BIORETS Demonstration of Hands-on Laboratory Teaching Module

Dark-Induced Leaf Senescence

July 22, 2022, led by Yan Lu and Alex Kolstoe

Learning Objectives:

(1) Understand leaf senescence mechanisms (e.g., intrinsic vs dark-induced leaf senescence).

(2) Learn basic techniques for dark-induced leaf senescence, and use controlled vocabularies to record leaf morphology.

Introduction

The main phases of the leaf life cycle include leaf expansion, maturity and senescence (**Figure 1**). **Senescence** is an energy-dependent, self-digesting process controlled by the interactions between environmental cures and developmental programs. It is a universal characteristic in biological systems. According to the level of the senescing unit, plant senescence could be classified into: **programmed cell death**, **organ senescence**, and **whole plant senescence**. All leaves, including those of evergreens (e.g., blue spruce), undergo senescence, in response to developmental factors (e.g., flowering and seeding; **Figure 2**), environmental factors (e.g., seasonal daylength and temperature changes; **Figure 3**), biotic stresses (e.g., pathogen attacks), or abiotic stresses (e.g., shading and wounding).

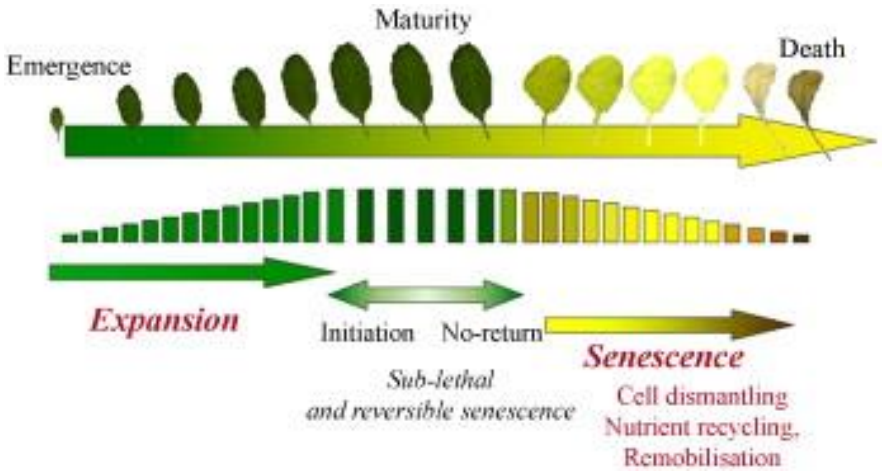

Figure 1. Schematic representation of the main phases of leaf life story (adapted from Figure 2 in Guiboileau et al. [2010]).

Figure 2. Senescence in response to flowering and seeding (adapted from Figure 22.26 in the Plant Physiology textbook). The soybean plant on the left senesced after flowering and producing fruits. The soybean plant on the right remained green and vegetative because flowers were continuously removed.

Intrinsic leaf senescence is a specialized form of programmed cell death, which permits remobilization of nutrients from source leaves to vegetative or reproductive sinks (Ma et al., 2010). The earliest structural change during intrinsic leaf senescence is chloroplast breakdown. Carbon fixation is thus replaced by the degradation and conversion of chlorophyll, proteins, and other macromolecules to exportable nutrients. Intrinsic leaf senescence is a normal developmental process.

Dark-induced leaf senescence is an extreme example of leaf senescence induced by shading. It results in a loss

of chlorophyll, disassembly of cellular components, and a reduction of photosynthetic activity (Sobieszczuk-Nowicka et al., 2018; Paluch-Lubawa et al., 2021). Dark-induced leaf senescence assays could be performed on whole plants, attached leaves, or detached leaves. This could be achieved by covering whole plants or individual leaves or by placing whole plants or detached leaves in the dark. Unlike whole plants or attached leaves, detached leaves are subjected to mechanical wounding and water-soaking, as they need to be excised from the plant and kept in an aqueous solution. Mechanical wounds may act as additional entry points to detached leaves for substances present in the aqueous solution.

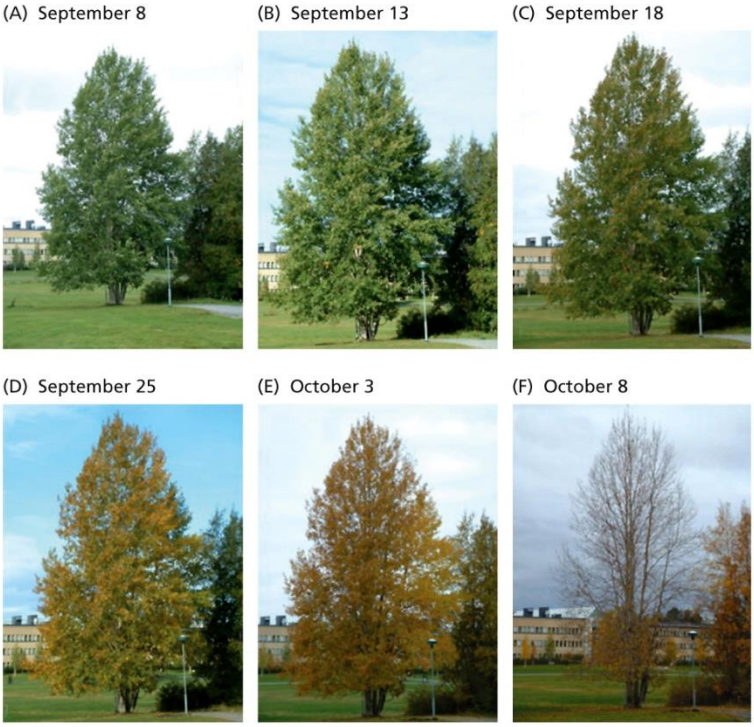

Figure 3. Seasonal leaf senescence in an aspen tree (adapted from Figure 22.11 in the Plant Physiology textbook).

Experiment Overview

In this exercise, you will perform dark-induced leaf senescence assays on attached and detached leaves. For the attached leaf assay, you may cover both sides of a few leaves from a plant of your choice with aluminum foils. For the detached leaf assay, you may remove a few leaves from a plant of your choice, keep them in aqueous solutions, and place half of the leaves in the dark and the other half under natural light (e.g., by a window). You will also supplement the aqueous solution with sucrose, alkali (e.g., sodium bicarbonate/baking soda), or acid (e.g., acetic acid in vinegar and citric acid in lemon juice) (**Figure 4**).

Figure 4. Supplementing the aqueous solution with sugar, baking soda, or vinegar.

Exogenous sugar treatment has been found to delay dark-induced leaf senescence in detached leaves (Li et al., 2020). A 6% sucrose solution was reported to be suitable for detached leaves or leaf segments. You are going to test whether supplying 6% sucrose to detached leaves delays their senescence.

Most plants thrive in the pH 6.0-7.0 (slightly acidic to neutral) range. pH is the negative logarithm of hydrogen ion (H^+) concentration (mol/L) in an aqueous solution: $pH = -\log10(H^+)$. A pH scale with popular items that are acidic or basic is shown in **Figure 5**. The tap water in the Kalamazoo area has a pH of 7.0. You are going to investigate the effect of pH on detached leaves by supplementing the solution with baking soda, which is sodium bicarbonate, or vinegar/lemon juice, which contains acetic acid or citric acid. A 6% baking soda solution has a pH of 8.0. A 6% vinegar solution has a pH of ~3.2. A 6% lemon juice solution has a pH of ~4.0.

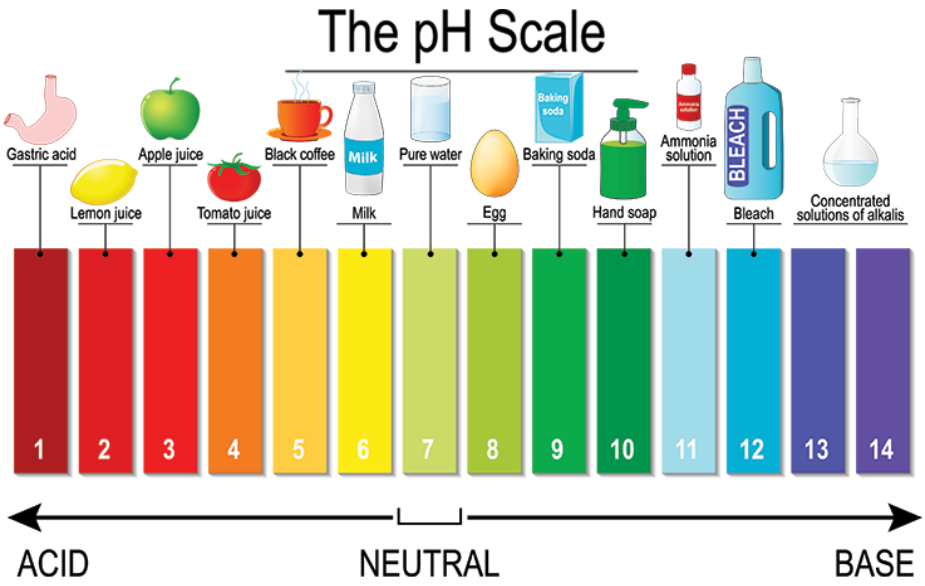

Figure 5. A pH scale showing popular items that are acidic or basic (adapted from https://www.sciencenewsforstudents.org/article/scientists-say-ph).

You are going to use controlled vocabularies to describe leaf morphology before and during the treatments. A list of controlled vocabularies is provided in **Table 1**.

Table 1. A list of controlled vocabularies to be used when observing leaf morphology

Category	Controlled vocabulary
Leaf color	Green, blue green, yellow green, yellow, brown, etc.
Leaf anatomy	Leaf blade, petiole, leaf margin, leaf tip, leaf base
Brown necrotic spots	Presence, absence
Percent leaf area (estimation)	100%, 75%, 50%, 25%, 0%
Leaf location in solution	Floating, sunken
Turbidity of solution	Clear, cloudy
Color of solution	No color, light yellow, or yellow

Definitions of some controlled vocabularies:
Leaf base: the lowest part of a leaf blade that is near the petiole.
Leaf blade: the broad, expanded area of the leaf.
Leaf margin: the perimeter of the leaf between leaf tip and leaf base.
Leaf tip: the part of the leaf blade farthest from the petiole.
Midvein: the vein in the center of a leaf.
Necrosis: death that is directly caused by physical damage, toxins, or other external agents.
Petiole: the leaf stalk that join the leaf blade to the stem.

Key Concepts:
Senescence
Programmed cell death, organ senescence, and whole plant senescence
Intrinsic leaf senescence and dark-induced leaf senescence
pH

Materials (for each student to perform the experiment at home or in the classroom)

- A roll of aluminum foil
- A large glass/plastic container that holds ≥1 cup
- Eight small clear glass/plastic containers that hold ≥ ½ cup (**Figure 6**; Mason jars, jam jars, yeast jars, baby food jars, small containers made of clear plastics [e.g., Viovia 5.5-oz/163-ml or 6.7-oz/198-ml **clear plastic** portion cups])
- 1-cup (237-ml) and 0.5-cup (118-ml) measuring glass/cups (or a 250-ml graduated cylinder if performing the experiment in a classroom)
- A 1-tbsp measuring spoon
- A marker pen
- Deionized water, purified water, or tap water
- Table sugar (i.e., sucrose)
- Baking soda (i.e., sodium bicarbonate)
- Vinegar, which contains acetic acid, or lemon juice, which contains citric acid
- Outdoor and/or indoor plants with green leaves (e.g., ground covers, herbaceous plants, shrubs, and trees)
- A pair of scissors to harvest leaves

Figure 6. Clear glass/ plastic containers, measuring glass, graduated cylinder, and measuring spoons

Procedure:

Activity A: Set up the attached leaf assay

1. Choose four non-senescing green leaves from a plant of your choice, take pictures of them, with at least one control leaf in the same picture (**Figure 7**). The five leaves should be developmentally and morphologically similar. Record the leaf morphology (leaf color, presence or absence of necrotic spots, and their percent leaf area) of the five leaves with controlled vocabularies.

Figure 7. Set up dark-induced leaf senescence assay with attached leaves.

2. Cover both sides of a dark-treatment leaf or leaf segment with a piece of aluminum foil (**Figure 7**), secure the foil by folding the four corners inward, label the foil with your name and date. Repeat this process for three other dark-treatment leaves or leaf segments. If you are concerned that the aluminum foil blocks the air and water vapor movements, make sure the foil at the tip and base sides are not folded.

3. 7-9 days later, remove the aluminum foil, take pictures of each uncovered dark-treatment leaf or leaf segment, with at least one control leaf in the same picture. Record the leaf morphology of the five leaves.

Activity B: Prepare the four aqueous solutions

4. Label the base and the lid of eight small clear containers with "**H_2O Light**", "**H_2O Dark**", "**Sucrose Light**", "**Sucrose Dark**", "**Alkali Light**", "**Alkali Dark**", "**Acid Light**", and "**Acid Dark**".
5. Measure 1/2 cup (118 mL) of water, pour it into the container labeled "**H_2O Light**". Repeat this process for "**H_2O Dark**".
6. Measure 1 cup (237 mL) of water, pour it into the large container that holds ≥1 cup. Measure 1 tablespoon of **table sugar (sucrose)**, pour it into the large container, and stir to dissolve sucrose completely. Split the sucrose solution equally into the two containers labeled "**Sucrose Light**" and "**Sucrose Dark**" (i.e., ½ cup/container or 118 ml/container), with a measuring glass/cup. Wash the large container, the tablespoon, and the measuring glass/cup with water. Dry the tablespoon with paper towels.
7. Measure 1 cup (237 mL) of water, pour it into the large container that holds ≥1 cup. Measure 1 tablespoon of **baking soda (sodium bicarbonate)**, pour it into the large container, and stir to dissolve baking soda completely. Split the baking soda solution equally into the two containers labeled "**Alkali Light**" and "**Alkali Dark**" (i.e., ½ cup/container or 118 ml/container), with a measuring glass/cup. Wash the large container, the tablespoon, and the measuring glass/cup with water. Dry the tablespoon with paper towels.
8. Measure 1 cup (237 mL) of water, pour it into the large container that holds ≥1 cup. Measure 1 tablespoon of **vinegar or lemon juice**, pour it into the large container, and stir to mix completely. Split the acid solution equally into the two containers labeled "**Acid Light**" and "**Acid Dark**" (i.e., ½ cup/container or 118 ml/container), with a measuring glass/cup. Wash the large container, the tablespoon, and the measuring glass/cup with water. Set the eight containers aside (**Figure 8**).

Figure 8. Eight containers of aqueous solutions.

Activity C: Prepare detached leaves

9. Harvest ~12 green leaves from the plant of your choice (exemplary plants in winter are shown in **Figure 9**). These leaves should be non-senescing and developmentally and morphologically similar. If you are interested in including leaves from another plant, please feel free to do so, multiple leaves could be incubated in each container.

Figure 9. Exemplary plants in winter.

10. Arrange the leaves according their size on a table, select 8 leaves that are non-senescing and most similar to each other developmentally and morphologically (**Figure 10**). Place one leaf per container and make sure all leaves face up.

Figure 10. Arrange the leaves according to their size on a table.

Activity D: Incubate and observe the detached leaves in aqueous solutions

11. Record the morphology (leaf color and percentage of the leaf in that color, number of brown necrotic spots and their percent leaf area) of each leaf and the turbidity and color of each solution, in a table (see **Table 2** below), with controlled vocabularies.

Table 2. Morphology of detached leaves in aqueous solutions						
Treatment	Category	Day 1	Day 3	Day 5	Day 7	Day 9
H_2O Light	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				
H_2O Dark	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				
Sucrose Light	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				
Sucrose Dark	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				
Alkali Light	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				
Alkali Dark	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				
Acid Light	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				

Acid Dark	1. Leaf color and percent leaf area	100% Green				
	2. Number of brown necrotic spots and their percent leaf area	0; 0%				
	3. Translucent or not	Not				
	4. Floating or sunken	Floating				
	5. Solution turbidity	Clear				
	6. Solution color	No color				

12. Take group and individual pictures of the capless containers with leaves on a white background (**Figure 11**).

Figure 11. Group picture of detached leaves in aqueous solutions.

13. Place the four containers labeled with "Light" under natural light (e.g., by a window) and the four containers labeled with "Dark" in the dark (e.g., in a drawer, cabinet, or closet). Capping the containers is optional during incubation.
14. Repeat steps 11-13 every 2-3 days until day 9.

Topics for Discussion:

1. Compare images and morphology of detached leaves in "**H_2O Light**" and "**H_2O Dark**". Leaves under which treatment showed signs of senescence and damage (leaf yellowing, translucent leaf blades, brown necrotic spots) first? Which aqueous solution showed yellowing (sign of chloroplast breakage and chlorophyll leakage) first? Use images you took yourself and leaf morphology you observed to support your answer (Exemplary images are shown in **Figure 12** below). Explain your observations.

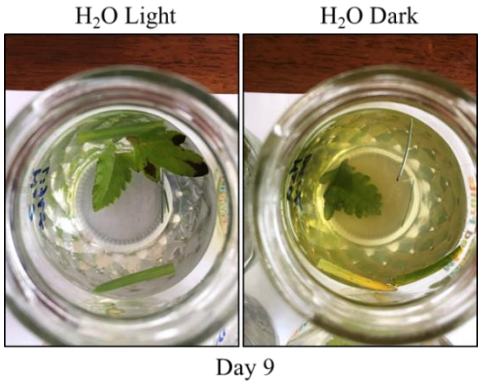

Figure 12. Detached leaves after 9 days' incubation in water under light or in the dark.

2. Compare images and morphology of detached leaves in "**H_2O Light**" and "**Sucrose Light**". Did supplementing water with 6% sucrose delay leaf senescence (e.g., leaf yellowing) under light? Use images you took yourself and leaf morphology you observed to support your answer (Exemplary images are shown in **Figure 13** below). Explain your observations.

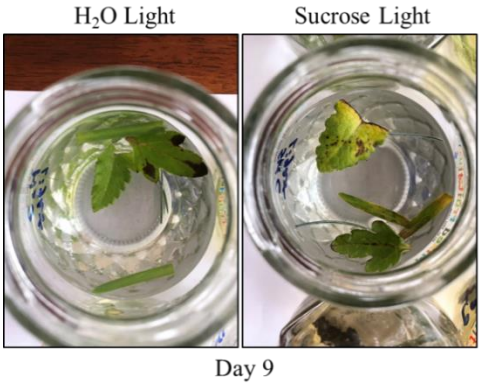

Figure 13. Detached leaves after 9 days' incubation under light in water or 6% sucrose solution.

3. Compare images and morphology of detached leaves in "**H_2O Dark**" and "**Sucrose Dark**". Did supplementing water with 6% sucrose delay leaf senescence and damage (e.g., leaf yellowing, translucent leaf blades, brown necrotic spots, and yellow of aqueous solution) in the dark? Use images you took yourself and leaf morphology you observed to support your answer (Exemplary images are shown in **Figure 14** below). Explain your observations.

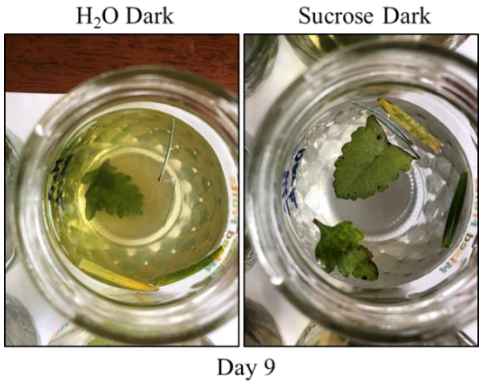

Figure 14. Detached leaves after 9 days' incubation in the dark in water or 6% sucrose solution.

4. Compare images and morphology of detached leaves in "**H_2O Light**" and "**Alkali Light**". Did supplementing water with 6% baking soda cause leaf damage (translucent leaf blades, brown necrotic spots, and yellowing of aqueous solution)? Use images you took yourself and leaf morphology you observed to support your answer (Exemplary images are shown in **Figure 15** below). Explain your observations. *(Hint: Consider the optimum pH range of plants.)*

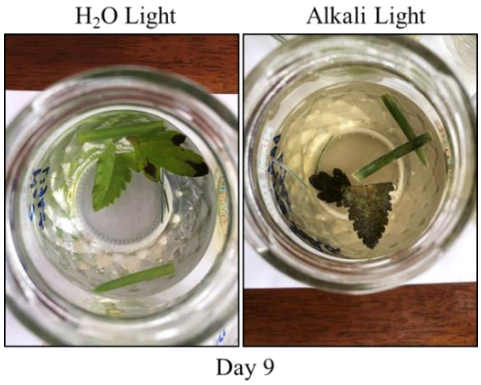

Figure 15. Detached leaves after 9 days' incubation under light in water or 6% baking soda solution.

5. Compare images and morphology of detached leaves in "**H_2O Light**" and "**Acid Light**". Did supplementing water with 6% vinegar or lemon juice cause leaf damage (translucent leaf blades, brown necrotic spots, and

cloudiness of aqueous solution)? Use images you took yourself and leaf morphology you observed to support your answer (Exemplary images are shown in **Figure 16** below). Explain your observations. *(Hint: Consider the optimum pH range of plants.)*

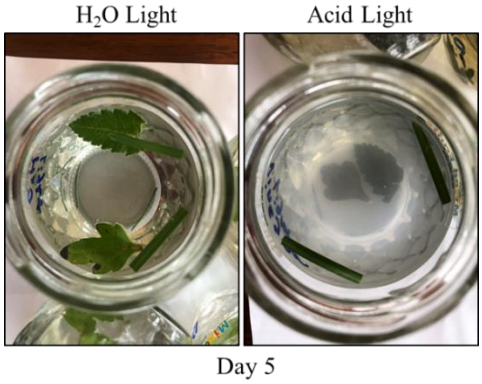

Figure 16. Detached leaves after 5 days' incubation under light in water or 6% vinegar solution.

Expected Results:

1. Detached leaves incubated in water in the dark are expected to show signs of leaf senescence earlier than those incubated in water under light. Dark treatment may result in a loss of chlorophyll, disassembly of cellular elements, and a decrease of photosynthetic activity.
2. Detached leaves incubated under light in sugar solution may show signs of leaf senescence earlier than those incubated under light in water. Detached leaves incubated under light can still perform photosynthesis; overaccumulation of sugar can trigger leaf senescence (Wingler et al., 2006).
3. Detached leaves incubated in the dark in sugar solution may show delayed leaf senescence than those incubated in the dark in water. Detached leaves incubated in the dark cannot perform photosynthesis; exogenous sugar treatment has been found to delay dark-induced leaf senescence in detached leaves (Li et al., 2020).
4. Detached leaves incubated in acid or alkali solution may show signs of leaf damage earlier than those incubated in water. Most plants thrive in the pH 6.0-7.0 (slightly acidic to neutral) range; supplementing the aqueous solution with 6% vinegar or baking soda changes the pH to ~3.2 or ~8.0, respectively.

Optional Evaluation Activities:

1. Ask students to illustrate the main phases of the leaf life cycle.
2. Ask students to tell the differences among programmed cell death, organ senescence, and whole plant senescence.
3. Ask students to list several signs of senescence and damage of detached leaves incubated in an aqueous solution.
4. Ask students to describe the importance of photosynthesis, the green pigment chlorophyll, the photosynthetic product (sugar) to plant growth and development.
5. Ask students to tell the optimal pH range of plants.

Potential Modifications:

1. This experiment was developed during the COVID19 pandemic for students to perform at home or in a classroom. If the students cannot find eight containers at home, they may drop the alkali or acid treatment.
2. If the students have other class duties, they may opt out leaf morphology observation and photographing on day 3 and 7 (or 9).

3. Students may compare the images and morphology of leaves incubated in aqueous solutions with those attached to the plant. Depending on the species, the developmental stage, the growth season, and solution ingredients, leaves incubated in aqueous solutions may show accelerated o delayed senescence, when compared to those attached to the plant. Most outdoor plants undergo leaf or whole plant senescence in late fall and winter. Therefore, the detached leaves or leaf discs incubated in aqueous solutions may showed accelerated senescence in late spring and summer but show delayed senescence in late fall and winter (**Figure 17**).

Figure 17. Early leaf senescence experiment showing the delayed senescence of a leaf disc cultured in the laboratory compared with the intact leaf of mock orange from which the disc was excised.

Potential Applications in Our Daily Lives:

1. Sequential and seasonal leaf senescence of outdoor plants (e.g., crops and trees).
2. Stress (e.g., pathogen attacks, shading, wounding)-induced leaf senescence of outdoor plants.

References:

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