INQUIRY & INVESTIGATION

An Inquiry-Based Lab Activity to Investigate Potential Effects of Arbuscular Mycorrhizal Fungi on Seed Germination

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Abstract

Incorporating research experiences into undergraduate education is an effective way to foster interest in science and introduce students to the scientific process. We describe an original research investigation into the effect of arbuscular mycorrhizal (AM) fungi on seed germination. Mycorrhizal fungi form symbiotic relationships with plants roots, and although they have been shown to benefit plant growth, their effect on seed germination is less well understood. In this lab activity, students incubate seeds in the presence and absence of AM fungal inocula and compare the germination rates (% seeds germinated) and germination times (days to germination) between experimental (+AM fungi) and control (–AM fungi) groups. Students present their results through a group-written report, in addition to individually submitted assignments. This lab activity provides students the opportunity to learn about species interactions, sterile technique, plant development, data presentation and interpretation, and scientific communication.

Key Words: *ecological restoration; germination; inquiry; mycorrhizal fungi; plant ecology; symbiosis.*

○ Introduction

An important role for introductory science courses is to introduce students to the scientific process. Research-based instruction, especially in laboratory-based courses, improves student understanding of the nature of science and promotes student persistence in science or interest in research (Auchincloss et al., 2014). Here, we describe an inquiry-based lab experiment that students in introductory biology complete to investigate the role of arbuscular mycorrhizal (AM) fungi on plant growth and development.

Mycorrhizae are symbiotic fungi that live on and in plant roots. The relationship between plants and mycorrhizae is context dependent but it is considered a mutualism under many environmental conditions. Fungal spores respond to root exudates from plants by increased branching of hyphae; researchers have identified compounds and signaling pathways important in this process (Bonfante & Genre, 2010). AM fungi enter cell walls and form a mass of hyphae between the cell wall and the cell membrane of the plant. AM fungi provide important benefits for plant nutrition, disease resistance, and tolerance of environmental stress (Delavaux et al., 2017).

The mycorrhizal fungal community is an important factor in successful ecological restoration. For example, AM fungi are usually depauperate in areas that have been used for agriculture (Oehl et al., 2004). AM fungi can enhance restoration efforts by promoting the growth and development of native plant species (Koziol et al., 2018; Cheeke et al., 2019).

Another factor in ecological restoration is the abundant and diverse seed bank present in degraded landscapes (Bakker et al., 1996). Dormant seeds can survive in soils for days, weeks, months, or years. The seed bank represents a source of native plant species that can recolonize an area but can also be a source of weedy non-native species that can delay or prevent restoration.

It would be interesting to know if AM fungi can promote seed germination of native seeds over those of non-natives, especially of invasive species. The effect of AM fungi on seed germination is under-studied for most plants. Mycorrhizae are required for seed germination in orchids (Rasmussen & Rassmussen, 2009), but AM fungi inhibit germination of other species (e.g., Fernández-Aparicio et al., 2010). Unlike signaling from plants to fungi, signaling from fungi to plants (roots or seeds) is poorly understood (Bonfante & Genre, 2010). The effect of AM fungi on seed germination is deserving of attention and can be easily tackled by undergraduate students.

Over several lab periods, students in a 100-level introductory biology course at Washington State University (WSU) Tri-Cities conduct an experiment to identify the effects of AM fungi on seed germination of common flowering plants found in eastern Washington. Students compare germination success and time to germination in seeds incubated with and without AM fungi. This laboratory activity addresses a number of standards for the *Next Generation Science Standards* (NGSS) (Table 1). The data generated by students' research also contribute to research on ecological restoration of eastern Washington habitats, a major focus of the Cheeke lab at WSU.

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Table 1. ***Next Generation Science Standards*** **(NGSS) relevant to this laboratory exercise (AAAS, 2011).**

Standard	Relation to This Exercise
HS-LS1-3: Planning and carrying out investigations.	Students work as a class to conduct original research on the effect of AM fungi on seed germination.
HS-LS2-2: Use mathematical representations to support and revise explanations based on evidence about factors affecting biodiversity and populations in ecosystems of different scales.	Students compare germination rates mathematically and evaluate how the presence of AM fungi could affect community composition and ecosystem function.
HS-LS2-6: Evaluate claims, evidence, and reasoning that the complex interactions in ecosystems maintain relatively consistent numbers and types of organisms in stable conditions, but changing conditions may result in a new ecosystem.	Instructors can choose species that address standards HS-LS2-6 and HS-LS2-7. For example, we focus on species that are relevant for ecological restoration, thereby highlighting human-caused ecological disturbance, ecological succession, and habitat restoration.
HS-LS2-7: Design, evaluate, and refine a solution for reducing the impacts of human activities on the environment and biodiversity.	

Because students work in teams and ultimately combine their data with those of their classmates, this lab demonstrates the interplay of individual and collaborative effort in science. Emphasizing the communal nature of science can promote positive attitudes about careers in STEM (Brown et al., 2018).

○ Materials & Preparation

We use regionally collected seeds of native and non-native plant species from eastern Washington that are purchased from local seed companies or collected from local habitats. Students clean, inspect, and sort locally collected seeds, which makes the experiment less abstract for students. We focus on testing the effect of AM fungi on seed germination of shrub-steppe plants because we want the experiment to be relevant to the students. Instructors or students could select seeds according to species of interest in their ecoregion.

For the substrate, we use locally collected field soil mixed with sand (1:1 by volume) or sand alone. Soil is sterilized by autoclaving twice at 120°C for two hours with a 24-hour resting period between autoclave runs. Teachers who do not have access to an autoclave could use commercially available pressure cookers (Swenson et al., 2018) or purchase commercially packaged sand or sterile seed-starting mix, which are unlikely to contain mycorrhizal spores. Half of the soil volume is reserved for the control treatment and half is thoroughly mixed with AM fungi (10% by volume) for the experimental treatment. It is important to keep control and AM fungi soil mixtures separated in different containers with lids; dust from the experimental soil can easily carry fungal spores to the control soil.

We considered including an equivalent amount of autoclaved inocula in the uninoculated controls. However, autoclaving can release nutrients from dead microbes (Razavi darbar & Lakzian, 2007), which could result in greater nutrient variability between the inoculated and uninoculated treatments. Additionally, the cost of the inocula would double. Although we opted to use only sterile substrate in the controls for the experiments presented here,

Table 2. Calculations for Petri dishes, seeds, soil, and AM fungi needed for an experiment. We have provided sample calculations based on our experimental design in spring 2020, when we used four species of plants. Different groups set up dishes for different plant species; across all lab sections, we had 24 replicates per treatment per species.

Item	What You Need to Decide	Sample Calculation for Spring 2020
Total # Petri dishes needed overall	• How many replicates per treatment? • How many treatments per species? • How many plant species?	We wanted 24 replicates per treatment and we had 2 treatments (control, experimental), so we needed 48 dishes per species (24 replicates per treatment * 2 treatments). We used 4 species, so we needed a total of 192 dishes (48 dishes per species * 4 species).
Seeds needed for each plant species	We recommend ≤5 seeds/dish. Multiply the number of seeds you want per dish by the number of dishes (replicates) per species (control and experimental groups combined).	We needed 48 dishes per species, so 5 seeds per dish meant we needed 240 seeds for each species (5 seeds per dish * 48 dishes per species = 240 seeds per species).
Soil (volume)	(total # dishes) * (40 cm^3 soil/dish) We recommend rounding up.	(192 dishes) * (40 cm^3/dish) = 7680 cm^3 (7.68 L of soil) (Round up to 8 L)
AM fungi (volume)	(Final volume of soil needed) * 10% We recommend rounding up.	(7680 cm^3) * 10% = 768 cm^3 AM fungi needed (Round up to 800 cm^3)

instructors could incorporate two controls (controls without inocula and controls with autoclaved dead inocula) to test for an effect.

There are a range of commercially available AM fungi products. We recommend products that contain a diverse mix of fungi rather than ones with only one or a few species. We use MycoBloom, a mix of seven AM fungal species that is available for purchase online (e.g., from Amazon.com).

For guidance in calculating the number or volume of Petri dishes, seeds, soil, and AM fungi required for the experiment, see Table 2. Additional materials required are ethanol (70–95%), gloves, forceps, squirt bottles of deionized (DI) or sterile water, beakers (30–100 mL), and magnifying glasses to inspect seeds for germination. Students check the seeds daily, so the dishes should be placed somewhere that they can access easily (e.g., greenhouse, well-lit bench space or shelving).

○ Procedure

We divide the experiment into five stages: observing germinated seeds (Stage 1), observing AM fungi-colonized roots (Stage 2), setting up the experiment (Stage 3), collecting and entering data (Stage 4), and analyzing and presenting the results (Stage 5). Stages 1, 2, and 3 could be combined into a single lab session.

Stage 1: Observing Germinated Seeds

Students spend 10–15 minutes observing seeds that have been germinated in 100 mm Petri dishes lined with damp filter paper. Students use these firsthand observations to develop an operational definition of *germination* and to select species for the experiment.

Stage 2: Observing AM Fungi-Colonized Roots

Students spend ~10 minutes looking at microscope slides of AM fungi-colonized roots and describing what they see. Slides can be prepared from AM fungi-colonized plants (DeMars & Boerner, 1995) or purchased from biological supply companies (e.g., Carolina Biological Supply no. 301952). This activity allows students to visualize the physical relationship between mycorrhizal fungi and plant roots.

Stage 3: Setting Up the Experiment

Students set up the experiment in a three-hour lab period. Sterile technique is critical at all stages of the experiment to prevent inadvertent inoculation of controls with AM fungal spores. Students sterilize all bench tops and gloves with ethanol or Lysol cleaner before the lab and between setting up control and AM fungi treatments. We set up control treatments first, then set up experimental treatments to minimize accidental carryover of spores between treatments.

Students work in groups to set up control and experimental Petri dishes for two plant species, with different groups setting up dishes for different plant species. Each replicate Petri dish (100 mm) consists of five seeds placed into 30–40 mL of substrate using forceps sterilized by dipping them in 95% ethanol and air drying before moving between Petri dishes. After moistening the soil in the Petri dish with ~30 mL of DI water, students gently press each seed into the substrate. Students sterilize gloves with 95% ethanol between Petri dishes. The position of each seed is numbered on the lid of the dish so that students can follow the fate of individual seeds (see Supplemental Material available with the online version of this article).

To reduce the risk of cross-contamination, we keep the two treatments physically separated at all times. Ideally, we would randomize the spatial distribution of dishes on the bench tops to control for environmental variation in the greenhouse, as this would intersperse control and experimental dishes. However, for this lab activity, we physically segregate the treatments to minimize the risk of cross-contamination (while acknowledging that doing so increases the potential for confounding environmental variation).

Stage 4: Collecting Data

At least one student from each group takes a few minutes every day to check on their group's seeds during the two-week experiment. When a seed germinates, the student records the date (month/day) on the datasheet (see Supplemental Material) and on the lid of the dish. The germinated seed is removed from the dish with sterile forceps and discarded to reduce confusion about which seeds have germinated. DI or sterile water is added as needed to keep the substrate evenly moist throughout the experiment. Adding parafilm around the edges of the Petri dish reduces water loss, especially if dishes cannot be checked over weekends.

We dismantle the experiment after two weeks. After accounting for the fate of all seeds, students discard their Petri dishes, then clean and sterilize the greenhouse benches. Each group enters their data into an Excel workbook and submits their original datasheets. The instructors check data for obvious mistakes and refer to the original datasheets from students for clarification, if necessary.

Stage 5: Compiling, Presenting & Analyzing Data

We devote a three-hour lab session to analyzing the data and constructing figures for the lab report. Students use data from all lab groups for their reports, not just from their own set of dishes. The students construct equations in Excel to calculate the mean percentage of seeds that germinate and the mean time to germination. We have students calculate standard error to estimate variation. For both dependent variables, replicates are individual Petri dishes, not individual seeds. Students present data in a bar graph (mean ± SE) for each dependent variable (mean % germination, mean number of days to germinate), grouped by species/treatment combination.

○ Student Assessment

We assess student learning through a project notebook, peer assessment, and written assignments. Students submit some assessments as individuals and others collectively as a group, thus reflecting the nature of scientific research as an individual and collaborative enterprise.

Students keep individual notebooks to record their activities and observations, following general guidelines for scientific notebooks (Caprette, n.d). After the first week, students reflect on their experience by recording answers to prompts provided by the instructor (see Supplemental Material). Students submit a group-written lab report at the conclusion of the experiment. In preparation for this final report, students individually submit drafts of the Introduction, Methods, and Results sections (including graphs), which are graded and returned before the final lab report is due. Groups are encouraged to draw from individual group members' drafts to develop the best collaborative report possible. At the end

of the project, each student provides a self- and peer-assessment for their group members.

○ Results & Discussion

We have completed three iterations of this lab, refining the experience each time (Table 3). Most notably, we simplified the lab over time to streamline the experimental design and to improve student understanding.

One improvement that we have made is to reduce the number of plant species tested, focusing on more replicates of fewer species. This simplification reduces the number of plant species that students need to compare, making the data analysis and interpretation less complicated. In addition, we now include only native plant species because using a second independent variable (native/non-native plant status) made the experiment challenging for our introductory students. Streamlining the experiment has also allowed us to devote more time to the writing process. Based on laboratory reports, these simplifications have helped students grasp the hypotheses, methods, and results more easily.

In the fall 2020 semester, COVID-19 safety measures affected how we completed this experiment. Classes on our campus, including labs, were conducted remotely through synchronous instruction. Students conducted the experiment in their homes over three weeks, using kits assembled by WSU staff. Because we had no direct oversight of the experiment and each student conducted the experiment under different environmental conditions, we have not included data from that semester.

In the other three semesters, we did not find strong effects of AM fungi on seed germination for most of the species that we have tested. However, there have been two exceptions, suggesting that results may be species specific. The addition of AM fungi reduced the germination rate for the non-native prickly lettuce (*Lactuca serriola*) in 2018 (average ± SE = 3.9 seeds ± 0.3 germinated/dish for controls; average ± SE = 2.7 ± 0.4 seeds germinated per dish for AM fungi treatments; one-way ANOVA: $F_{1,28} = 6.16$, $p = 0.019$). The addition of AM fungi increased the germination rate for the native bluebunch wheatgrass (*Pseudoroegneria spicata*) in 2020 (average ± SE = 4.5 seeds ± 0.1 germinated/dish for AM fungi treatments; average ± SE = 3.8 ± 0.3 seeds germinated per dish for control treatments; one-way ANOVA: $F_{1,46} = 4.97$, $p = 0.031$). Time to germination appears to be unaffected by the addition of AM fungi for the species we have tested.

Some factor other than the addition of AM fungi might be able to explain the few cases in which we obtained significant

Table 3. Iterations of this laboratory activity over three semesters, including species used, experimental design, and changes implemented or planned. We report common names of plant species; scientific names are available in the online materials.

Semester & Enrollment	Plant Species Used by Entire Class & Number of Replicates	Details of Experiment	Changes Made or Planned for Next Iteration
Fall 2018 73 students 4 lab sections	Eight plant species (5 native, 3 non-native) Native species: Idaho fescue, sand penstemon, aster species, yarrow, basin wild rye Non-native species: cheatgrass, prickly lettuce, goatsbeard 10–30 replicates of each	Students worked in groups of 3–4 students. Each group set up 5 control and 5 experimental (AM fungi treatment) replicates for two species from the list, assigned by the instructor.	• Streamlined lab prep and setup
Spring 2019 49 students 2 lab sections	Eight plant species (4 native, 4 non-native) Native species: Idaho fescue, bluebunch wheatgrass, nine-leaf biscuitroot, yarrow Non-native species: cheatgrass, green foxtail, knapweed, puncture vine 9–18 replicates of each	Students worked in groups of 3–4 students. Each group set up 3 control and 3 experimental (AM fungi treatment) replicates for two species from the list, assigned by the instructor.	• Reduce the total number of species used • Increase replication for each species and treatment • Use native species only
Spring 2020 49 students 2 lab sections	Four native species: globe mallow, sand penstemon, Idaho fescue, bluebunch wheatgrass 24 replicates each	Students worked in groups of 3–4 students. Each group set up 3 control and 3 experimental (AM fungi treatment) replicates for two species from the list, assigned by the instructor.	Plans for fall 2020: Extend experiment to allow three weeks for germination.

effects of AM fungi on seed germination. We did not process germinated seeds to quantify AM fungi colonization of the seed radicle, though that is an assay we could do in the future. However, colonization is likely not necessary for AM fungi to affect seed germination. As mentioned previously, communication from fungi to plants is poorly understood for this system (Bonfante & Genre, 2010) but "myc" factors (chemical signals produced by AM fungi) have been identified that may impact seed germination (Genre et al., 2013).

Even though we have not seen a clear-cut relationship between AM fungi and seed germination thus far, students have responded positively to the lab experience. Based on our observations and conversations with students, they are enthusiastic about the experiments, taking care while setting up the dishes, checking seeds, and recording data. They are excited to know that their work contributes to how plant–fungal interactions could promote ecological restoration of habitats in eastern Washington, an area of interest for the Cheeke lab.

The lab activity described here is also highly flexible. The near ubiquity of associations between mycorrhizae and plants means that this exercise is suitable for biology, ecology, environmental science, botany, or horticulture courses. Instructors could scale the experiment up or down, depending on their interests and time availability. It could be condensed to one or two lab periods or expanded to fill an entire academic term. By choosing different plant species, instructors could make the experiment relevant to local issues or student interests.

This activity allows students to investigate an open question about interactions between plants and fungi: Do symbiotic mycorrhizae affect seed germination? Students respond favorably, displaying enthusiasm for studying a question without a canned answer. This fun, relevant, low-tech activity incorporates biological content (plant ecology, microbial ecology), improves scientific skills (sterile technique, record keeping, data analysis), and builds scientific community (collaboration and cooperation with classmates).

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