on positive control medium. The shoot was cut off at the root shoot junction and photographed. Distal and proximal ends of the shoot are indicated and the leaves at each node are numbered sequentially from the shoot base up. The orange circle shows the position of the third node. The cotyledons are simple and lanceolate while the foliage leaves are pinnately-compound with multiple leaflets per leaf.

Appendix 9: Sample Visual Deficiency Data – Tabular

Table 6. Typical visual deficiency symptoms affecting the shoots of tissue-cultured seedlings of tomato (*Lycoperiscon esculentum* cv. Micro-Tom) as assessed 8-10 weeks after seed sowing. Bold, italicized font denotes symptoms that have not been reported for tomato previously, to the best of our knowledge, and may be unique to the cultivar and/or the tissue culture environment.

Deficiency	Symptoms (Shoot)
Positive Control	- Chlorosis of the lower (older) leaves, but only affecting a small number of leaves; generally the cotyledons and the lowest-most two to five foliage leaves, and of modest severity. The cotyledons diffusely chlorotic and light green to yellow-green in color and the foliage leaves with interveinal chlorosis to medium or light green in color - Stem base sometimes was a faint purple coloration, but very minor in degree
Nitrogen[1] (Negative Control)	- Strong growth inhibition; about 25% as tall as the positive controls on average; only two to three foliage leaves, all smaller than the cotyledons - Diffuse chlorosis (to yellow), appearing first in the oldest leaves (cotyledons) and subsequently in the younger, more distal, leaves - Stem and leaves with a strong purple (anthocyanin) coloration, the amount of pigmentation greatest at the stem base
Phosphorous	- Moderate shoot growth inhibition, seedlings 65-75% as tall as the positive controls, leaves 25-50% as large as for the positive controls - Leaves darker green than for the positive controls, at least initially - Leaves and stems with moderate to severe purple coloration; the veins and bases (lower surfaces) of the leaves especially affected; older (lower) leaves, and stem regions, most strongly impacted - Leaves exhibiting a rapid turnover, becoming dry (withered) and ultimately abscising from the stem base (cotyledons) up; generally at least 50-75% of the leaves abscised by the point of data collection
Potassium	- Moderate shoot growth inhibition, seedlings about 50-75% as tall as the positive controls and leaves about 50% as large as for the positive controls - Lower (older) leaves generally both chlorotic and necrotic. The necrosis developing prior to the chlorosis, as a series of small spots (stippling) or splotches. The chlorosis interveinal to diffuse and generally starting in the leaf interior and progressing towards the margins - Leaves wilting (dying) and abscising prematurely starting at the stem base. Up to 25-50% or more of the leaves may be abscised by the time of data collection, but in our experience the severity of leaf abscission has been highly variable between individual experiments and thus difficult to predict a priori
Calcium	- Height growth terminated prematurely, generally within three-four weeks of seed sowing; the stems swollen basally but tapered, almost to a point, distally. The shoot tips generally chlorotic or necrotic but the ‘discoloration’

	generally highly localized or entirely absent. The seedlings only about 50% at tall as the positive controls. - ***The middle and lower regions of the shoots swollen (syn. hypertrophic), with stem diameters two to three times that of the positive controls at the point of maximal swelling (generally the lowest node with branching, see below).*** - Branching induced from the swollen regions of the stems, generally from the cotyledonary node and/or first foliage node. Multiple shoots are possible for each leaf axil, but they generally exhibit poor elongation and may appear as little more than clusters of leaves. If the axillary shoots are elongated they may exhibit hypertrophy and shoot tip necrosis, in turn, producing additional 'generations' of axillary shoots. - - Leaf symptoms (interveinal chlorosis, tip necrosis, veinal necrosis, epinasty) variable and primarily limited to the largest leaves.
Magnesium	- Growth not obviously inhibited relative to the positive controls - Interveinal to spotty leaf chlorosis, starting first in the lower leaves and spreading up the stem, by the time of data collection the most severely affected leaves yellow to even yellow-white in color. The affected leaves also often with purple coloration around their veins, giving way to necrosis over time - ***Chlorosis generally preceded by leaf epinasty (downward curling), or both symptoms developing at more or less the same time*** - Lowermost leaves may start to abscise precociously but the rate and degree of leaf abscission less than for the potassium and phosphorous deficiency treatments, generally only the cotyledons and possibly one or two of the lowest foliage leaves abscised
Sulfur	- Moderate to strong growth inhibition, seedlings 30-40% as tall as the positive controls, leaves 50-75% as large as for the positive controls - Diffuse to splotchy chlorosis, starting first in the oldest leaves or at nearly the same time across all leaf types (sulfur is immobile to weakly mobile in most plants), by the time of final data collection the most affected leaves yellow to yellow-white in color - Stems and leaves with a strong purple coloration, especially at the stem base - Leaf abscission of the cotyledons and possibly one or at most two of the lower foliage leaves possible by the time of data collection
Iron	- Growth inhibition absent or difficult to detect by visual inspection alone - Interveinal chlorosis affecting the distal (younger) leaves. The chlorotic regions light green to yellow at first but becoming white and spreading across all of the leaf regions with time - Slight to moderate purple coloration affecting both the leaves, particular the

	leaf veins and stalks (petioles) and also the stem. The purple color confined to the distal leaves and stem and develops subsequent to the chlorosis

[1]The seedlings from the negative control and nitrogen deficiency treatments exhibit similar deficiency symptoms and are difficult to distinguish without quantitative data, with the rate of symptom development and amount of growth inhibition being greatest for the

Appendix 10: Sample Visual Deficiency Data – Photoplate

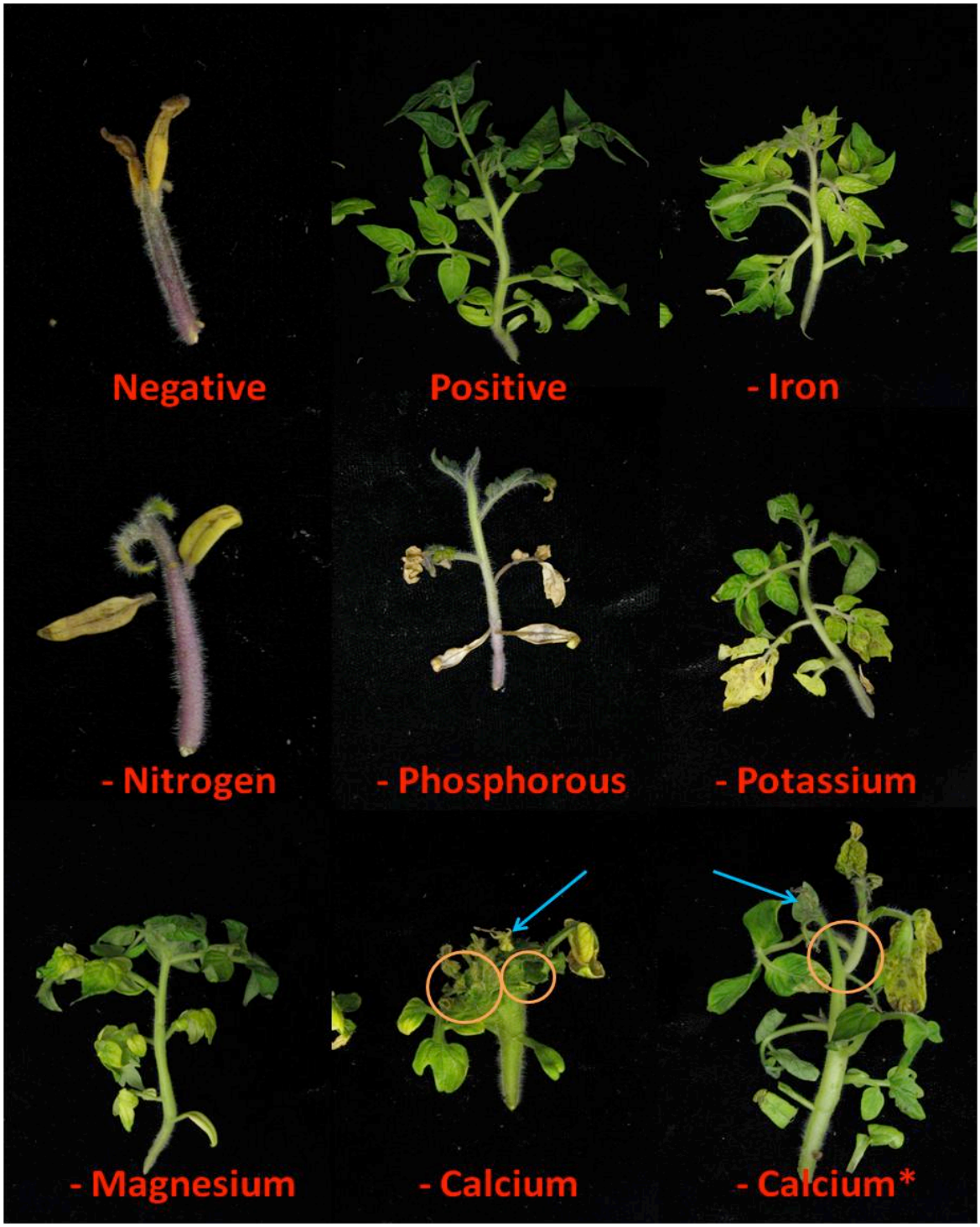

Figure 2. Typical nutrient deficiency symptoms for tissue-culture tomato seeds (*Lycopersicon esculentum* cv. Micro-Tom) as evaluated 8-10 weeks after sowing. For the calcium deficiency

seedlings the shoot tips are indicated with blue arrows and axillary shoots are indicated with orange circles.

Appendix 11: Sample Shoot Growth Data

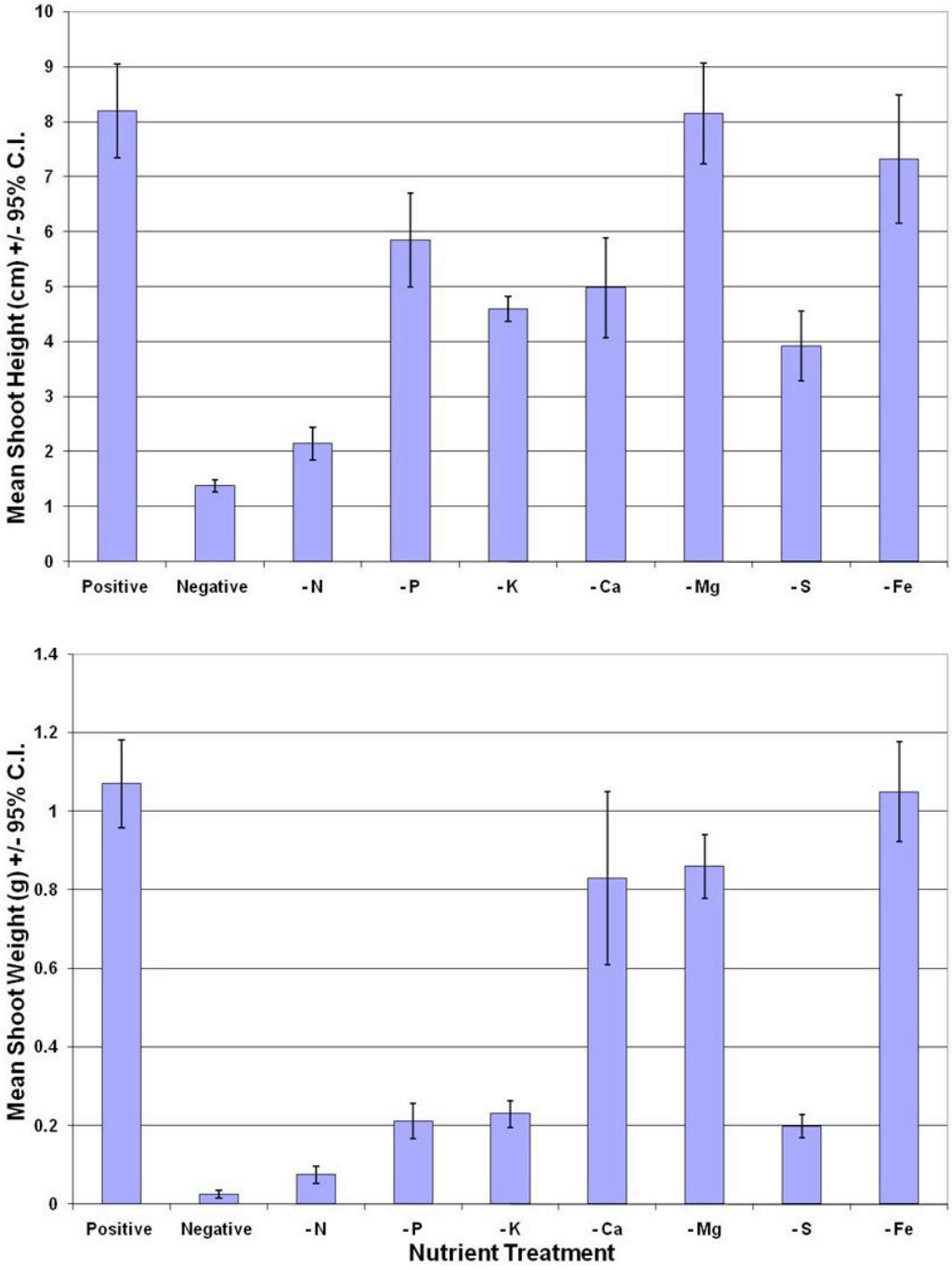

Figure 3. Shoot growth data for tissue-culture tomato seeds (*Lycopersicon esculentum* cv. Micro-Tom) as evaluated 8-10 weeks after sowing. Six seedlings were evaluated per nutrient treatment. The negative control medium was prepared with distilled water.

Appendix 12: Sample Nutrient Deficiency Key

Key for the Identification on Nutrient Deficiency Symptoms in Seedling Tissue Cultures of Tomato (cv. Micro-Tom) – As evaluated 8-10 weeks after sowing:

1. Seedlings with strong purple (anthocyanin) coloration, affecting both the stems and leaves .....… **2**
1. Seedlings with little or no purple coloration, as for the positive controls ……..……..…….... **3**

2. Leaves, at least the lower leaves, diffusely chlorotic and yellow in color; shoot growth strongly inhibited, shoots about 25% as tall the positive controls…..... **Nitrogen**
2. Leaves with little or no chlorosis, in fact the leaves unusually dark green, but exhibiting precocious abscission starting at the stem base and affecting most of the leaves after 8-10 weeks of growth; shoot elongation only slightly inhibited, seedlings75-80% as tall as the positive controls on average ….……..…… **Phosphorous**

3. Shoot growth strongly inhibited, seedlings about 50% as tall as the positive controls; stems and/or leaves with premature necrosis or tip dieback ……...…….…..... **4**
3. Shoot growth not strongly inhibited, seedlings at least 75-80% as tall as the positive controls on average, necrosis absent or not a primary (early-developing) symptom ……..... **5**

4. Necrosis of the shoot tip, and sometimes also the leaf tips, or in some cases just stunting and chlorotic without obvious necrosis; stems distinctly swollen (two to three times the diameter of the positive controls), branching induced but the axillary shoots poorly elongated and appearing as clusters of leaves …..... **Calcium**
4. Necrosis of the leaves and sometimes also the stem (over longer periods of time), the leaves with many small spots of necrosis (stippling) and subsequently becoming chlorotic and exhibiting precocious abscission. Symptoms developing first in the lower leaves; stems of normal diameter; axillary branching not apparent …….…….. **Potassium**

5. Interveinal chlorosis, limited to or most severe in the oldest (lowermost) leaves, the chlorotic tissues yellow in color; the affected leaves also curled downwards (epinastic) with the epinasty developing with or before to the chlorosis ……..…….. **Magnesium**

5. Interveinal chlorosis, limited to or most severe in the upper (youngest) leaves, the chlorosis yellow to white in color; leaf epinasty not apparent ..... **Iron**

Appendix 13: Online Plant Tissue Culture Resources

http://www.agritechpublications.com/ - An online 'Plant Tissue Culture Bookstore'. Subscriptions to Agricell Report, a monthly journal that summarizes major advances and new technologies in the fields of plant tissue culture and biotechnology as gleaned from the scientific literature, trade publications, patents, interviews, meetings and the internet are also available through the website.

http://www.kitchencultureeducation.org/ - Kitchen Culture Education Technologies Inc. (KCET) is a non-profit organization formed by Dr. Carol Stiff that is dedicated to promoting and improving plant science education, particularly plant tissue culture education, by providing training and basic supplies to K-14 teachers, schools, and community groups. It subsidiary company, the Home Tissue Culture Group (http://www.hometissueculture.org/) sells plant tissue culture kits and supplies including the 'Kitchen Culture Kit' which includes reagents and instructions for performing tissue culture in a non-research setting using basic kitchen equipment (pressure cookers, microwaves, etc.)

http://tech.groups.yahoo.com/group/hometissueculture/ (Home Plant Tissue Culture Group) - founded by Carol Stiff (Kitchen Culture Education Technologies) the Home Plant Tissue Culture Listserv provides a venue for the sharing of ideas, protocols, seeds, cultures, and reagents between plant tissue culture hobbyists.

http://plant-tc.cfans.umn.edu/listserv/- (Plant Tissue Culture ListServ) - Established in November of 1994, the *PLANT-TC* ListServ is an international forum for discussing the scientific, educational, and organizational aspects of plant tissue culture. The membership consists primarily of professional researchers, both from academia and the private sectors, but anyone can join. The ListServ is a good source of advice for practical problems related to plant tissue culture. The Listserv archives are searchable and provide a wealth of information and perspectives..

http://aggie-horticulture.tamu.edu/tisscult/tcintro.html (The Plant Tissue Culture Education Exchange) - Website created and maintained by Dr. Dan Lineberger in the Horticulture Department at Texas A&M University. Designed as a repository for basic information related to plant tissue culture.

http://www.phytotechlab.com/ - Phytotechnology Labs is the largest vendor of plant tissue culture supplies and reagents. The technical information section of their website includes instructions for a wide variety of basic tissue culture protocols as well reference materials (metric conversions chart, tissue culture terminology, etc.). all available as downloadable pdf files. They also sell kits for the propagation of specific groups plants (orchids, violets, etc.).

http://www.carolina.com, http://wardscientific.com both companies (Carolina Biological, Ward's Scientific) sell plant tissue culture teaching kits and sterile cultures (rose, potato, banana etc.).