TIPS, TRICKS & TECHNIQUES

Plants Are Not Independent Organisms: Introducing the Role of Mycorrhizal Fungi in Plant Roots with a Safe Method for Visualization

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Abstract

In plant science education, students can develop their perception of plants as independent living organisms. However, the accumulated studies testify that plants are not living alone in nature. The current paradigm in plant science explains a plant as a holobiont (plant and interacting microorganisms), instead of a single organism. In the center of this new paradigm, there is a symbiosis between plant and arbuscular mycorrhizal fungi (AMF). About 80% of terrestrial plant species form mutualism with AMF that act as an extension of roots. The symbiosis is crucial for plant nutrient uptake from the soil as well as resistance against biotic and abiotic stresses. Although the symbiosis is crucial for plant physiology and ecology, the efforts for summarizing current understanding and formulating it for teaching are relatively poor. For efficient introduction in class, it is important to visualize the AMF living inside roots. However, the widely used methodology for staining requires a carcinogenic chemical. Based on the current state of knowledge in AMF biology, I introduce AMF and a safer methodology for visualizing AMF in plants. Furthermore, I provide suggestions for teachers to design class activities that could inspire students to learn and think about plant physiology and ecology together with AMF.

Key Words: *arbuscular mycorrhizal fungi; plant holobiont; symbiosis; plant physiology; ecology; primary; secondary; undergraduate.*

○ Introduction

Plants are autotrophs. The sentence in biology textbooks describes one of the fundamental features of the organism. As an autotroph, a plant can produce its source of energy using photosynthesis. The description, together with the term "producer" in ecology, has led teachers to introduce a plant as an independent organism living and functioning alone in nature. However, plants are not independent organisms in nature. Besides the carbohydrates generated by photosynthesis, a plant requires various macro- and microelements to grow and maintain its metabolism. The acquisition of various nutrients for a plant is associated with water uptake from the soil and has been known to be realized by the interaction between root and abiotic components of soil. However, recent studies revealed that the mineralization of plant-absorbable forms of nutrients as well as the absorption from soil is mediated by various soil microbes rather than roots in nature (Berlanga-Clavero et al., 2020; Vandenkoornhuyse et al., 2015). Furthermore, resistance to environmental changes, crucial for plant homeostasis, is constituted by the various microbes interacting with the plant (Berlanga-Clavero et al., 2020; Vandenkoornhuyse et al., 2015). From the below-ground rhizosphere to the above-ground phyllosphere exposed to air, plants are multi-organismal systems functioning as one unit of life through intimate and continuous inter-kingdom interactions between the plant and the resident microbes (Berlanga-Clavero et al., 2020; Lee et al., 2019; Lyu et al., 2021; Smith & Read, 2008). The symbiotic meta-organisms that may have evolved together under natural selection and form functional units are termed holobionts (from the Greek *holos*, whole or entire) (Margulis & Fester, 1991; Mindell, 1992). Plants co-evolved interacting with microbes for nutrient uptake and homeostasis maintenance, represents holobionts that are not only physically associated but also have evolved to function together during most of their lifetime (Lee et al., 2019; Margulis & Fester, 1991; Mindell, 1992). Recent advancements in next-generation sequencing have allowed the study of plants in their natural form, rather than in sterile artificial conditions, which has been previously the case. Numerous studies of microbes interacting with plants have revealed intimate functional associations between the plant and microbes (Berlanga-Clavero et al., 2020; Bordenstein & Theis, 2015; Hoysted et al., 2018; Lee et al., 2019; Lyu et al., 2021). There has been a paradigm shift in plant sciences to consider plants as holobionts, not individual organisms (Berlanga-Clavero et al., 2020; Lee et al., 2019). The plant holobiont concept is becoming a new standard to study and understand plant physiology and ecology (Figure 1). For teachers in biology, it is therefore important to introduce the concept clearly to students. Introduction and demonstration of one good example of a mutualistic symbiosis, at the core of the plant holobiont, can be helpful for teachers to introduce the concept and inspire students.

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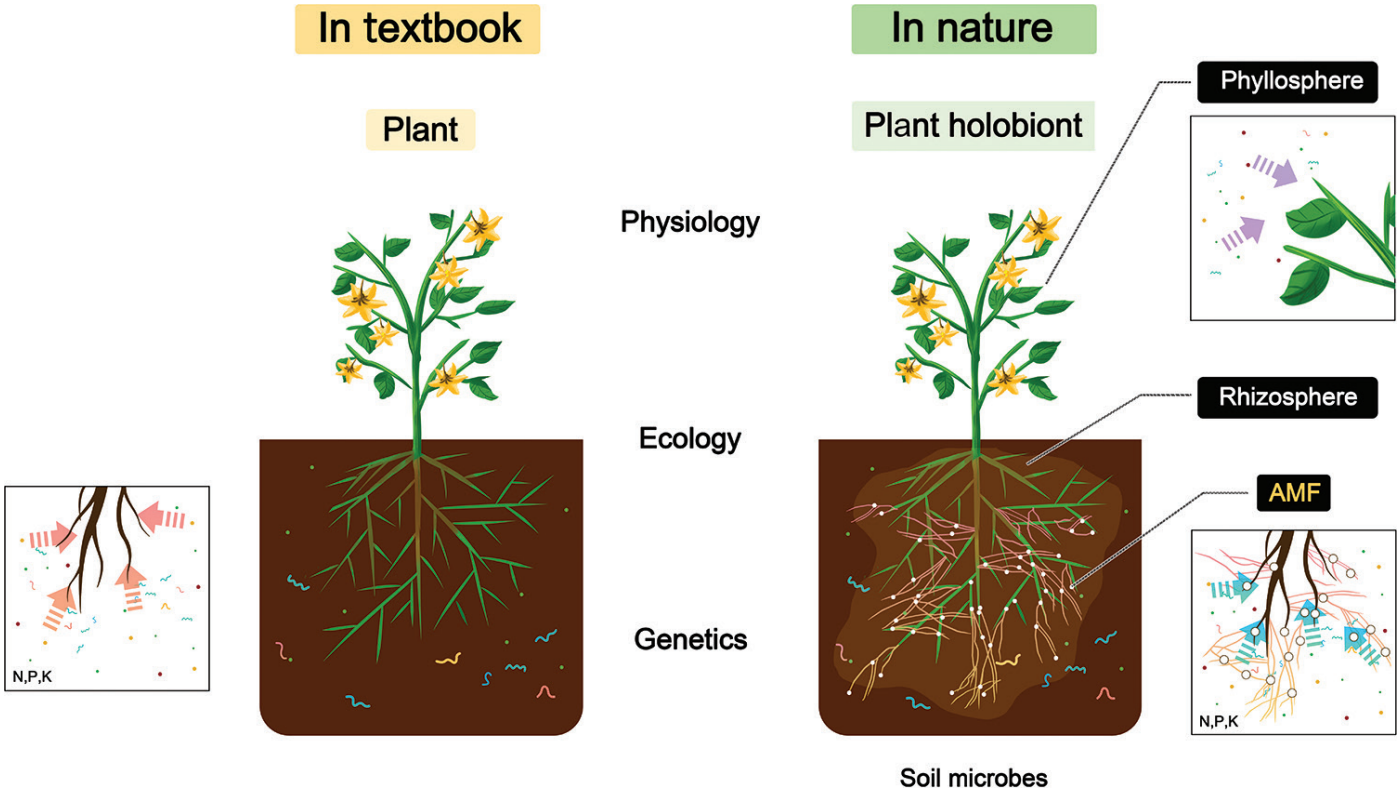

Figure 1. The plant holobiont concept in plant science. Traditionally, a unit for understanding physiology, ecology, and genetics of plants was plant itself. However, the recent paradigm of plant science considers plant holobiont as the unit. Co-evolved microorganisms of plant rhizosphere and phyllosphere form a complementary single system of life together with host plant. The plant holobiont concept can explain how plants can absorb nutrients in soil and maintain homeostasis in nature. AMF stands for arbuscular mycorrhizal fungi.

The symbiosis between plant and arbuscular mycorrhizal fungi (AMF) is one of the oldest and most successful mutualistic partnerships on Earth. The mutualism is understood to have evolved due to the limitation of plant root capacity for foraging soil nutrients, especially phosphate and nitrogen, which are crucial for plant growth (Smith & Read, 2008). Fossil records testify that the history of the symbiosis began with the first appearance of terrestrial plant ancestors about 450 million years ago (Ordovician) (Field et al., 2015). Since then, AMF have co-evolved with plants and helped plants to thrive and colonize the land. Unlike most of the other mutualisms found on Earth, there is no host-symbiont specificity in AMF-plant symbiosis. From liverworts to angiosperms, about 80% of terrestrial plant species form a symbiosis with AMF that occur in almost all terrestrial ecosystems (Field et al., 2015; Hoysted et al., 2018). All AMF share a common ancestor, forming the monophyletic fungal phylum Glomeromycota (Smith & Read, 2008). The plant-AMF symbiosis initiates with AMF colonization of plant root cortex cells. During root cell colonization, a unique morphological structure called the arbuscule (often described as "a tree-like structure" and giving the name of symbiosis) forms which functions as a hub for nutrition exchange between two symbiotic partners. After the colonization of root cells, AMF extend the mycelium toward the soil. The developed network of mycelium in soil absorbs various soil nutrients (especially phosphate and nitrogen) and transports them to the host. As a reward, AMF acquire their source of carbon in the form of sugar and lipids, and rely exclusively on plant photosynthesis for carbon. Although soil nutrition acquisition is the core function of AMF for the plant, there are other effects of AMF colonization of plants. The root colonization of AMF can improve plant resilience against abiotic (e.g., drought, flood, or salinity) as well as biotic (e.g., pathogen or herbivory attack) stresses (Begum et al., 2019; Li et al., 2019; Stratton et al., 2022). Consequently, symbiosis with AMF influences plant growth, yield (Felfoldi et al., 2022; Pena Venegas et al., 2021), as well as the interaction with other microorganisms in the environment (Real-Santillan et al., 2019), ultimately affecting the phosphate, nitrogen, and energy flux of food webs in the ecosystem (Nuccio et al., 2013; Smith & Read, 2008). The plant sequestration of recent photosynthates for plant holobiont maintenance is mainly mediated by AMF (Kaiser et al., 2015), while various soil nutrients processed by microorganisms are delivered to the plant via AMF (Nuccio et al., 2013). The symbiosis between plant and AMF forms the backbone of the plant holobiont (Lee et al., 2019). Also, it should be highlighted that AMF can form a symbiosis with more than one plant individual simultaneously. It has been shown that AMF can colonize the roots of the same plant species as well as different plant species (Allen & Allen, 1986). Therefore, it is feasible that AMF can mediate nutrition allocation between the interconnected individuals and affect the competition as well as collaboration between neighboring plants. Across the network of AMF hyphae, the absorbed nutrients from soil can be allocated from rich to poor patches (Whiteside et al., 2019). Indeed, it was demonstrated previously that below-ground AMF diversity can determine above-ground plant growth and diversity (van der Heijden et al., 1998; van der Heijden et al., 2003). The AMF-plant symbiosis is

key to understanding plant physiology and to explaining the survival and reproduction of plants in the environment.

Considering the ubiquitous nature of AMF in ecosystems and their importance in plant physiology and ecology, it is crucial to introduce AMF to students studying biology. Unfortunately, an introduction to microbes without hands-on observation can be unintuitive for students as they can find it difficult to draw the organisms in their minds and consequently fail to understand how the symbiosis functions. Since AMF are fungi, it will be important for teachers to organize class activities to help students visualize plant colonization of AMF for the effective introduction of plant holobiont. However, a widely used methodology for staining AMF requires a carcinogenic chemical, Trypan blue (PubChem CID: 6296) (Gange et al., 1999). For the health of students, especially those who are not fully familiar with handling of hazardous chemicals, it is important to introduce alternative methods for achieving the same education goals. Here, I introduce a safer and simpler methodology for visualizing AMF colonization in plant roots. Furthermore, I suggest some considerations for teachers to help design class activities to introduce the plant-AMF symbiosis jointly with plant physiology and ecology.

○ A Safe Staining Method for Classroom Activity to Introduce the Plant-AMF Symbiosis

The principle of AMF staining is to distinguish the AMF structures from the plant root tissue. To achieve this, staining reagents target the fungal cell wall component, chitin. As an azo dye, the binding capacity of common ink for chitin is similar to Trypan blue (Shimizu et al., 1995), therefore ink can replace Trypan blue. Both chemicals are soluble in water and should be applied with an acidic bath. In this method, there is no need to prepare the stain solution with hydrochloric acid or lactic acid. Vinegar, which is safer and easier to apply, can be used for stain preparation. Overall the process is composed of the following three steps: (1) cleaning of tissue, (2) staining, and (3) destaining.

Sampling of plant materials for class activities

Sampling roots colonized by AMF in nature is simple, as approximately 80% of terrestrial plant species on Earth have mutualistic interaction with AMF (Smith & Read, 2008; Veiga et al., 2013). Some plant species from Brassicaceae, Caryophyllaceae, Cyperaceae, and Proteaceae families are non-mycorrhizal. They are not the primary hosts for colonization, although occasionally their roots can still be colonized by AMF if there are nearby AMF host plants. Therefore, plants from the four families are not recommended for sampling. Plants from Fabaceae and Poaceae, the two dominant plant families in both urban and rural ecosystems form symbiosis with AMF (Smith & Read, 2008). The major crops, such as rice, wheat, maize, potato, carrot, and onion can also be the good samples. The choice of plant species for root sampling will be dependent on the aim of the course or the scientific research question. It will be also interesting to introduce that the most of root crops people consume daily have AMF colonization. People are not only eating the plants, but also AMF in the root. The entire root system of the target plant should be carefully dug-up from the soil. Since there is more active AMF colonization occurring in younger and active roots, it is important to minimize the damage in the younger part of the root system. Sampled roots can be rinsed with tap water 3–4 times to remove the soil and neutralize the pH in the root surface. The washed material can be stored in a plastic bag or 50mL falcon tube with 50% ethanol in a regular fridge (4°C) for up to a year. When it is time to stain AMF, the stored samples are removed from the ethanol and rinsed several times with tap water to completely remove the ethanol. After washing, the younger part of the roots can be sampled by tweezers and a scalpel then stored in a container (2mL Eppendorf tube is recommended) for following the staining process.

Cleaning of sampled tissue

For the observation of AMF structures, it is important to make a good contrast between plant tissue and stained fungal structures. Plant roots are, in general, not white or transparent as various phytopigments in roots such as tannins exist. Also, to effectively stain AMF structures, it is important to soften the root tissue to allow penetration of the stain. For this, 10% Potassium hydroxide (KOH) (w/v) can be used. The sampled root pieces can be placed in 10% KOH solution for 20–180 minutes in a 70-90°C water bath. It is also possible to incubate the root pieces with KOH solution at room temperature over 24 hours. It is important to regularly check the condition of samples to avoid tissue damage. The fully cleaned root should be almost transparent and very soft. Carefully decant the 10% KOH solution and rinse the root with tap water several times to neutralize the pH. Depending on the plant species, the time of 10% KOH solution incubation can vary. Woody roots with more tannins should be incubated for longer with several replacements of the 10% KOH solution.

Staining

In order to stain the roots, previously described protocol (Vierheilig et al., 1998) can be further modified and used. A solution of 3-5% ink and vinegar (v/v) should be prepared. Depending on the preference and availability in the classroom, various types of ink and vinegar can be used. Considering the accessibility and handling convenience, black or blue colored pen inks can be used. The pH range variation of vinegar (pH 2–3) is an adequate pH level for staining. Therefore, the choice of vinegar is not critical for the staining. For the same reason, students can use fresh lemon juice (approximately pH 2) instead of vinegar. The cleaned root should be placed in the staining solution and incubated for 20–30 minutes in 90°C water bath. Alternatively, for few days courses, samples with staining solution can be stored in room-temperature overnight.

Destaining

Destaining is a step to adjust the contrast between stained AMF structures and non-stained plant tissue. Approximately 100mL of tap water with two droplets of vinegar can be used for rinsing the stained root. The stained roots can be placed in the destaining solution and incubated for 2 minutes at room temperature. It is not recommended to incubate in a 90°C water bath. Depending on the plant species, simple tap water without vinegar can be enough for destaining. After the destaining, roots can be stored in 50% ethanol for 2–3 months.

○ Observation of AMF Structures

The AMF structure inside the stained root can be observed with a light microscope (x200 to x400 magnification). Root pieces are

placed on a slide glass and covered with a cover-slip. The cover-slip should be gently pressed with a finger to flatten the root tissue and ensure a flat surface for microscopic observation. A soft paper towel can be placed on top of the slides to avoid direct touching by the finger while pressing. For long-term storage of the slides, apply the transparent nail polish solution to seal the space between the cover-slip and slide glass.

In general, students can observe three different structures of AMF in a root as shown in Figure 2. First, students can observe fungal hyphae without septa (cell wall-like structure crossing the cytoplasm), which is a distinct characteristic of AMF compared with other fungi. However, with old mycelium, septa can be occasionally formed and observed. The second structure that can be observed is an arbuscule. This structure is formed by the mutual folding of cell membranes between the plant and AMF. It is not the result of invasive colonization with digestion of plant cells. The arbuscule is the structure for nutrient exchange between the two symbiotic partners. The last structure that can be observed is the vesicle, which is the structure used for carbon storage. Here, the AMF stores carbon in the form of lipids. These three structures are observable in x200 magnification with a light microscope. However, depending on the plant species and the age, clearer observation of the AMF structures can be made in higher magnification.

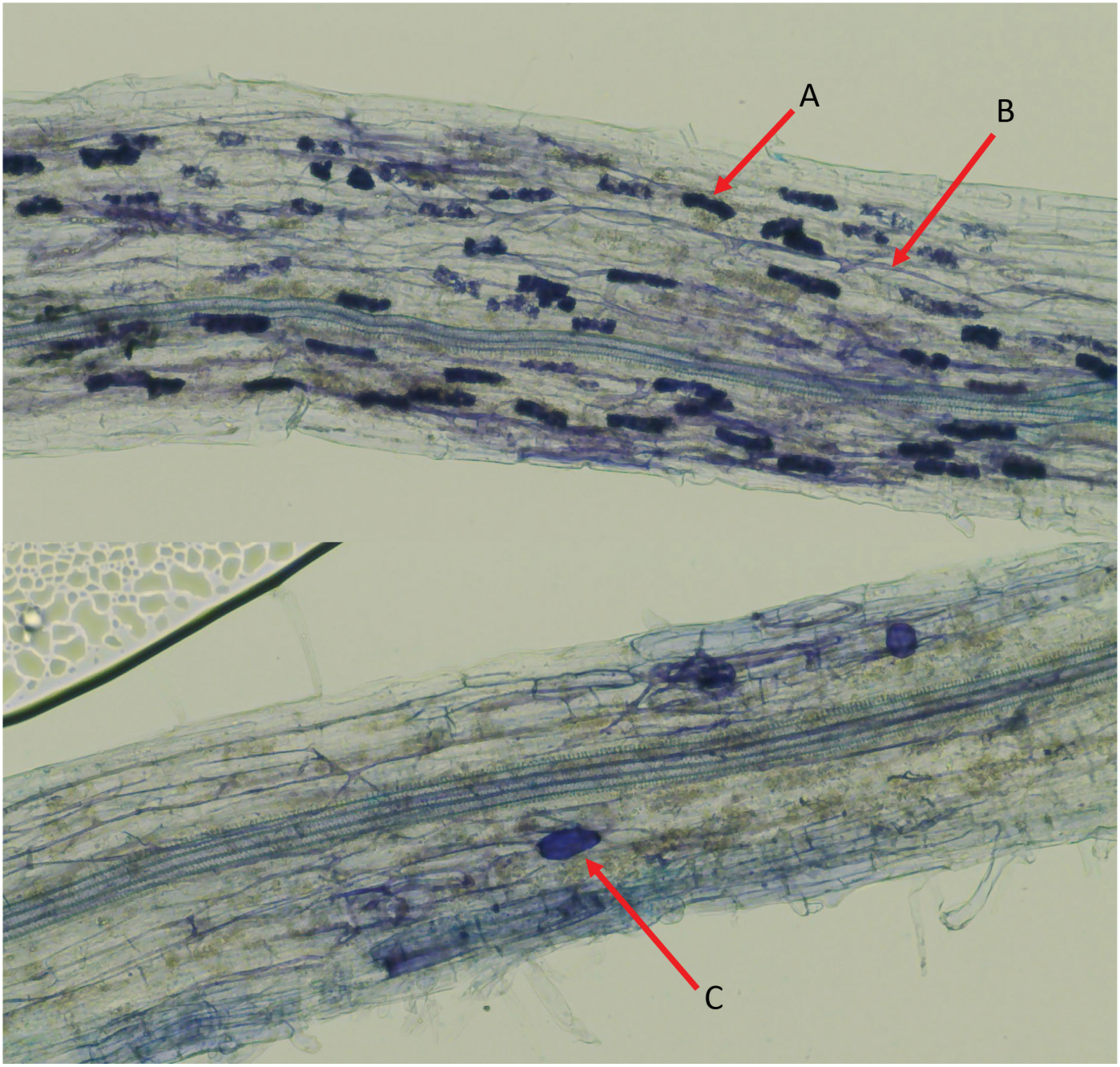

Figure 2. Stained root of *Pulsatilla alpina* with present method. Magnification x250. Fungal structure was stained in blue. The dense cluster inside of the plant cell (A) is arbuscule, the structure of nutrition exchange between plant and AMF. Intraradical mycelium without septa (B) can be observed. Carbon storage structure called vesicle (C) can be observed.

○ Consideration Points for Developing Class Activities

Plants live in symbiosis with a multitude of microorganisms in nature. In this article, I have introduced the AMF-plant symbiosis and the role of the symbiosis, a key part of the plant holobiont. Furthermore, I introduced a safe methodology for the visualization of AMF that can be used to design class activities. Teachers can show how prevalent the symbiosis between plant and AMF is in nature, while students can think and question what will be the natural form of plant roots in the soil. Here, I further suggest two teaching goals that can be designed by teachers for class activities: (1) to emphasize how ubiquitous AMF are in nature and (2) to let students realize the importance of AMF in plant physiology and the associated ecology.

For the first teaching goal, it is important to design the class activity to include field sampling. Teachers can lead students to sample as diverse plant individuals as possible, to highlight that the plant-AMF symbiosis can be observed in almost all cases. The class can be designed together with botany courses to allow students to think about different lineages of plants and their association with AMF. The AMF structures will have morphological variation among different plant species. In general, plant root cell size can vary both interspecifically and intraspecifically. Consequently, the cortex cell occupying AMF structure, such as arbuscules, can have different morphology depending on plant cell structural variation. Designing a class activity for describing morphological variation in AMF structure along different lineages of plants could be interesting. It will also be interesting to design the class to contrast the two common groups of plant, monocots and eudicots. Eudicots have taproots while monocots have fibrous root systems. Consequently, the two types of plants have different foraging strategies in the soil as well as associations with AMF. The activity will make a good transition from the first teaching goal to the second teaching goal, by linking plant physiology and ecology with AMF.

As an extension of plant roots, AMF mycelium forages the part of soil that plant roots cannot reach and absorbs various nutrients. Due to the differences in the root system, photosynthesis type, age, stage of development, and nutrition requirement, different plant species have different degrees of association with AMF (Weishampel & Bedford, 2006). Therefore, designing a class activity for tracking AMF root colonization along the different plant physiological traits will be useful for students to understand the potential role of plant-AMF symbiosis. For this, the quantitative measurement of AMF colonization will be required. The measurement can be accomplished with the introduced method of staining. Here, I am introducing the modified methods for AMF colonization measurement from a previous publication (McGonigle et al., 1990). To measure the degree of colonization, one can randomly cut and sample the 10–20 pieces of 1–3 cm sized root from the stained root system of a plant individual. One slide with aligned root pieces can be made per each plant individual to measure the AMF colonization. With a light microscope, one can make 4–5 observations along the length of each root piece. The number of observations and the total number of positive observations of AMF structures can be counted and noted for each plant individual. With the formula: (The number of positive observations for AMF structure)/(The total number of observations) x 100, AMF colonization percentage per plant can be calculated. The calculated percentage can be used for the comparison of different groups of plant morphological traits such as plant height, total leaf area, or shoot and root biomass.

It is also possible to further design the class activity for plant ecology. Together with the plant diversity study, the root colonization of AMF can be measured and compared. For example, the AMF colonization difference between dominant species of the site compared with non-dominant species can be measured. Different habitats as well as seasonal change can be further considered. It might also be interesting to design an activity to measure abiotic factors of soil together with AMF colonization of plant and plant diversity. It has been documented that soil physicochemical characteristics can affect the symbiosis between a plant and AMF and modulate the mycorrhizal benefit to the plant (Jansa et al., 2002; Pena Venegas et al., 2021). Detailed soil physicochemical characteristic investigations (for example, electric conductibility, or macro-/microelement analysis) require lots of time and resources to be realized in the classroom. However, it can be possible to check the pH of the soil by sampling a small amount of the soil from the site and by using general pH meter with soil wash. The measured pH can be further investigated together with AMF colonization and plant diversity.

○ Conclusion

To address the new paradigm in plant sciences and introduce students to the plant holobiont concept, education in plant science should be changed. The introduction of the symbiosis between AMF and plant, the backbone of the plant holobiont, can be an effective way to introduce the new concept to students. By introducing the current state of knowledge of the plant-AMF symbiosis, and combining it with a safe methodology for visualizing AMF in plants from nature, teachers can design classroom activities for introducing AMF biology jointly with other courses such as botany and ecology. Considering the importance of AMF for the physiology and ecology of plant holobiont, further efforts by teachers are required to design experiments to inspire and engage the students.

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