Networks of Communication: Defense-related signal transfer between tree seedlings via mycorrhizal networks and an educational mycorrhizal-focused video game

by

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Abstract

The majority of terrestrial plants associate with fungi in symbiotic resource-exchange relationships called mycorrhizae. Mycorrhizal networks (MNs) arise when the same fungus is connected to multiple plants, allowing for interplant resource transfer and impacting ecosystem functions. Recent work suggests MNs also transfer defense-related information from pathogen-, herbivore-, or mechanically-damaged plants to unharmed neighbors. I investigated the defense pathways involved in defense-related signal transfer in ectomycorrhizal systems. Paired Douglas-fir seedlings were grown with varying levels of belowground connectivity (soil water only; soil water and MNs; soil water, MNs, and roots), and a defense response was stimulated in donor seedlings by methyl jasmonate. After 24 and 48 hrs, I measured expression of two regulatory genes on the jasmonate and ethylene pathways. Receiver response was unrelated to hormone treatment of donors in either gene, but the jasmonate response of donor and receiver pairs was correlated across treatments. Positive expression of both genes across donors and receivers and pervasive presence of spider mites suggested signal transfer may either have not occurred or been masked by already ongoing defensive responses. Results indicate the complexity of these systems, and further work is needed to better characterize defense signal transfer via ectomycorrhizal networks.

Because of the importance of these mycorrhizal systems to ecosystem functioning, it is crucial that resource managers and scientists have a good understanding of mycorrhizal ecology. However, lower student interest in plants and fungi combined with difficulties visualizing belowground processes present
challenges for teaching and learning mycorrhizal concepts. To address this, I co-created the digital plant-centric action-based game Shroomroot for use in lower level postsecondary settings. I conducted a pre-test/post-test evaluation of Shroomroot in a 2nd year postsecondary Introduction to Soil Science course. Students’ knowledge of mycorrhizal ecology increased after playing Shroomroot, and engagement with mycorrhizal content tended to increase after gameplay. These exploratory results suggest positive potential for action-based plant-oriented digital games in the higher education classrooms.

Both studies focus on improving our understanding of mycorrhizae and mycorrhizal networks, ecologically and pedagogically. Greater understanding of mycorrhizae has the potential to improve our multi-faceted relationships with the ecosystems upon which we depend.
Preface

This thesis is an original, unpublished product of the author, Julia Amerongen Maddison (JAM). The projects herein were identified and designed by Dr. Suzanne Simard and JAM. The experimental specifics were designed by JAM and Dr. Suzanne Simard in conjunction with committee members, Dr. Maja Krzic, Dr. Sue Grayston, and Dr. Rik Blok. JAM established and led greenhouse experiments and lab work with the exception of combustion elemental analysis and VHP closed vessel microwave acid digestion for foliar nutrient analysis, performed by the British Columbia Ministry of Environment Analytical Laboratory. JAM co-created Shroomroot with Christopher Adderley and Alex Catamo of Area Denial Games, and wrote educational assessments with suggestions from course instructors. Statistical analyses were conducted by JAM with suggestions from Dr. Suzanne Simard and Dr. Valerie Lemay. Ethical approval for the study in Chapter 2 was obtained from the Behaviour Research Ethics Board (BREB) under the code H14-02382, project name “Educational Efficacy of Root-Mycorrhizae Action-based Mobile Game,” nickname “shroomroot.”
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List of Abbreviations

AOC – Allene oxide cyclase (gene on jasmonate pathway)
ACS2 – Aminocyclopropane carboxylic acid (gene on ethylene pathway)
DGBL – Digital Game-Based Learning
ET – Ethylene
JA – Jasmonic acid
KS – Kolmogorov-Smirnov
MeJA – Methyl jasmonate
MN – Mycorrhizal network
SR – Shroomroot
TEF – transcription elongation factor
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Dedicated to all of my parental units.
Chapter 1: Introduction

When we walk through a forest, most of what we experience is aboveground, but there is a rich world of complex belowground activity that involves plant roots, nutrients, water, fungi, microorganisms, and many other factors. This belowground activity affects forest functioning, and understanding these interactions is crucial to holistic and successful forest management. “Mycorrhiza” are symbiotic relationships between a fungus (“myco”) and a plant root (“rhiza”) that are formed by an estimated 90% of terrestrial plants (Fortin et al., 2009). The core aspect of the symbiosis is a resource exchange, in which the fungus gives the plant resources from the soil such as nitrogen, phosphorus, and water, and the plant gives the fungus carbon derived through products of photosynthesis (photosynthates). The fungi in these symbioses have filaments called “hyphae” growing throughout the soil in a complex matrix called a “mycelium”, and the same fungus can connect multiple plants at the same time. These networks of fungal mycelium allow resource exchange to occur indirectly between neighbouring plants, and are known as “mycorrhizal networks” (MNs). Mycorrhizal networks are important in ecosystems worldwide, impacting nutrient cycling, soil aggregation, forest stand dynamics, and many other ecosystem processes (reviewed in Simard et al., 2012, 2015; van der Heijden et al., 2015). Our developing understanding of mycorrhizal ecology is changing the way we practice forestry (Schwartz et al., 2006, Azul et al., 2014, Puettman et al. 2009), and changing the way we think about the balance of relationships between species (van der Heijden and Horton, 2009; Simard et al.,
Recent work has suggested that MNs may also facilitate the transfer of defense-related information between plants (reviewed in Gilbert and Johnson, 2015; Gorzelak et al., 2015).

In this thesis, I aim to elucidate the nature of this defense-related information transfer in a particular kind of mycorrhizal system, and also to present and evaluate a plant-centric, action-based game I co-created about mycorrhiza for use in lower level postsecondary courses. The first study in this thesis is a greenhouse experiment investigating MN-mediated defense signalling between paired Douglas-fir seedlings. The second study presents the mycorrhizal-focused game entitled Shroomroot, as well as an exploratory evaluation of Shroomroot’s impact on students’ content knowledge and content engagement in 2nd year Introduction to Soil Science course.

In this chapter, I provide general background for each of these two studies. For the greenhouse experiment, I provide background on the mycorrhizal symbiosis, MNs, plant defense signalling, and interplant communication, and then narrow in on how mycorrhizal networks may facilitate interplant communication and how I aim to contribute to our understanding. For the educational game study, I provide background about the field of digital game-based learning (DGBL), and distinguish between types of games and associated learning and affective outcomes, with a focus on studies that involve ecology-centered games.

Chapter 2 details the MN-mediated defense signal transfer experiment and Chapter 3 the educational game study. Chapter 4 summarizes the main findings of both studies and presents concluding thoughts.
1.2: Interplant signal transfer via ectomycorrhizal networks

1.2.1: Mycorrhizae and mycorrhizal networks

More than 90% of land plants are known to associate with mycorrhizal fungi (Fortin et al., 2009), and this association is thought to have been an important development in land colonization by plants (Pirozynski and Mallock, 1975, Fortin et al., 2009). The fungal partner in the association has a higher surface area of hyphae in the soil than the plant roots, giving the fungus an advantage when it comes to soil resource collection. In contrast, the plant is a photosynthetic autotroph, meaning the plant can use energy from sunlight to convert inorganic CO$_2$ into energy-storing carbohydrates usable in the rest of metabolic activities. The plant benefits from associating with the fungus by receiving better access to soil resources, while the heterotrophic fungus benefits by receiving food in the form of plant photosynthates.

There are many types of mycorrhiza, including arbuscular, ericoid, arbutoid, and ectomycorrhiza, but the two most prominent groups are arbuscular mycorrhizas (AM) and ectomycorrhizas (EM). These two groups are primarily differentiated by the physical structure of the symbiosis, and tend to involve different taxonomic groups of fungi and plants (Smith and Read, 2008). Arbuscular mycorrhizal fungi are in the group Glomeromycota, and while exceptions exist, AM plant hosts tend to be grasses and shrubs. In an AM symbiosis the hyphae penetrate the plant cell walls and form complex tree-like structures in between the plant cell wall and the plant cell plasma membrane called “arbuscules.” These are the sites for
nutrient exchange between the plant and fungus. Ectomycorrhizal mycorrhizal fungi are primarily Ascomycota and Basidiomycota, and EM plant hosts tend to be conifers and other woody plant species (again, with exceptions). In EM symbioses, fungal hyphae remain outside of plant cell walls, but grow between the cells of the outermost layers of root tissue in a structure called a “Hartig net”. EM fungi also form an often visible and relatively thick (compared to AM fungi) layer of hyphae on the surface of the root known as the “mantle.”

Both AM and EM fungi form mycorrhizal networks that have been observed to transfer resources between neighboring plants (reviewed in Newman, 1988; Simard et al., 2015; van der Heijden et al., 2015). However, there is the potential for resource transfer in EM networks to be more efficient and/or occur in greater volumes due to structures called “strands” or “rhizomorphs”. These structures, which I will refer to only rhizomorphs, are formed by certain groups of EM fungi, and are thick tubes of differentiated hyphae with hollow centers, in comparison to thinner filaments of undifferentiated hyphae (Agerer, 2006). Rhizomorphs last longer in the soil (Bledsoe et al., 2014) and have a much greater potential for long-distance and more efficient transport of resources (Agerer, 2006). This is associated with differences in the magnitudes and consequences of resource transfers compared with AM networks, as discussed by Simard et al. (2002, 2012). Presumably, the same could apply to defense-related signal transfer, as discussed in section 1.2.3 below, making it possible that EM networks including rhizomorphs may have increased potential for long-distance or more efficient signal transfer than AM networks or EM networks without rhizomorphs. The distinct structures of the
AM and EM symbioses, evolutionary history, fungal and plant species involved, and potential for different transfer behaviour via AM vs EM networks all make it important to investigate each mycorrhizal group distinctly with respect to MN-mediated interplant signal transfer. The majority of MN-mediated interplant signal transfer has been studied with respect to defense-related signalling (as opposed to abiotic stress or allelochemicals, which plants use to directly hurt herbivores or neighboring plants). Below I provide context for plant defenses and then describe previous work regarding interplant defense-related signalling via volatile organic compounds (VOCs).

1.2.2: Plant defense

Like animals, plants have a variety of mechanisms by which to combat damage from pathogens and herbivores. These mechanisms can be constitutive (i.e. always active) or induced by stimuli – either a natural threat such as an herbivore or an artificial threat such as manual defoliation treatment. Because defense-related interplant signal transfer examines plant behaviours occurring in response to stimuli, investigations focus primarily on induced responses. Induced responses are diverse, interlinked, and are often grouped by the nature of the threat (i.e., pathogen or herbivore) and associated with particular plant hormones. Biotrophic pathogens (that feed while the plant is alive) are mainly associated with the salicylic acid (SA) pathway, while herbivores and necrotrophic pathogens (that kill the plant cells and then feed on them) primarily involve the jasmonate (JA) pathway and the ethylene
(ET) pathway. Another plant hormone implicated in defense responses is abscisic acid (ABA), but its exact role is still unclear (Derksen et al., 2013).

These different plant hormone pathways act as signal cascades (signalling pathways with many steps) within a plants’ tissues - they link recognition of a threat with response to that threat. For example, when plants recognize wounding and/or insect-derived compounds called “elicitors” (bits of protein, peptides, etc), the jasmonate pathway is triggered. Linolenic acid is released from membrane lipids (from the chloroplast membrane and others), and through a series of steps is converted to the hormone jasmonic acid (Figure 1.1a). Jasmonic acid is then converted into a variety of different conjugates (JA + another compound), of which only a few currently have an understood biological function (Kombrink, 2012; Zhou and Memelink, 2016). The jasmonic-amino-acid conjugate JA-isoleucine (JA-Ile) appears to be the most bioactive, triggering the majority of JA-related downstream defense responses, including production of phenols, terpenoids, alkaloids, and other defensive compounds (reviewed in Zhou and Memelink, 2016). Jasmonic acid can also be converted to methyl jasmonate (MeJA), which is a volatile form of the hormone that is released outside of the plant tissues. JA and MeJA can also stimulate the JA pathway in a positive feedback loop, thereby promoting further propagation of the jasmonate response and making it a strong candidate for long distance (i.e. beyond a single leaf) signal transfer within plant tissues (Heil and Ton, 2008).

Ethylene (ET) is another plant hormone which has a comparatively simpler biosynthetic pathway (Figure 1.1b), and in general tends to operate synergistically with the JA pathway (i.e., activate or deactivate in concert with JA, Zhu et al., 2014).
While JA and its derivatives (collectively called jasmonates) have been implicated in long-distance signal transfer within plant tissues and in volatile form, ET is considered less likely to operate as a signal over these longer distances (Heil and Ton, 2008). This is due to ET's comparatively weak ability to stimulate its own biosynthesis – suggesting that signal propagation would be difficult (but this is still somewhat speculative, Heil and Ton, 2008).

Because most volatile and MN-mediated defense signal transfer has focused on stimuli related to necrotrophic pathogens and herbivore or mechanical wounding, I will only briefly touch on the salicylic acid (SA) pathway. Like the JA pathway, the biotrophic-pathogen-associated salicylic acid pathway is also implicated in long-distance signaling (Heil and Ton, 2008), and has a volatile form of methyl salicylate (MeSA). For both the JA pathway and SA pathway, the speed of systemic movement of hormones via vascular tissue is considered to be slow relative to other forms of systemic signalling (hydraulic or electric), putting into question the influence of JA and SA on vascular-based long-distance signalling (Huber and Bauerle, 2016). However, release of volatile defense-related compounds like MeJA or MeSA is thought to allow for faster propagation of the defense signal within an individual plant when compared to the same signal travelling via vascular tissue (Heil and Ton, 2008; Huber and Bauerle, 2016).

### 1.2.3: Plant communication

It is now well-established that the release of defense-related volatile organic compounds (VOCs) by individual plants can also affect the defensive status of their...
neighbors (Ramov, 2013; Das et al., 2013). In one of the first studies to show this, Baldwin and Schultz (1983) found increased phenolic concentrations in leaves of poplar (*Populus x euroamericana*) and sugar maple seedlings (*Acer saccharum*) that were sharing airspace with mechanically damaged neighbors (but not sharing soil compartments). Seedlings that were at a similar distance but in isolated airspaces showed proportionally less phenolic response (Baldwin and Schultz, 1983). Since then, multiple studies have demonstrated the potential for this effect to impact defense-related leaf chemistry and gene expression (e.g., Arimura et al., 2001; Paschold et al., 2006; Godard et al., 2008) as well as herbivory – such that plants that receive “warning” from their neighbors show decreased damage from herbivores (Dolch and Tscharntke, 2000; Arimura et al., 2001; Karban et al., 2006).

This defense-related signal transfer has been broadly deemed communication in many studies (Baldwin and Schultz, 1983; Heil and Karban, 2010; Ramov, 2013), but this terminology has been controversial, for two main reasons: (1) disagreement around what “communication” means and whether this sort of chemical signalling can be labelled as communication and (2) because it was argued that it was unusual for plants to be “warning” their nearest neighbors, which are known to compete for light and nutrients (Heil and Karban, 2010).

One definition of communication provided by the Oxford dictionary is “The imparting or exchanging of information by speaking, writing, or using some other medium.” In the case of defense-related interplant signalling, defense-related information is being exchanged between different individuals. Because the focus of my ecological work is on the transfer of information, and the word “communication"
is commonly used in the field of both VOC and MN-mediated interplant signal transfer (e.g., Heil and Karban, 2010; Babikova et al., 2013), I will use both “plant communication” and “interplant signal transfer” in this thesis.

The second criticism of plant communication is that it seems evolutionarily disadvantageous – why would plants “help” their immediate competitors? The current thinking tends to be that plants are “eavesdropping” on neighbors rather than that plants are “purposefully” sending early warning signals to neighbors (Ramov, 2013; Heil and Karban, 2010). In this context, the expectation is that the advantage of using volatiles for quick systemic defense signalling within an individual plant outweighs disadvantages from potentially improving neighbors’ fitness. However, as work within the field of mycorrhizal networks has suggested, the forest may be more mutualistic than we once thought (van der Heijden and Horton, 2009). Despite the uncertainty around the boundaries of the term “communication” and the balance of evolutionary pressures, there now exists a solid body of evidence for airborne transfer of plant defense-related signals (Heil and Karban, 2010, Huber and Bauerle, 2016).

1.2.4: MN-mediated interplant signal transfer

One of the most recent advances in understanding plant communication is in signal transfers via mycorrhizal networks (MNs) (Song et al., 2010; Barto et al., 2011; Babikova et al. 2013a; Alchatz et al., 2013; Song et al., 2014; Song et al., 2015). Included in discussion of MN-mediated signal transfer is the transfer of plant allelochemicals (i.e., compounds plants use to harm each other, as in Barto et al.,
2011; Achatz et al., 2013), but here I restrict my discussion to the transfer of defense-related signals. The transfer of resources across MNs has been relatively well-studied (reviewed in Simard et al., 2012; van der Heijden et al., 2015), but investigation of defense-related signal transfer via MNs is relatively new, and presents interesting possibilities with respect to MNs’ influence on ecosystem function.

Interplant signal transfer via mycorrhizal networks has the potential to follow different dynamics than airborne forms of communication, and influence ecosystem functioning in unique ways. Mycorrhizal networks would provide a primarily aqueous form of transport, and when rhizomorphs are present in ectomycorrhizas, MNs can transport numerous compounds, such as protein monomers, N ions, phosphates, lipids, and fungal carbohydrates (reviewed in Simard et al., 2015). This could provide opportunities for different chemicals to be transported than through the air, opening signal transfer to less stable, non-volatile, and potentially larger signal molecules. Rhizomorphs can also connect trees up to 20m apart (Beiler et al., 2015), creating potential for more long-distance communication relative to the narrow range observed in airborne communication (Karban et al., 2006). Finally, MNs could provide more targeted communication between trees than airborne pathways, restricting signal transfer to mycorrhizally-linked individuals (as in Simard et al., 1997).

MN-mediated signal transfer could impact spatial patterns of disease resistance, regeneration of seedlings, and generally contribute to the self-organization of forests as complex adaptive systems (Gorzelak et al., 2015). This is
currently speculative and while work on belowground communication has successfully demonstrated rapid (within 24 hours) and significant signal transfer in greenhouse settings (reviewed in Babikova et al., 2013b; Gorzelak et al., 2015), more research is needed to examine the mechanisms of this transfer. Moreover, the ecological significance of signal transfer needs to be investigated, including whether it occurs in field settings, whether it is important to forest health, and whether there is influence and interplay between MN-mediated versus airborne interplant communication.

What is known about MN-mediated interplant signal transfer has been primarily established in AM systems (Song et al., 2010; Babikova et al., 2013a; Song et al., 2014), with one study showing evidence for transfer in EM systems (Song et al., 2015). Song et al. (2010) were the first to demonstrate signal transfer in AM systems; they investigated whether “donor” diseased AM tomato plants (Solanum lycopersicum) could transfer defense signals through MNs to “receiver” neighboring, healthy plants, thus providing an early warning system to prepare for pathogen infection. Song et al. (2010) grew pairs of tomato plants in a sterilized field soil/sand mixture (2:1 by weight) separated by mesh barriers, the different sizes of which controlled for the development of a MN. They inoculated MN treatments with the mycorrhizal species, Glomus mosseae (an AM fungus), and did not inoculate non-MN treatments (controls). Separating paired plants aboveground with airtight bags, they treated one tomato plant (the donor) with tomato blight (Alternaria solani) and observed increased expression of defense genes, both upstream (LOX, AOC, PAL) and downstream (PR1, PR2, and PRS3), and related enzymes (peroxidase, chitinase,
LOX, by a variety of assay methods) in the neighboring plant (the receiver). Their findings indicated that the presence of a MN significantly facilitated the belowground transfer of defense signals from infected plants to healthy ones.

To more deeply explore the nature of this defense signal transfer, Song et al. (2014) investigated the involvement of the jasmonate pathway (Figure 1.1a) in MN-mediated interplant signal transfer. They performed a similarly structured experiment to the one described above, but with different combinations of transgenic tomato plants that were either “deaf” in the jasmonic pathway (spr2 tomato mutants are unable to create Linolenic-acid, a precursor to JA (Li et al., 2002)) or “mute” (in jai1 mutants, JA fails to trigger changes in gene expression and enzyme production(Li et al., 2002)). They showed that these mutations along the jasmonate pathway resulted in a failure of signal transfer, strongly suggesting jasmonate pathway, and potentially jasmonic acid, involvement.

Song et al. (2010 and 2014) and Babikova et al. (2013a) focused on AM systems, while a recent study by Song et al., (2015) investigated this effect in an EM system. Song et al. (2015) grew pairs of Douglas-fir (Pseudotsuga menziesii) and ponderosa pine (Pinus ponderosa) seedlings, and treated the 4-month old donor Douglas-firs with mechanical defoliation or insect defoliation by spruce budworm (Choristoneura occidentalis). They measured a suite of defense enzymes (peroxidase, polyphenol oxidase, and superoxide dismutase, using spectrophotometric assays) in both ponderosa pine receiver and Douglas-fir donor plants and found a significantly higher increase in all three enzymes where the plants were linked by a MN (i.e., in the mycorrhizal network treatment (35µm mesh)). In both the soil-only treatment
and the treatment that allowed roots to interact (in addition to mycorrhizal hyphae), the receivers had lower defense enzyme activity (Song et al., 2015). This provides the first evidence that this interplant signal transfer may occur in ectomycorrhizal networks.

Thus far, interplant signal transfer via MNs has been tested with stimuli in the form of necrotrophic pathogens (Song et al., 2010), herbivores (Babikova et al., 2013a, Song et al., 2014, Song et al., 2015) and mechanical wounding (Song et al., 2015), all stimuli typically associated with JA and ET pathways. Because Song et al. (2015) demonstrated early evidence of defense transfer in EM systems by measuring downstream defense enzymes, little is known about the upstream defense pathways involved in EM-mediated defense signal transfer. My thesis is therefore focused on examining gene expression along the ET and JA pathways in EM host, interior Douglas-fir (*Pseudotsuga menziesii var. glauca*), with the goal of improving our understanding of pathways involved in defense signaling from injured to healthy plants through connecting MNs. While it would be interesting to also examine the dynamics of SA in MN-mediated interplant signal transfer, that is outside the scope of this thesis.

### 1.2.5: Study species

In the context of mycorrhizal network ecology, one of the most well-studied EM hosts is Douglas-fir, particularly interior Douglas-fir (*Pseudotsuga menziesii var. glauca*). Interior Douglas-fir is a common, economically-, and culturally-important conifer species. It is used widely for timber production, and is part of the grassland-
forest interface that is used for cattle grazing, urban development and recreation. Douglas-fir is known to associate with *Rhizopogon*, an ectomycorrhizal genus of fungi known for forming rhizomorphs, making it a particularly interesting target for signal transfer via EM networks. Song et al. (2015) performed their experiment on signal transfer via EM networks with a Douglas-fir donor and ponderosa pine receiver, but I will be restricting my study to Douglas-fir only. Because I am measuring defense pathway involvement using gene expression, having annotated sequence information was crucial to performing the study. Ponderosa pine does not yet have a complete transcriptome (Lesser et al., 2012), while Douglas-fir has a complete, annotated transcriptome (Howe et al., 2013, ref from 2015). In order to maintain comparability to the Song et al. (2015) experiment and be able to measure expression of target defense pathway genes, my experiment involved all interior Douglas-fir donor and receiver pairs.

1.2.6: Summary

Both AM and EM mycorrhizal networks have the potential for numerous ecological impacts on the functioning of ecosystems (van der Heijden et al., 2015, Simard et al., 2015), and this may extend to facilitating belowground interplant communication. AM and EM mycorrhiza are structurally different, and the potential for rhizomorphs in EM networks is an interesting aspect that may impact signal transfer dynamics. Interplant signal transfer via EM networks is just beginning to be explored, and very little is understood about the potential mechanisms involved. Work in AM systems to understand the mechanisms has demonstrated involvement
of the jasmonate pathway, a plant hormone involved in defense responses to
necrotrophic pathogens and herbivores. Jasmonic acid is a promising candidate for a
signal molecule, given its ability to stimulate the jasmonate pathway and well
known role in defense signalling within plant tissues. No similar investigations have
been carried out targeting the importance of the jasmonate pathway or any other
plant hormone pathway in EM-network-facilitated signal transfer. This part of the
thesis aimed to re-assess the ability of EM networks to facilitate signal transfer in
the context of gene expression responses, and to further our understanding of the
defense pathways involved. To address these general objectives, I performed a
greenhouse study involving paired Douglas-fir trees with a gradient of belowground
connectivity with potential for transfer through (1) soil water only, (2) mycorrhizal
network and soil water, and (3) roots, mycorrhizal network, and soil water. After
treatment (or not) of methyl jasmonate (MeJA) to donor seedlings, I measured JA-
and ET-pathway-related gene expression of both donors and receivers, and
morphotyped all seedling roots for EM structures to examine mycorrhizal network
potential.

A comprehensive overview of the thesis objectives is provided following the
game literature review, presented below.
1.3: Shroomroot: an educational, simulation-style, digital game to enhance postsecondary teaching and learning about mycorrhizal networks

1.3.1: The importance of learning about mycorrhizal ecology

Mycorrhiza and MNs are a crucial part of ecosystem functioning around the world. Our developing understanding of mycorrhizal ecology is changing the way we practice forestry (Schwartz et al., 2006, Azul et al., 2014, Puettman et al., 2009) and changing the way we think about the balance of relationships between species (van der Heijden and Horton, 2009; Simard et al., 2015). Future forest managers, forest ecologists, and soil scientists will be more effective and adaptive if they can develop a tangible and holistic understanding of mycorrhiza and MNs. In addition, in an era of global change and renewed acknowledgement of the importance of our interactions with natural systems, fostering better understanding of ecosystem dynamics is important for all members of the general public (which all undergraduates are and will continue to be).

Mycorrhizal systems are inherently complex, and practically impossible to interact with in reality. One can dig up mycelium or mycorrhizal root tips and look at them, but growth of hyphae through the soil, resource-seeking, resource-exchange, and other important processes cannot be viewed in real-time or in many cases in any real way at all.

In addition to the challenges presented by mycorrhizal ecology as a subject area, plant and fungal biology tend to be less appreciated across natural science
disciplines, with postsecondary textbooks biased towards animals and with enrollment in botany-related majors dropping (Uno 1994, Uno 2009). “Plant blindness” is a term coined by Wandersee and Schussler (1999) for this underappreciation of plants. Initially attributed to biases in textbooks and curricula, there is new evidence that this may be the result of cognitive or perceptual biases and not purely a cultural phenomenon (Schussler and Olzak, 2008; Balas and Momsen, 2014). While botany competes with zoology for the spotlight, “fungal blindness” is not a term in general use (Googling it reveals one hit - a person on twitter mentioning it), perhaps indicating an even deeper level of underappreciation for mycology.

This paints a dour picture of interest around plant-fungal interactions, but the specific field of mycorrhizal-mediated plant communication has recently captured the attention of the general public, with new popular science articles (Pollan’s “The Intelligent Plant”, 2013; Cossins’s “Plant Talk”, 2014), books (Wohlleben, 2015), and TED talks (Paul Stamets in 2008, Suzanne Simard in 2016) discussing the topic. The rising popularity of this field makes now a good time to develop engaging resources presenting the basic principles of mycorrhizal ecology, both to teach basic mycorrhizal concepts in their own right, but also to inform and enrich discussion about MN-mediated plant communication. Fervor around mycorrhizal networks also has the additional potential to encourage general interest in plants and fungi.

For a subject area that involves the dynamic interplay of underappreciated living organisms that interact on effectively inaccessible timelines and scales – an
interesting potential tool is the digital game. Digital games can provide experiential learning about systems that are otherwise inaccessible, and multiple studies have demonstrated their potential to increase learning and engagement with previously challenging or complex content (reviewed in Li and Tsai, 2013; Boyle et al., 2016).

1.3.2: Digital Game-Based Learning

The use of digital games in educational settings was initially controversial due to associations of violence and a-sociality, but there is now general consensus on the potential for digital games to contribute positively in educational settings (Boyle et al., 2016). Well-designed games have many of the main features of motivated learning, including challenge, fantasy, rules, and curiosity (Malone and Lepper, 1987; Garris et al., 2002), and many games are inherently structured as learning experiences – the game must teach the player about the game world by slowly presenting new principles, skills and terminology in progressively challenging ways (Gee, 2008). Games can affect multiple kinds of outcomes, including but not limited to affective outcomes – regarding student engagement and attitudes – and knowledge acquisition outcomes – regarding the learning of content by students. In many studies, games used in education show a positive impact on student affective outcomes, increasing student engagement toward the subject area (e.g. Wrzesien and Alcañiz Raya, 2009; Hwang et al., 2013). There are also many cases in which digital games have been shown to have a positive impact on student cognitive outcomes, with an increase in knowledge acquisition over traditional
Game structures can be quite diverse, directly affecting student experience with the game and therefore educational outcomes. One way to distinguish games is by their genre, and a common framework used is from Herz (1987). There are, however, many other frameworks for categorizing game genres (Wolf, 2000; Arsenault, 2009) and the task of categorizing games has been considered by some to be subjective (Penelle et al., 2008). As seen in recent systematic reviews by Connolly et al. (2012) and Boyle et al. (2016), game genres are also often divided into many categories. Rather than select a categorization framework and describe each genre individually, here I briefly define a few selected traits that can be used to distinguish digital game structures, primarily drawing on trait descriptions in Rieber (1996), Garris et al. (2002), and Paul Gee (2008). Throughout the discussion, I reference some commonly used game genres, but focus primarily on distinguishing games in a trait-based manner.

**Game traits**

As mentioned previously, fantasy is one of the main motivational aspects that digital games are well-positioned to provide. Fantasy can take two main forms – endogenous or exogenous (Rieber, 1996, Garris et al., 2002). Games with exogenous fantasy are situated in a world that is unrelated to the content being presented. An adventure game that involved a storyline about fighting enemies with sword and shield but presented concepts about chemistry would be exogenous fantasy (many
math games have exogenous fantasy). In contrast, if the same adventure game were
designed to present content about medieval battle practices, and players had to
make historically relevant decisions about equipment, fighting moves, etc.
throughout the course of the game – that would be an endogenous fantasy.
Endogenous fantasies are typically seen as preferable, because the connection
between fun and learning is more direct (Rieber, 1996), but exogenous fantasy can
also be a successful tool (e.g. Kebritchi et al., 2010).

Another trait is whether or not the player’s perspective is a “bird’s eye view”
or an experience-oriented first- or third-person view (Paul Gee, 2008, Huang et al.,
2013). Games that allow players to manipulate multiple components of a system,
such as simulation games like SimCity or Age of Empires, are often from the bird’s
eye view perspective. Rather than playing as a particular individual, players make
decisions and work with concepts at a system level, aiming for system-level goals.
Experience-oriented games or role-playing games tend to be first or third person,
and allow a player to more directly inhabit a single perspective or character (e.g.,
Fallout, Super Mario). These games require the player to make decisions and
understand concepts at this smaller scale. Neither of these styles is necessarily
better than the other, but emphasize different aspects of target content and player
experience (Gee, 2008).

Another key motivational aspect of games is challenge (Garris et al., 2002),
which can also take many forms, from cognitively-oriented to action-oriented (such
as in Feng et al., 2007). Puzzle games tend to present challenges as cognitive
problems that require solving, with no time limit or physical dexterity involved. On
the other end of the spectrum, action games require players to address challenges that are primarily physical in nature – using the game’s physical controls to react quickly and accurately to virtual stimuli (e.g., Mario Cart, Guitar Hero). The same game can involve both puzzles and action, and require involved problem-solving while also requiring skilled manipulation of physical controls to carry out tasks. Again, neither style is necessarily better, but each emphasize different aspects of the gameplay experience and encourage mastery of different skills.

This is a restricted description of specific traits that can be attributed to digital games, but presents a backdrop with which to discuss the use of digital games to present fundamental concepts in mycorrhizal ecology. Games that are bird’s eye view and are cognitively-oriented have been more commonly studied in the educational game literature (Connolly et al., 2012, Boyle et al., 2016), but success in knowledge and affective outcomes have also been found in action-based, experience-oriented games (Feng et al., 2007, Bailey and West, 2013). Previously studied ecological games have been few, and have been almost entirely cognitively-oriented and from the first- or third-person, either as an animal or human (Sarab et al., 2009; Hickey et al., 2009; Schaller et al., 2009; Wrzesien and Alcañiz Raya, 2010). For example, Schaller et al. (2009) presented and evaluated the game Wolfquest, in which players control a lone wolf exploring a 3D world and learn how to survive as a wolf – hunting elk, finding a mate, cooperating with packmates, and other activities. They found an increase in players’ knowledge of wolves and emotional connection to wolves, reaffirming the potential for success in first-person-oriented ecologically-focused games.
There has been widespread popularity of simple action-oriented entertainment games like Angry Birds, to the extent that there is now a major motion picture about Angry Birds (and a trilogy of movies planned for Tetris). These games can be easy to begin playing, and tend to reach a broader audience than digital games previously did (particularly adults and females; ESA, 2015). Even in these simple games, learning about the game world occurs – in order to play, game rules, mechanics and distinguishing symbols have to be learned, and are learned rather automatically. Simple action-based games tend to be less studied as educational tools (Boyle et al., 2016), but are an interesting avenue for presenting basic concepts and terminology in an engaging and easily accessible way.

For ecology, simulation games can be successful at promoting learning (e.g., Sarab et al., 2009, Hickey et al., 2009) but they can also be overly complex. Wrzesien and Alcañiz Raya (2010) found that student learning was not significantly improved in the game group over traditional instruction, when the cognitive load was too high. “Cognitive loading” refers to how much mental effort is used to interpret and understand a certain learning material or interactive lesson (Sweller, 1994; Kalyuga, 2007). If a game presents too much information in the form of rules and game dynamics, the effort of learning how the game works can overwhelm content learning. This means that digital game design must be carefully balanced to create desirable features such as challenge and fantasy, but also simple enough to allow students to focus on content learning.
Cognitive load does not come only in the form of obviously cognitive tasks – physics-based action games that require careful manipulation of controls also require additional cognitive effort that can impede learning (Kalyuga, 2007). Even for what appears to be a simple game, a balance must be struck between the challenge of the physical aspects of the game and the complexity of concepts introduced. For simple, action-based games to be used in an educational setting, it is crucial that most of the gameplay and symbolism is directly relevant to the content to be learned, so that the use of mental effort is as efficient as possible.

To explore the ability of an action-based, plant-centric game to increase student understanding of and attitudes towards mycorrhizal ecology, I co-created and evaluated the simple, experience-oriented action game called Shroomroot.

1.3.4: Shroomroot

Shroomroot is an action-based game that allows students to “be a root” and connect with some of the more animal-like aspects of plant behavior, such as finding and collecting nutrients underground, deciding whether to interact with fungi as part of a mycorrhizal symbiosis, and distinguishing between beneficial versus costly interactions with other plants in the mycorrhizal network. Learning is meant to happen in somewhat subtle ways, in which the student learns by “doing” and success in the game depends on immediate feedback and visual, physical understanding of the dynamics of the root-fungus system. Shroomroot also has an achievements system that allows players to unlock “rewards” after reaching certain goals within the game (for example, collecting 30 units of a given resource), and
these rewards contain additional content information in the form of pictures and text.

In this thesis, I briefly describe development of Shroomroot, summarize Shroomroot as a game, and present results from an exploratory evaluation of the game with respect to learning acquisition and student engagement using a quantitative pre-test/post-test design.

1.4: Thesis objectives

This thesis aims to investigate the nature of defense signal transfer between Douglas-fir seedlings via EM systems (Chapter 2) and to evaluate student affective and learning outcomes of the mycorrhizal-focused game, Shroomroot (Chapter 3).

The objectives within the investigation of MN-mediated defense signal transfer via EM networks were to:

(1) determine whether mesh treatments allowing formation of MNs would result in a significantly higher defense response in receiver seedlings in MeJA-treated pairs relative to treatments without MNs, and

(2) determine whether this defense response is more pronounced in AOC, the jasmonate pathway gene, or in ACS2, the ethylene pathway gene.

The objectives within the creation and evaluation of Shroomroot were to:
(1) build Shroomroot, an action-based, plant-centric game that is situated in the world of belowground ecology and aimed for lower level postsecondary curriculum in an introductory soil science course.

(2) perform an exploratory evaluation of Shroomroot as an educational tool in a 2nd year Introduction to Soil Science course using a quantitative pre-test/post-test design. Within this second objective, there are four sub-objectives, which are to:

(2a) assess the impact of Shroomroot on student knowledge acquisition about mycorrhiza.

(2b) assess the impact of Shroomroot on students’ attitudes towards learning about mycorrhizal content.

(2c) explore the relationship between knowledge acquisition and content attitudes.

(2d) to explore metrics related to student game play (student gameplay proficiency and engagement) and how these relate to knowledge acquisition and content attitudes.
Figure 1.1: Diagram of (a) jasmonate (JA) and (b) ethylene (ET) defense pathways. Substrates are in boxes, enzymes are listed in italics next to arrows. Dark boxes on the left contain main steps of the JA pathway, including immediate downstream steps; light box on the right indicates the main steps of the ET biosynthesis pathway. An asterisk along each pathway marks the step targeted by the genes in this pathway – AOC for the JA pathway and ACS (specifically ACS2) for the ET pathway. Grey ovals show steps along the jasmonate pathway at which transgenic tomatoes in Song et al. (2014) were dysfunctional. spr2 plants cannot produce ω-3 fatty acid desaturase, preventing the release of lipid substrate for biosynthesis of α-linolenic acid (Li et al., 2003); jai1 plants are unable to produce coronatine-insensitive1, which triggers multiple downstream defense responses (Li et al., 2004). Linkage between JA and ET pathways is shown by the presence of JA-ACC, a conjugate of jasmonic acid and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). Diagram was made based on information from Ralph et al. (2006), Wasternack and Hause (2013), Van der Poel and Van Der Straeten (2014), and Booke et al. (2015).
Chapter 2: Investigating defense-related signal transfer between Douglas-fir via ectomycorrhizal networks

2.1: Introduction

Mycorrhizal networks (MNs) are webs of fungal mycelia (analogous to the roots of a plant) that connect plant roots belowground. MNs are considered common in forest and other ecosystems worldwide, and may be important to forest dynamics and resilience (reviewed in Simard et al., 2012). The fungi that make up MNs form a symbiotic relationship with plant roots in which the fungus forages for soil nutrients to exchange for photosynthetic carbon with their partner plants. MNs arise when the same fungus also colonizes the neighbor’s roots, and hence links the plants together. A new and exciting discovery about MNs is their ability to shuttle not only water, carbon, and nutrients between plants, but also informational chemical signals related to defense and competition (Song et al., 2015, Song et al., 2010; Barto et al., 2011; Babikova et al., 2013). The shuttling of informational signals is considered by many to be a form of plant communication (Heil and Karban, 2010). Communication is defined by the Oxford dictionary as “The imparting or exchanging of information by speaking, writing, or using some other medium.” In the case of defense-related interplant signalling, defense-related information is being exchanged between different individuals.

Plant communication is known to occur through the air (reviewed in Das et al., 2013) and through the ground (Chamberlain et al., 2001; Dicke and Dijkman, 2001; Falik et al., 2011; Barto et al., 2012), and there is building evidence that MNs
may facilitate plant communication via belowground pathways. Barto et al. (2011) found that plants connected by an MN showed a greater ability to harm each other with antagonistic allelochemicals, both synthetic and natural. The MNs in their study were formed by arbuscular mycorrhizae (AM), which can have different ecological properties than ectomycorrhizal (EM) fungi (Smith and Read, 2008). Arbuscular mycorrhizae typically penetrate the cell walls of their hosts, forming intricate nutrient exchange structures in between the cell wall and the plasma membrane called “arbuscules.” EM fungi have the prefix “ecto-” for their tendency to remain outside their hosts’ cell walls, growing in between the cells of the epidermis of the root. This in-growth of hyphae between the root cells forms the “Hartig net”, while other hyphae covering the outside of the root cells form the “mantle” (Smith and Read, 2008).

Specifically with respect to defense warning signals, only a few studies have investigated the effects of MNs on belowground communication (Song et al., 2010; Babikova et al., 2013; Song et al., 2014, Song et al., 2015). Song et al. (2010) first showed this effect, investigating whether diseased AM tomato plants (Solanum lycopersicum) could transfer defense signals through MNs to neighboring, healthy conspecific plants, thus providing an early warning system to prepare for pathogen infection. They grew pairs of tomato plants separated by mesh barriers, the different sizes of which controlled for the development of a MN. They inoculated MN treatments with the AM mycorrhizal fungus (Glomus mosseae) and did not inoculate non-MN treatments. Separating paired plants aboveground with airtight bags, they treated one tomato plant (the donor) with tomato blight (Alternaria solani) and
observed regulation of downstream defense genes and related enzymes in the neighboring plant (the receiver). Their findings indicated that the presence of a MN significantly facilitated the belowground transfer of defense signals from infected plants to healthy ones, with most increase in gene expression and enzymatic activity beginning between 18 and 65 hours after treatment of donor plants.

To more deeply explore the nature of this defense signal transfer, Song et al. (2014) investigated the involvement of the jasmonate pathway in MN-mediated interplant signal transfer. The jasmonate pathway involves the synthesis of the phytohormone jasmonic acid (JA), which is a signalling molecule that triggers defensive responses to mechanical damage, necrotrophic pathogens, and herbivory (Wasternack and Hause, 2013). Due to JA’s role as a signalling molecule, and its structure as a relatively small molecule, JA is a promising candidate as a potential signal molecule moving via MNs. Song et al. (2014) performed a similarly structured experiment to the one described above, but with different combinations of transgenic tomato plants that were either “mute” in the jasmonic pathway (spr2 tomato mutants are unable to create Linolenic-acid, a precursor to JA (Li et al., 2002)) or “deaf” (in jai1 mutants, JA fails to trigger changes in gene expression and enzyme production(Li et al., 2002)). They showed that these mutations along the jasmonate pathway resulted in a failure of signal transfer, strongly suggesting jasmonate pathway, and potentially jasmonic acid, involvement.

Song et al. (2010 and 2014) and Babikova et al. (2013) focused on arbuscular mycorrhizal systems, while a recent study by Song et al., (2015) investigated this effect in an ectomycorrhizal system. Song et al. grew pairs of interior Douglas-fir
(Pseudotsuga menziesii var. glauca) and ponderosa pine (Pinus ponderosa) seedlings, and treated the 4-month old Douglas-firs with mechanical defoliation or insect defoliation. They measured a suite of defense enzymes (peroxidase, polyphenol oxidase, and superoxide dismutase) in both receiver and donor plants and found a significantly higher increase after around 24 and 48 hours in multiple defense-related enzymes where the plants were linked by a MN (i.e., in the mycorrhizal network treatment (35µm mesh)). In both the soil-only treatment and the treatment that allowed roots to interact (in addition to mycorrhizal hyphae), the receivers had lower defense enzyme activity. This provides the first evidence that this interplant signal transfer may occur in ectomycorrhizal networks, but whether the jasmonate pathway is involved in EM-mediated signal transfer has not previously been studied.

To delve deeper into defense signal transfer in ectomycorrhizal systems, I investigated the involvement of two main defense pathways in MN-mediated signal transfer between paired Douglas-fir seedlings: the jasmonate pathway and the ethylene pathway. The ethylene pathway was included for two reasons: (1) as a control, with the expectation that the jasmonate pathway would be more involved based on Song et al. (2015); and (2) to expand our observations of this phenomenon into another defense pathway. Established defense-related mutant lines of Douglas-fir do not exist, and so it was not feasible to perform an experiment similar to the transgenic tomato plant experiment in Song et al. (2014). Instead, to investigate the involvement of the JA and ET pathways, I measured gene expression in receiver and donor Douglas-fir seedlings in key regulatory genes closely upstream from JA and ET synthesis (JA and ET pathway diagram in Figure 1.1).
**Jasmonate regulatory gene: AOC**

In conifers, one of the best characterizations of defense-induced expression upstream of jasmonate biosynthesis was done in Sitka spruce (*Picea sitchensis*) by Ralph et al., (2006). They showed that allene oxide cyclase is the most directly upstream regulated step in jasmonic acid synthesis. AOC is the first step of the octadecanoid pathway which produces jasmonic acid, converting 12,13-epoxy-octadecatrienoic acid into 12-oxo-phytodienoic acid (or OPDA). Ralph et al. (2006) confirmed that further steps on the octadecanoid pathway leading to JA synthesis were not regulated in Sitka spruce, and expression was likely constitutive. To target AOC in Douglas-fir, I worked with the putative AOC transcripts from the Douglas-fir transcriptome (Howe et al., 2013).

**Ethylene regulatory gene: ACS2**

Defense-induced expression of genes involved in ethylene synthesis was described in Douglas-fir by Ralph et al. (2007), who found that aminocyclopropane carboxylic acid synthase 2 (ACS2) was the regulated step most directly upstream of ethylene synthesis. ACS2 converts S-adenosyl methionine into 1-aminocyclopropane 1-carboxylic acid (ACC), which is then converted by ACC oxidase (ACO) into ethylene (reviewed in Rodriguez et al. 2014). ACO is not responsive to defense stimulus by wounding or herbivory and is therefore not considered a regulated step in ethylene synthesis (Ralph et al., 2007).

Most previous studies in interplant communication via MNs have focused on natural stimuli – either mechanical wounding or application of pathogens or herbivores. Methyl jasmonate (MeJA) is the volatile form of jasmonic acid, and has
been used in many studies to induce a defense response in conifers comparable to responses to natural stimuli (Moreira et al., 2009, Moreira et al., 2012). In their experiment with transgenic tomato plants, Song et al. (2014) used MeJA to stimulate a defense response in donor tomato plants as a way to target the jasmonate pathway. As a simple chemical application, it can be easier to work with and control dosage of MeJA, in contrast to application of pathogens or herbivores.

The objective of this study was to investigate the potential role of mycorrhizal networks as pathways for defense signaling between injured and healthy Douglas-fir seedling neighbors. I tested two main hypotheses. The first hypothesis was that mesh treatments allowing formation of MNs would result in a significantly higher defense response in receiver seedlings in MeJA-treated pairs relative to treatments without MNs. The second hypothesis was that this defense response would be more pronounced in AOC, the jasmonate pathway gene than in ACS2, the ethylene pathway gene. The jasmonate pathway includes positive feedbacks in which the synthesis of jasmonic acid can be stimulated upstream by jasmonic acid (Wasternack, 2007), and the expectation was that, as a regulated step in jasmonate synthesis, AOC would be more stimulated than ACS2 if jasmonic acid signals were moving across the MN.

To test these hypotheses I performed a greenhouse experiment investigating whether defense-signaling between EM plants involves the jasmonate or ethylene pathway, or both. To do so I grew pairs of Douglas-fir seedlings in pots separated by differently-sized mesh barriers that control the formation of a MN (Figure 2.1). Similar to Song et al. (2010), after sufficient growth of the seedlings, I isolated the
airspace of individual plants aboveground and applied defense-inducing methyl jasmonate (MeJA) to a “donor” seedling and measured defense-related gene expression in both the donor and “receiver” seedling. To infer the likelihood of a common mycorrhizal network between paired seedlings, I also morphotyped root tips on all seedlings for mycorrhizal identification. Finally, to confirm that mesh barriers did not have a substantial impact on seedling foliar nutrient concentrations, I measured the foliar nutrient concentrations in a subset of seedlings.

2.2: Methods

2.2.1: Experimental Setup

The greenhouse experiment was carried out in a 3x2x2 completely randomized design with paired donor and receiver seedlings and 3 biological replicates per treatment combination (72 seedlings total). The main factors involved three mesh sizes (0.5µm mesh, which only allows transmission of soil water; 35µm mesh, which also allows development mycorrhizal networks but not passage of roots; and 250µm mesh, which also allows passage of plant roots), two time steps (24 hr and 48 hr), and two hormone treatments (MeJA and control). The two time steps were selected based on the timing of most activity for defense responses in receiver plants that have been seen in other studies in gene expression (Song et al., 2010, Song et al., 2014), enzymatic activity (Song et al., 2010, Song et al., 2015), and release of defense-related volatile organic compounds (Babikova et al., 2013), which
in the respective studies tended to show strong differentiation from control receiver responses within the 24hr to 48 hr time window.

The study was performed in the UBC Horticulture greenhouse under a consistent temperature (day 20-24º, night 14-16º), moisture (2-18 HD), and lighting regime (16H days). Pot locations were randomized once every month to control for any spatial variation in greenhouse conditions (such as shading or edge effects).

Soil was collected from Interior Douglas-fir forest near Kamloops, BC, and is a Dystric Brunisol with a sandy clay loam texture. The soil was kept at 4º C for 4 months prior to planting. At planting, the wild soil was mixed with sterile potting soil to increase porosity and improve drainage, with a ratio of 70% wild soil to 30% potting soil. Mycorrhizal inoculum comes from the spores and hyphae present in the natural soil, and this is a reliable method of establishing a mycorrhizal network for ectomycorrhizal systems (Melanie Jones, pers. comm; Massicotte et al., 1994.).

Plastic one-gallon pots were filled with soil both inside and outside of paired mesh bags, such that there was soil also separating the two bags (Figure 2.1). Interior Douglas-fir seeds (Seedlot no. 39330, British Columbia Ministry of Forests and Range, Tree Seed Center, Surrey, BC) were planted five per mesh bag, and the surface of all pots was covered with a fine layer of silica sand with the goal of reducing seedling death from “damping off” fungus. Watering occurred daily for two weeks, then was steadily decreased to once every 8 days. The water regime was based on previous experience growing Douglas-fir in the UBC greenhouse and is designed to ensure healthy germination and survival, but to avoid excessive watering that would discourage seedlings from forming mycorrhizal associations.
Emergence was recorded for the first month of growth, until no new germinants emerged. Emergence was considered the moment seeds visibly rose above the sand layer.

2.2.2: Treatment and harvest

One year after planting, seedlings were subjected to hormone or control treatment and harvested for gene expression, ectomycorrhiza morphotypes, and foliar nutrient analysis. Seedlings were watered within 5 days before hormone treatment to ensure moist soil (to avoid water stress and closed stomata). Seedling characteristics were measured as close to hormone treatment as possible (about 24 hrs). Height (to the needle tip of the leader of highest apical branch) was measured. Due to unexpected spider mite (*Tetranychus urticae*) infestation at the time of harvest and potential effect on expression, the degree of infestation was rated. Spider mite infestation was rated from 0-3 (none, minor presence, moderate presence, extensive presence). Due to perceived variations in seedling vigour (based on colour, shape and orientation of needles, and presence of mites) at the time of harvest, a vigour rating was assigned to each seedling. The vigour rating is described in more detail below.

Vigour was measured using a qualitative index from 1-5, with 1 being very low vigour and 5 being high, taking into account needle colour, level of wilting, presence of spider mites, and presence of needle distortion. In borderline cases, intermediate values were assigned (e.g. 3.5). The index that was used is as follows:

1. Appearing to be in the process of dying (very wilted, brown needles that fall
off easily – no seedlings were in this rating

2. One or multiple signs of extensive needle distortion, yellow or red needles, wilting or spider mite presence

3. Only one of moderate needle distortion, yellow or red needles, wilting, or spider mite presence, or multiple signs of mild needle distortion, yellow needles, wilting, or spider mite presence

4. Only one of mild needle distortion, yellow or red needles, wilting, or spider mite presence

5. No needle distortion, yellow or red needles, wilting, or spider mite presence

**Treatment**

To prevent volatile communication between donors and receivers or neighbors in other pots, all seedlings were covered with plastic bags made from Foodsaver® vacuum sealing rolls. Bags were 27 cm wide by 56 cm tall and had an air-tight valve 30 cm above the base through which ambient air could be restocked to avoid suffocation of seedlings. Bags were sealed around seedling trunks using Craftsmart modelling putty (non-toxic) and Tuck® Contractors Sheathing Tape. Before hormone treatment of donor seedlings, receiver bags were inflated from the top with ambient air using an Intex 120 V wall plug-in air pump and sealed shut.

Donor seedlings received treatment following methods used by Richard et al (2000), without the ethanol (an earlier trial of 1-year old Douglas-fir seedlings encased in plastic bags showed that including ethanol resulted in dead seedlings after 24 hours, regardless of MeJA presence). Cotton swabs containing 50µl of 95%
liquid MeJA (Sigma-Aldrich) or dry cotton swabs (controls) were taped to the inside of donor seedling bags. Donor bags were inflated with ambient air, and sealed completely. All seedlings were kept in a well-lit and temperature-controlled zone of the greenhouse for the 48 hours of the experiment. To avoid seedlings running low on CO₂, seedling bags were filled with air by pumping ambient air through the valves using a 500ml syringe at the beginning and end of each day from when seedlings were bagged until harvest. Locations with respect to main factors were randomized, except that MeJA-treated seedlings were paired with a control-treated seedling in otherwise the same treatment combination (e.g. 250μm-24hr) to ensure that any hormone leakage or airborne MeJA contamination would affect both the MeJA-treated receivers and the control receivers. This ensured validity of relative comparisons between MeJA-treated and control receivers.

2.2.3: Harvest

Aboveground stem tissue was harvested after 24 hours or 48 hours and was separated into three main tissue types. Young, flushing foliage was separated from more mature tissue and discarded. While young needles would normally be the best tissue for gene expression, seedlings were not at consistent development stages and inconsistencies in tissue developmental stage could produce misleading gene expression results. Mature needles and stem tissue from lateral branches were separated, flash frozen into separate 50ml Falcon tubes, stored on dry ice, and moved within 6 hours to a -80 freezer to preserve RNA (only stem tissue was used in later gene expression work, as described below). Mainstem tissue and extra
lateral branch tissue was stored at 4º C for foliar nutrient assessment.

Roots were harvested for later morphotyping no more than 48 hours and 72 hours after initial donor treatment, as mycorrhizal tip structures were not expected to change within 24 hours and harvest of aboveground structures was time-consuming. Roots were removed from pots and washed clean of soil. Before removal from the pot, basal diameter of all seedlings was measured. Mesh bags were checked for penetration by roots. Clean, moist roots were placed into ziploc bags and stored at 4ºC.

2.2.4: Gene expression

*Douglas-fir AOC transcript selection*

As stated above, at the time of this study, there was an annotated Douglas-fir transcriptome, but no experimental evidence of the functional defense role of the allene oxide cyclase transcripts putatively annotated in the transcriptome (TreeGenes project, Wegrzyn et al., 2008). Based on results from Ralph et al. (2006) showing the successful amplification of AOC in response to weevil and budworm feeding in aboveground tissues of Sitka spruce, the Sitka spruce AOC transcript (WS0039_A09) used in Ralph et al. (2006) was blasted against the Douglas-fir transcriptome. Two distinct putative transcripts for allene oxide cyclase (isogroup01292 and ESTgi47150793) were the closest hits. Isogroup01292 was closer in the blast (eValue = 0) than ESTgi47150793 (eValue = 1.59487E-137), and ESTgi47150793 is a putative transcript for AOC4 (based on annotation provided by
the TreeGenes project), which in *Arabidopsis* is a gene copy of AOC that primarily expresses in roots (Stenzel et al., 2012). For these reasons, Isogroup01292 was the transcript targeted for measurement of AOC expression in my samples.

**RT-qPCR**

After thorough review of the conifer defense literature revealed that most studies focus on stem tissue, only stem tissue was used in gene expression analyses in this study to retain comparability to other work. Frozen stem tissue was ground in a TissueLyser II and RNA was extracted using the Sigma Aldrich Total Plant RNA kit, followed by DNase treatment using RTS DNase Kit by Mo Bio. RNA concentration was measured post-DNase treatment using a Nanodrop. Reverse transcription to create cDNA was performed using ThermoFisher’s High-capacity Reverse Transcription Kit, following kit instructions. cDNA was diluted as necessary to bring the solution to a 2ng/μl concentration. 10ng (5μl) of cDNA was added to final qPCR reactions. 20μl qPCR reactions were carried out using the Power SYBR Green Master Mix at the following ratios: 10μl SYBR, 0.2μl bovine serum albumin (BSA), 4.8μl H20, and 5μl of sample. qPCR was done in an Applied Biosystems StepOnePlus machine, with a thermoprofile of 95° for 10min, 45 cycles of 95°C for 15s, 60°C for 30s, 72°C for 30s, and then 72°C for 10min. The reference gene transcription elongation factor (TEF) was used to standardize target gene expression, and was selected based on its previous use as a housekeeping gene (Islam et al. 2010) and on a lack of differential response to hormone treatment in selected samples in this study. Four technical replicates were performed and the best (i.e. where most samples amplified
successfully in target amplicons) 2 or 3 runs were used in analysis. Success in amplifying the desired target and reference genes was confirmed for selected samples by sequencing through NAPS (UBC, Vancouver, BC), and for all samples by melt curve analysis.

Primers were designed using the BLAST primer designer and Primer3 software, with a Tm of between 57 and 61°. Four primer pairs (forward and backward) were tested across a selection of representative samples (control and MeJa-treated donors). The primer pair with the most consistent amplification, lack of multiple bands showing on gels following regular PCR and melt curves following qPCR, and strongest sequence results were chosen for use in the final analysis. Primers for ACS2 were designed based on the ACS2 gene described in Douglas-fir by Ralph et al. (2007). Primers for AOC were designed based on two distinct putative AOC transcripts from the Douglas-fir transcriptome (Howe et al., 2013). Primers for TEF were copied from Islam et al., 2010) and were confirmed for success with the samples in this study. Primer information is shown in Table 2.1.

Raw fluorescence data (Rn, before baseline-correction) were imported into LinRegPCR to calculate amplicon-specific PCR efficiency and corresponding starting concentration (N0) and Cq values. Samples with failed amplification were assigned a crossing point (Cq) value of 45, which was the maximum number of cycles. After averaging technical replicates, relative expression of target genes was calculated using the delta-delta Ct (ddCt) method after calibrating for amplicon-specific PCR efficiency (Livak and Schmittgen, 2001; Ruijter et al., 2009). This ddCt method calibrates target gene expression against the reference gene (TEF), and then takes
the ratio of the expression of seedlings in the MeJA treatment to paired control seedlings to generate expression values that represent the fold-change of expression in MeJA-treated seedlings (and receivers connected to MeJA-treated donors) compared to control seedlings.

2.2.5: Morphotyping

Roots were examined for mycorrhizal structures on all seedlings (Goodman et al., 1998). 50 root tips were randomly sampled per seedling and these were sorted into unique ectomycorrhizal structures (morphotypes). Representatives of each morphotype will be sent for DNA sequencing to confirm mycorrhizal species identifications, but due to this process being incomplete, these results are not included in this thesis.

2.2.6: Foliar nutrients

To investigate effect of mesh bag treatments on foliar nutrient concentrations, foliar nutrients were measured in a subset of seedlings, with 6 seedlings per mesh treatment. Analyses done targeted key macronutrients (by combustion elemental analysis) and micronutrients (by VHP closed vessel microwave acid digestion). Macronutrients measured were C, Ca, K, Mg, N, P, S_ICAP, and S_Comb. Micronutrients measured were Al, B, Cu, Fe, Mn, Na, and Zn. Analyses were performed by the British Columbia Ministry of Environment Analytical
Chemistry Laboratory, Victoria, BC. Foliar nutrient results are reported in the Appendix.

2.2.7: Data analysis

Relative expression values were log-transformed to meet normality and variance assumptions. All comparisons between relative expression values, seedling characteristics, mesh pore size, and mycorrhizal colonization were performed using ANOVA when variables were continuous (post-transformation, in the case of gene expression data) and Fisher’s exact test when variables were ordinal. Mycorrhizal morphotypes were also compared among mesh sizes using non-metric multi-dimensional scaling (NMDS) and vigour was tested for significant correlation with the ordination. Donor and receiver “dCt” expression, as opposed to “ddCt” expression (i.e. before taking the ratio of MeJA-treated expression to control expression) was also compared using non-linear regression. All analyses were performed in R Studio (R version 3.2.2, R Core Team 2015).

Results are split into two main groups. Descriptive statistics about survival and germination, as well as relationships between mesh and other seedling health variables, were performed using three replicates of each treatment combination and across donors and receivers (n=72). Gene expression results are limited to a subset of seedlings, with only duplicates for the donor seedlings. Two receiver seedlings failed to be amplified in either the reference or the target genes, and one donor seedling failed to be amplified in ACS2. These samples were removed from gene expression analyses (n=34 receivers, 23 (ACS2) or 24 (AOC) donors). All gene
expression means are reported as fold change unless otherwise stated, and all means are reported ± the standard deviation.

2.3: Results

2.3.1: Seedling characteristics

Emergence and size

Overall average emergence was 75%, and the 250µm treatment had significantly lower emergence (66%) than the 0.5 and 35µm treatments (78%, p=0.02; 79%, p=0.01, respectively). Seedling mean height was 33.7cm±8.32 and mean basal diameter was 5.28mm±0.95. Height did not differ significantly across mesh sizes (p>0.05), but basal diameter was lower in the 250µm (4.89±1.05mm, p=0.0026) and 35µm (5.16±0.80mm, p=0.04) treatments than in the 0.5µm (5.80±0.79mm) mesh treatment (p-values adjusted with TukeyHSD, ANOVA Table 2.2). Height:diameter ratio tended to increase with mesh size, with 250µm treatment (6.72±1.79) tending to have a higher ratio than the 35µm (6.42±1.27) or 0.5µm (6.11±1.03) treatments.

Vigour and spider mite presence

The average vigour rating across all seedlings was 3.87±0.62. An average of 71% of seedlings had a vigour rating of 4 or above, 26% had a vigour rating of 3 or 3.5, and only 3% (2 trees) had a rating of 2.0. The distributions of vigour ratings among mesh pore sizes was significantly non-random based on Fisher’s exact test
Frequency of lower vigour ratings increased with decreasing mesh size, with 54% of seedlings in 0.5µm mesh being rated 3.5 or lower, versus 25% in 35µm mesh and 8% in 250µm mesh. The distribution of differences in vigour rating between paired donor and receivers among mesh sizes was also significantly non-random (p=0.0006). The 0.5µm mesh treatment had no donors and receivers with the same vigour rating, while in both treatments that allowed networks – 35µm and 250µm – 58% of donor/receiver pairs had the same vigour rating (Figure 2.2). A summary of vigour frequencies across different mesh sizes is shown in Table 2.3.

Spider mites were found on 81% of seedlings, and their abundance was moderate or extensive on 71% of seedlings. There was no significant relationship between mesh size and spider mite severity (p>0.05).

2.3.2: Mycorrhizal network

All seedling donor and receiver pairs had at least one instance of common mycorrhizal morphotypes. Estimated mycorrhizal colonization of root tips averaged 59% (range: 6% to 80%). Among mycorrhizal root tips, the majority (54%) were morphotypes suspected to be mycorrhizal fungi primarily in the group Pyronemataceae. The next biggest group of morphotypes were harder to distinguish in terms of color, being from dying or dead roots – but morphologically were also similar to Pyronemataceae. Morphotypes suspected to be from the genus Rhizopogon were the next most abundant group, at 6%. A small number of morphotypes were suspected to be Cenoccocum (0.2%) and others were unknown or in transition but showed definite signs of affecting root morphology (2%).
Mycorrhizal colonization was significantly associated with vigour, with higher colonization associated with higher vigour ratings (p=0.003, adj $r^2=0.10$, Figure 2.3). Percent mycorrhizal colonization was also significantly higher in the 250µm mesh treatment (66%±12%) than in the 35µm treatment (55%±15%, p=0.02) and non-significantly higher than the 0.5µm treatment (57%±12%, p=0.07, ANOVA Table 2.4).

The NMDS for morphotypes versus mesh size is shown in Figure 2.4. There is substantial overlap between samples of different mesh sizes, but based on the centroids of each mesh type, there is some separation in mycorrhizal community structure particularly between the 0.5 and 250µm mesh sizes. The 0.5µm mesh samples are more closely associated with non-mycorrhizal root tips, while the 250µm mesh seedlings are more associated with Rhizopogon-like morphotypes. Vigour was significantly correlated with the distribution of morphotypes (p=0.004, blue arrow in Figure 2.4).

### 2.3.3: Belowground root interactions

In 250µm mesh treatments, all but one seedling demonstrated root penetration through the mesh bag. 28% of 0.5 and 35µm treatment seedlings had small roots growing through the seams at the bottom of the mesh bags, but in only one case were both seedlings in the same pot exhibiting this behaviour. This one case was a 0.5µm control donor seedling that was itself not measured for gene expression, though its partner receiver was. This receiver was not an outlier (nor close to being one) in terms of gene expression of either target gene.
2.3.4: Gene expression

Allene oxide cyclase (AOC)

Relative donor AOC expression averaged a 6.89 fold increase in MeJA-treated donors relative to control donors (p=0.044). Relative expression was not significantly related to time step or mesh treatment (p>0.05), but average relative expression was insignificantly higher in the 48 hr time step (12.06 fold-increase) than the 24hr time step (3.93 fold-increase). Receiver AOC expression was not significantly increased in receivers paired with MeJA-treated donors (4.18 fold-increase, p>0.05). Without removing outliers, no other main factor was significantly related to receiver expression. After removing one major outlier (Cook’s distance >0.5), time step was significant (p=0.041). No pairwise comparisons were significant with or without the outlier, but as in donors, the 48 hr relative expression (1.10 fold-increase) tended to be higher than the 24 hr relative expression (0.25 fold decrease). Receiver seedlings in the 0.5µm mesh treatment tended to have higher mean AOC expression (1.15 fold increase) than those in the 250µm (0.56 fold decrease) or 35µm (0.20 fold decrease) treatments. AOC relative expression across the main factors is shown in Figure 2.5(a and b), and a summary of ANOVA results can be found in the Appendix (Donors: Table A2.1, Receivers: Table A2.2).

Receiver dCt AOC expression (0.01, arbitrary units, before taking the MeJA/CTL ratio) across factors was not significantly different than either control
(0.004) or MeJA-treated (0.03) donor dCT AOC expression (p>0.05). After log transformation, donor dCt expression across factors was significantly positively related with receiver dCt expression in a non-linear fit (p=0.03, adj $r^2=0.16$, Figure 2.6).

**Aminocyclopropane carboxylic acid (ACS2)**

Relative donor ACS2 expression averaged a 4.48 fold increase in MeJA-treated donors relative to control donors (p=0.028), but was not related to time step or mesh treatment (p>0.05). Seedlings in the 48 hr treatment tended to have higher relative expression (23.10 fold increase) than those in the 24 hr treatment (2.25 fold increase). Relative receiver ACS2 expression was not significantly affected by any main effect or treatment combination (p>0.05). The 0.5µm mesh treatment tended to have higher expression (1.47 fold increase) than the 250µm (1.17 increase) or 35µm (0.81 fold decrease) mesh treatments. There were no major outliers (Cook’s distance <0.5 for all samples). ACS2 relative expression comparisons across the main factors are shown in Figure 2.7(a and b), and a summary of ANOVA results can be found in the Appendix (Donors: Table A2.1, Receivers: Table A2.2).

Receiver dCt ACS2 expression (0.001, arbitrary units, before MeJA-CTL subtraction) across factors was not significantly different than control (0.0004) or MeJA-treated (0.003) donor dCT ACS2 expression (p>0.05). No significant relationship was found between across-factor Donor dCt ACS2 expression and receiver dCT ACS2 expression.
**Covariates**

Due to the relationships between vigour, mesh, and mycorrhizal colonization, vigour and mycorrhizal colonization were tested as covariates in the models for donor and receiver expression. Mycorrhizal colonization did not contribute significantly to any of the models. In the case of AOC relative expression in receivers, including MeJA seedlings’ vigour ratings did improve the model (p=0.01, AIC of 30.6 vs 41.5), but did not change the results. Time step was the only significant term in the model, but even after correcting for vigour, the 24hr and 48hr time step did not differ according to pairwise comparisons.

**2.4: Discussion**

Donor seedling expression was higher in both genes following hormone treatment, suggesting successful stimulus of a defense response in both the ethylene and jasmonate pathways by volatile MeJA treatment. Evidence for the transfer of defense signals to receivers was not seen, as receiver expression was not differentiated by hormone treatment of donors. A significant positive correlation was found between donor and receiver AOC dCt expression (across both control and hormone-treated seedlings). There were mycorrhizal morphotypes in common between all donor and receiver pairs, suggesting potential for mycorrhizal networks to form in the 35µm and 250µm mesh treatments. Mycorrhizal colonization and mesh size were significantly related to the vigour of seedlings, suggesting greater network-forming potential may have improved seedling health.
Hypothesis 1: Evidence for signal transfer differentiated by networks

My first hypothesis, that receiver seedlings would express a significantly higher defense response when networked with donor seedlings than when isolated, was not supported. Relative receiver expression of neither the jasmonate pathway gene, AOC, nor the ethylene pathway gene, ACS2, was differentiated by hormone treatment of donors. After removal of an outlier, AOC expression was significantly related to time step, suggesting a change in AOC expression through time. In both genes, receiver seedling expression was not significantly different from control or MeJA-treated donor seedlings, suggesting receiver seedlings were undergoing a jasmonate response, but not one distinguished by MeJA-treatment of donors.

The results from this study are inconsistent with past work in MN-mediated interplant signal transfer studies (reviewed in Gorzelak et al., 2015, Johnson and Gilbert, 2015). There are two potential explanations for this. The first is that some amount of signal transfer did occur, but was either not strong enough to stimulate a response in receivers or the response triggered was masked by a system-level stress response to spider mites or other stressors. The second possibility is that signal transfer did not occur, either due to the complex structure of the mycorrhizal network and/or soil system, or to a failure to stimulate a strong enough or correct type of response in donor seedlings.

Signal transfer may have occurred but have been masked by a system-level stress response to spider mites. If the pairs of potted seedlings were already stressed due to spider mites or other unseen agents, their ET and JA pathways may have been activated prior to the hormone treatment application. Spider mites have
been seen to activate both JA and ET biosynthesis pathways in other plants (Zhurov et al., 2014; Martel et al., 2015). Expression related specifically to MeJA treatment may have been somewhat masked by this ongoing stress response in both donors and receivers, but with direct treatment of MeJA on donors still causing a minimally visible increase in expression. In contrast, receivers would already be expected to show a lower response as a result of signal transfer compared to direct treatment by MeJA, and this lower response may not have been visible against already high stress levels. While receiver expression was not differentiated by hormone treatment, it was also not different from MeJA-treated donor expression for either gene, suggesting that receivers had activated JA and ET pathways regardless of hormone treatment in donors. In comparison, Song et al. 2015 found that receiver enzyme responses were around 50% of donor responses. In this study, the 7-fold increase in MeJA-treated donor expression AOC may have been too low to generate a response in receivers that could be detected above expression due to other stressors. This underlines the importance of having baseline expression measurements, at time step zero and/or pre-hormone treatment, to have a more robust understanding of treatment effects without confounding by external factors.

The increase in MeJA-treated donor expression relative to control seedlings (just under a 7-fold change in AOC and about 4.5-fold in ACS2) was lower than other AOC and ACS2 responses in conifers towards herbivores. Ralph et al. (2007) found that ACS2 was 48.2-fold higher than controls in two-year-old Sitka spruce trees 48 hrs after weevil-feeding. Similarly, Ralph et al. (2006) found expression of AOC, also in two-year-old Sitka spruce (*Picea sitchensis*), increased 34.11-fold due to weevil
feeding after 48 hours. In contrast, Yaqoob et al. (2012) found that 100mM of MeJA painted on a 1cm section of stem in 2-year-old Norway spruce trees had no impact on the expression of ACS. While not measuring gene expression, Lombardero et al. (2013) found that MeJA treatment failed to alter the terpene production of both mature Pinus pinaster and Pinus radiata trees, while a native herbivore, the pine processionary moth, had a significant impact on terpene production. This points to the potential for MeJA to have mixed, complex responses on conifer defense systems despite many previous studies showing similarity of chemical and physical defense characteristics (such as traumatic resin ducts, terpene synthesis, enzyme assays) between MeJA and natural defense threats (Miller et al., 2005, Moriera et al., 2012).

It is possible that while MeJA succeeded in stimulating the target genes in donor plants, it was not enough to mimic the natural level of defense response caused by pathogens or herbivores, and therefore failed to stimulate signal transfer where other studies in this area have succeeded. This underlines the importance of measuring the donor response in order to understand the context in which receivers are responding (or not) to treatment. It also points to the need for further research on the thresholds involved in these sorts of signal transfers – at what level of stress does a plant begin to impact its neighbors via MN-mediated signals?

The structure of the network and/or soil system may also have prevented, slowed down, or masked signal transfer. Support for the presence of a common mycorrhizal network was found in the form of common morphotypes between all pairs of donor and receiver seedlings. The majority of morphotypes were Pyronemataceae-like, with more minimal Rhizopogon-like morphotype presence.
Song et al. (2015) saw signs of successful signal transfer within 24 hours in a MN of entirely *Wilcoxina rehmi*. The structure of the MN in this study was therefore expected to have been sufficient to demonstrate signal transfer, but networks can be highly variable in structure and response. Resource transfer has been found to vary depending on many factors including environmental conditions, EM community structure, and current resource status of involved partners (reviewed in Simard et al., 2012), and it is likely that signal transfer depends on many factors as well. Networks are spatially diverse and constantly in flux, with changes in community composition and structure on short and long time scales (Tuffen et al., 2002; Johnson et al., 2005; Twieg et al., 2007). It is possible that there were aspects of the soil structure, invertebrate presence, or other unmeasured factors that caused the mycorrhizal networks that formed to be less capable of transporting defense signals in this study.

**Hypothesis 2: Comparison of AOC and ACS2 genes**

My second hypothesis, that the defense response would be more pronounced in the jasmonate than ethylene pathway, was only partially supported. Because there was no evidence for signal transfer, it is impossible to judge the influence of the ethylene versus jasmonate pathway on signal transfer. However, the positive significant relationship between donor and receiver seedlings in AOC expression indicates that there may be a relationship between donor and receiver jasmonate pathways or that donor and receiver jasmonate pathways are both responding to a common influence. Spider mites were common on all seedlings, and are known to
primarily stimulate a jasmonate response in angiosperms (Zheng et al., 2009, Zhurov et al. 2014). If the jasmonate pathway is involved in interplant signal transfer via ectomycorrhizal networks, as in arbuscular mycorrhizal networks (Song et al., 2014), the positive correlation in AOC expression may be due to bi-directional signal transfer between donor and receiver. In contrast, if the ethylene pathway is not directly involved in MN-mediated signal transfer, a lack of crosstalk between donor and receiver seedlings could result in uncorrelated expression of ACS2, especially while seedlings were closed-off from each other’s airspace aboveground. However, it is also possible that spider mites were stimulating a jasmonate response in all affected seedlings more strongly than an ethylene response. This could lead to shared donor and receiver responses due to similarity in spider mite severity by pot. Similarity in spider mite severity by pot was not perceivable statistically, but the metric for measuring severity was relatively crude.

2.4.1: Vigour and seedling size

Seedling vigour was significantly related to mycorrhizal colonization as well as mesh size. Improved vigour was likely a response to increased mycorrhizal colonization of roots in greater mesh sizes (250µm in particular) as well as the increased potential for mycorrhizal network formation, allowing the potential for cooperation and resource transfer between paired seedlings. Relative expression in receivers of both AOC and ACS2 tended to be highest in the 0.5µm treatment and lowest in the 35µm treatment suggesting that 0.5µm receivers may have been more stressed overall. This may have been a lasting effect of isolation from neighbours
throughout the growing period. Mycorrhizal networks have been seen to facilitate seedling survival and growth in laboratory settings (Bingham and Simard, 2011).

In contrast, basal diameter and emergence were both lower in the 250µm mesh size than in the other mesh treatments. Seedlings also tended to be sturdier in smaller mesh treatments, having a lower height to basal diameter ratio. Sturdiness has been seen in other studies to increase in response to greater spacing between trees (Wonn and O’hara, 2011) and may have resulted from a lack of or lower degree of root competition in the 0.5µm and 35µm treatments. The lower emergence in the 250µm treatment is somewhat mysterious given it occurs before one would expect any effects arising due to root competition. Other studies have found little to no short-term effect of mesh bags on soil characteristics (Teste et al., 2009, Asay et al., 2013), but perhaps in this study there were some early microclimate differences that affected seedling emergence.

2.5: Conclusion

My first hypothesis was unsupported and my second only partially supported. Receiver AOC and ACS2 expression did not increase in response to MeJA-treatment of donors nor was expression significantly related to MN presence. This may have been due to lack of signal transfer, to masking of signal transfer by spider-mite-driven defense responses, or to undetectable levels of signal transfer – either by this experiment or by the receivers themselves. However, donor AOC expression was correlated with receiver AOC expression across factors, while ACS expression was not. This may be due to an interaction between donors and receivers, perhaps
due to ongoing jasmonate signalling belowground, or may be the result of similarity in jasmonate responses of paired seedlings to spider mite presence.

In this experiment, I did not find evidence for signal transfer via MNs, highlighting the complexity of these systems. Measuring donor response is an important aspect of understanding both sides of these signal transfer systems, and can aid in interpretation of receiver responses. Including baseline sampling is also important and would have improved interpretation of my results in light of the ongoing stress response observed across seedlings in both control and MeJA treatments. Investigating thresholds for successful MN-mediated signal transfer may be an interesting future avenue for this field – especially given the importance of thresholds and non-linear behaviour in complex adaptive systems such as this.

I have demonstrated successful defense stimulus by MeJA of a putative AOC transcript in Douglas-fir (isogroup01292), which may be a useful gene to target in future studies of interplant signal transfer in Douglas-fir. I have also shown that seedling vigour was significantly related to mycorrhizal colonization and mesh size, pointing again to the importance of baseline measurements to allow removal of variation from stress related to growing conditions, but also to the positive impact of mycorrhizal networks on seedling health.

Further work is needed to elucidate the involvement of defense pathways, and particularly JA, in ectomycorrhizal-mediated interplant signal transfer. In future studies, care should be taken to induce a substantial donor response and to potentially apply more than one stimulus where possible (e.g., having both natural and artificial induction treatments). Work is also needed to investigate whether this
phenomenon is observed in field settings. MN-mediated signal transfer has the potential to play a role in community-level responses to disease and stress, the balance of competition and facilitation between inter-and intra-specific neighbours, and the dynamics of complex plant-fungal evolutionary relationships.
Figure 2.1: Diagram of experimental setup showing cross-section of a pot. Trees are shown in mesh bags with pathways available depending on mesh pore size. 35µm mesh treatments were expected to result in the greatest signal transfer (i.e., increased defense gene expression in receivers connected to MeJA-treated donors).
Figure 2.2: Difference in vigour between donor and receiver seedlings versus mesh size of seedling pairs (n=24 pairs). Calculated as the vigour rating (value from 1 to 5) of the receiver seedling subtracted from the rating of the donor seedling (value from 1 to 5). Vigour rating is described in section 2.2.1. Boxes represent individual seedling pairs at a given difference value. Distribution of differences was significantly non-random based on Fisher’s exact test (p=0.0006).
Figure 2.3: Plot of vigour rating versus percent of root tips that were colonized by mycorrhiza for all seedlings (n=72). Boxes represent individual seedlings. Vigour rating was from 1 to 5 with 5 being the most healthy. A detailed description of vigour rating can be found in section 2.2.1. Regression was significant (p=0.003, adjusted $r^2=0.10$).
Figure 2.4: 2-dimensional Non-metric multidimensional scaling (NMDS) plot showing relationship between mycorrhizal morphotypes and mesh bag treatments (stress = 0.13791). Orange labels are morphotype names, and black (0.5µm), green (35µm), and red (250µm) labels are seedling sample names. Samples that are closer together have more similarity in distribution of morphotypes. Centroids around mesh treatments are shown, with the same colour scheme as sample names. Vigour correlated significantly with the ordination (p=0.004), and is overlain as a blue arrow, with the arrowhead pointing towards increasing vigour.
Figure 2.5: AOC relative expression in (a) donors and receivers by time step and (b) receivers alone by mesh size and time step, shown as ddCt (one unit on the y-axis is approximately a 2-fold difference of expression). Relative expression values represent fold-change between hormone-treated and control seedlings and were calculated using the ddCt method after calibrating for amplicon-specific amplification efficiencies in LinRegPCR (2009). Asterisk indicates significant difference in fold-change of expression (p=0.044). Error bars show standard error of the mean.
Figure 2.6: Non-linear regression showing correlation between donor and receiver AOC dCt expression (before taking the ratio of MeJA-treated/control seedlings), shown as ddCt (one unit on the y-axis is approximately a 2-fold difference of expression). Regression is significant at p=0.03, adjusted $r^2=0.16$. Equation for log-transformed dCt expression is $y=5.05737+0.03167x^2$. 
Figure 2.7: ACS2 relative expression in (a) donors and receivers by time step and (b) receivers alone by mesh size and time step, shown as $\text{ddCt}$ (one unit on the y-axis is approximately a 2-fold difference of expression). Relative expression values represent fold-change between hormone treated and control treatments and were calculated using the $\text{ddCt}$ method after calibrating for amplicon-specific amplification efficiencies in LinRegPCR (2009). Asterisk indicates significant difference in fold-change of expression ($p=0.028$). Error bars show standard error of the mean.
Table 2.1: Primer information for Interior Douglas-fir (*Pseudotsuga menziesii var. glauca*) genes. AOC = allene oxide cyclase, ACS = aminocyclopropane carboxylic acid, TEF = transcription elongation factor.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Tm</th>
<th>sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmACS2_1-F</td>
<td>59°</td>
<td>GAAGATATTGGATAGACAACCCAAC</td>
</tr>
<tr>
<td>pmACS2_1-R</td>
<td>63°</td>
<td>ATCTTCGTGGTTCTTGAGATG</td>
</tr>
<tr>
<td>pmAOC_2-F</td>
<td>58°</td>
<td>GCCCACGAACACTCCAGTGAAT</td>
</tr>
<tr>
<td>pmAOC_2-R</td>
<td>58°</td>
<td>GCCCTGCTTTACCTTGCCTTC</td>
</tr>
<tr>
<td>pmTEF-F</td>
<td>60°</td>
<td>CAGAATTGGGTGCTTGATCG</td>
</tr>
<tr>
<td>pmTEF-R</td>
<td>60°</td>
<td>CCTCACACTCCAACCTGCATA</td>
</tr>
</tbody>
</table>
Table 2.2: ANOVA results of seedling characteristics by mesh treatment (n=72).
Each variable was run as a separate ANOVA, $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SS</th>
<th>df</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>68</td>
<td>2</td>
<td>0.487</td>
<td>0.616</td>
</tr>
<tr>
<td>Basal diameter</td>
<td>10.27</td>
<td>2</td>
<td>6.516</td>
<td>0.00256 *</td>
</tr>
<tr>
<td>Emergence</td>
<td>30.02</td>
<td>2</td>
<td>5.468</td>
<td>0.00592 *</td>
</tr>
</tbody>
</table>
Table 2.3: Distribution (in number of seedlings) of vigour rating across mesh sizes (n=72). Vigour rating was from 1 to 5 with 5 being the most healthy. A detailed description of vigour rating can be found in section 2.2.1.

<table>
<thead>
<tr>
<th>Rating</th>
<th>0.5μm</th>
<th>35μm</th>
<th>250μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>3.5</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>4.5</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.4: ANOVA results of mycorrhizal colonization by mesh treatment. $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Factor</th>
<th>SS</th>
<th>df</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesh</td>
<td>359.1</td>
<td>2</td>
<td>4.036</td>
<td>0.02201 *</td>
</tr>
<tr>
<td>Residuals</td>
<td>3069.8</td>
<td>69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3: Shroomroot - an action-based digital game to enhance postsecondary teaching and learning about mycorrhiza

“I want to argue that game design is not accidentally related to learning, but rather that learning is integral to it. Game design is applied learning theory.” – James Paul Gee

3.1: Introduction

The functioning of forests depends on the belowground activity of plant roots and their environment, which includes fungi, microbes, nutrients, water and many other factors. An estimated 90% of plants in terrestrial ecosystems form a symbioses called a “mycorrhiza” (Fortin et al., 2009), which is a mostly mutualistic relationship between a fungus and a plant root where the fungus helps the plant acquire resources from the soil such as nitrogen (N), phosphorus (P), and water, and the plant gives the fungus photosynthates (products of photosynthesis) that contain carbon (C). Mycorrhizal fungi cover a wider surface area within the soil than do plant roots, and are able to gather N, P, and other nutrients from both organic sources (such as plant litter) and inorganic ones (nitrate, phosphate), whereas plant roots primarily absorb inorganic forms of nutrients (Smith and Read, 2008). The fungi can also provide a form of pathogen protection for the plant (reviewed in Jung et al., 2012).

The fungi in these symbioses have filaments called “hyphae” growing throughout the soil in a complex matrix called a “mycelium”, and the same fungus can be connected to multiple plants at the same time. These networks of fungal
mycelium allow resource exchange to occur indirectly between neighbouring plants, and are known as “mycorrhizal networks.”

Mycorrhizal networks are important in ecosystems worldwide, impacting nutrient cycling, soil aggregation, forest stand dynamics, and many other ecosystem processes (reviewed in Simard et al., 2012, 2015; van der Heijden et al., 2015). Our developing understanding of mycorrhizal ecology is changing the way we practice forestry (Schwartz et al., 2006, Azul et al., 2014), the way we think about the balance of relationships between species (van der Heijden and Horton, 2009; Simard et al., 2015), and how we understand plant communication (Ramov, 2013; Gorzelak et al., 2015; Johnson and Gilbert, 2015). Understanding these mycorrhizal interactions is key for holistic and adaptive forest management, which is more crucial today in the face of global change (Puettmann et al., 2009). Yet, mycorrhizal ecology is complex and difficult to observe in action, making it a challenging subject to teach and learn, especially in lower level university courses in which basic mycorrhizal ecology is often covered (Collins, 2008).

Today’s undergraduate students and future forest managers and soil scientists are part of a generation comfortable with and enthusiastic about use of media in the classroom (Prensky, 2001) and technology in general (Tapscott, 2009). In particular, the idea of incorporating digital games into the classroom has generated a flurry of interest over the past 20 years under the umbrella of “digital game-based learning”, or DGBL. Various empirical studies (reviewed in Boyle et al. 2016) have demonstrated DGBL’s potential for positive learning and affective (beliefs and attitudes) impacts. However, not all attempts to use DGBL show
improvement over traditional instruction, sometimes with poorer long-term effects on learning acquisition (Rondon et al., 2013) or a lack of effect on student attitudes towards science (Barko and Sadler, 2013). This means that context is key, and further research is needed to explore how specific game features, content areas, and styles of integration (e.g., providing additional instructional support surrounding gameplay) impact game success (Huang et al., 2013; and reviewed in Boyle et al., 2016).

In ecology, a game that provides an interactive window into complex, hard-to-visualize worlds has the potential to greatly improve students’ understanding of the complex biological relationships (Lo et al., 2008; Speelman and Garcia-Barrios, 2010). Despite this, application of games focused on ecology has been somewhat limited in scope. While there are examples of successful ecology-based games (Sarab et al., 2009; Schaller et al., 2009), most focus on human or animal characters, and take place in the ocean or on land (as opposed to belowground). Studies investigating plant-centric games have also shown positive results (Hwang et al., 2012; Su and Cheng, 2014), but these games are also either centered on human characters or have no first- or third-person characters (i.e., they are God’s eye view simulation-style games, as described in Gee, 2008). Character perspective in games can impact how well players feel situated within the game world (Gee, 2008, Moreno and Mayer 2004). To my knowledge, there are no studied examples of plant- or fungus-focused ecology games in which the player plays a plant experientially in the first- or third-person – controlling plant growth in a physically explicit way. A game that offers players the opportunity to be intimately situated in
the plant world, especially the belowground world, has the unique potential to present key concepts about mycorrhizal ecology to undergraduate students in motivating and tangible ways.

In this study I co-created and evaluated Shroomroot, an action-based personal computer (PC) and mobile game in which students control a Douglas-fir root growing through the soil, acquiring water and nutrients and interacting with fungi. There were two main overarching objectives of this study. The first objective was to build Shroomroot, an action-based, plant-centric game that is situated in the world of belowground ecology and aimed for lower level postsecondary curriculum in an introductory soil science course. The second objective was to perform an exploratory evaluation of Shroomroot as an educational tool using a quantitative pre-test/post-test design. This second objective is includes the following four sub-objectives to: (2a) assess the impact of Shroomroot on student knowledge acquisition about mycorrhiza, (2b) assess the impact of Shroomroot on students’ attitudes towards learning about mycorrhizal content, (2c) explore the relationship between knowledge acquisition and content attitudes, and (2d) explore metrics related to student gameplay (i.e., student gameplay proficiency and engagement) and how these relate to knowledge acquisition and content attitudes.
3.2: Methods

3.2.1: Creation of Shroomroot

General Narrative of Shroomroot

Shroomroot is an action-based game of 12 levels targeted at the lower level undergraduate courses, in which the player directs the growth of a Douglas-fir root through a Podzolic soil (i.e., a typical soil type of Western North America where Douglas-fir is a native species). As time passes, the plant drains its resources (water, N, and P), and the player’s main goal is to control the growth of the root in order to acquire resources from the soil. The player must acquire resources quickly enough to prevent plant starvation and to allow their Douglas-fir seedling to grow bigger. Halfway through the game, the player can interact with mycorrhizal fungi and form mycorrhizal networks, expanding the resource-gathering dynamic. After completion of the 12th level, the game presents a graphic of a small Douglas-fir tree in a forest along with selected statistics about the player’s success in the game (e.g., how many mycorrhizal links they formed, as described below). A description of level-specific goals can be found in Table 3.1, with selected screenshots in Figure 3.1.

Alongside the root-growing gameplay, players can accumulate “Rewards” that become progressively unlocked for various achievements within game. These can be viewed in a separate “Rewards” screen of the game, and include rewards for number and type of resources collected, distance of root grown, and number and types of mycorrhizal relationships. For example, after the player forms at least 5 mycorrhizal links with seedling roots, the 5 seedling links reward is unlocked, with a small picture and description of the source-sink concept (Figure 3.2).
**Development of Shroomroot**

Shroomroot was developed using the 3D Unity game engine (game-building software) by Christopher Adderley (programming, user interface, game design) and Alex Catamo (sound) of Area Denial Games (Vancouver, BC), and myself (graphic design, in-level gameplay, game design). Construction of the game occurred from June to November 2014, and took approximately 750 person-hours. Shroomroot is playable through Firefox or Safari web browsers with the free download of the Unity Web plug-in, can be found at [http://shroomroot.com/game.html](http://shroomroot.com/game.html) and is also available for free on Google Play (Android only). A more in-depth description of the process of collaboration and development can be found in the Appendix.

Throughout design and development, a balance was sought between the following three main goals – to make Shroomroot (1) meet mycorrhizal-oriented learning objectives for 2nd year undergraduate students with some science background, (2) be engaging and functional as a game, and (3) be scientifically accurate within the target content.

**Goal 1: Providing educational content**

Through a combination of gameplay and the text in the Rewards section, Shroomroot aims to present biological concepts about the mycorrhizal symbiosis and mycorrhizal networks to meet learning objectives in lower level postsecondary curriculum. Vocabulary used and concepts presented were structured based on the expected knowledge base of 2nd year students who have had some, but limited, biology background. This was done by consulting the corresponding instructors for both Introduction to Soil Science and Forest Ecology courses at the University of
British Columbia, Vancouver. Because this intervention was performed in a 2nd year Introduction to Soil Science course, more focus was given to the mycorrhiza-centered learning objectives in that course (as described in section 3.3.2). The specific concepts that are addressed in Shroomroot are presented in Table 3.1 (gameplay) and Table 3.2 (selected reward text), while full reward text can found in the Appendix.

**Goal 2: Functioning as an engaging game**

Shroomroot includes progressively complex physical and strategic challenges to give a sense of skill-building and continued practice, and self-competition based on the potential to achieve varying degrees of success within each level (one can finish levels with fewer or greater resources – there is more detail on this in the Appendix, section A3.2.2). Shroomroot also includes progressive rewards for achievements within-game, an extrinsic motivation approach that has been found to be effective at increasing game engagement (King et al., 2011). Efforts were also taken to create pleasing graphics, animations, and smooth functioning of the game, aspects that Huang et al. (2013) found to be important elements of engagement.

**Goal 3: Ensuring scientific accuracy**

Efforts to ensure scientific accuracy included a mixture of literature research on my part and the consultation of experts in the relevant fields. Some particular aspects of the game may not be completely accurate due to simplifications needed to improve gameplay, but misleading or incorrect game aspects were avoided wherever possible. In the text for some of the Rewards, particular detailed gameplay
dynamics are clarified as to how our true understanding of mycorrhizal ecology differs from the way in which Shroomroot represents the concept (e.g., in the “Collected 1st Organic Phosphorus!” Reward, Table 3.2).

3.2.2: Evaluation of Shroomroot

Following completion of the game, an exploratory evaluation was conducted to examine whether the game was able to successfully meet mycorrhiza-focused learning objectives and encourage engagement with both the mycorrhizal content and engagement in the game itself.

Introduction to Soil Science course

About halfway through Term 2 in the 2014-15 academic year, Shroomroot was played by undergraduate students during one 50-min lecture session in the 2nd year Introduction to Soil Science course offered in the Faculty of Land and Food Systems at the University of British Columbia (UBC), Vancouver, Canada. I will use “intervention” as a general term encapsulating the integration of Shroomroot into the Introduction to Soil Science course. The Introduction to Soil Science course (offered during a 13 week academic term) provides students with a broad introduction of basic soil principles, including physical, chemical, and biological properties of soils, soil formation, classification, use and conservation. Mycorrhizal ecology is covered at an introductory level as part of one lecture on soil biology. The basic concepts of the mycorrhizal symbiosis are explained (such as the exchange of nutrients for C compounds synthesized during photosynthesis), but mycorrhizal
networks are not typically addressed. The course is required by three majors in Faculty of Forestry and two majors in the Faculty of Land and Food Systems at UBC. The Introduction to Soil Science course has two lecture sections that run at the same time in different rooms. In the 2014-15 academic year, the course had 232 students, with 119 in one section and 113 in the other. For the 2014-15 term, Shroomroot was used to deliver the initial lesson on mycorrhiza, and was played in both sections concurrently. Students played Shroomroot two days after a lecture session providing an overview of soil biology that did not include any information on mycorrhiza.

**Delivery of assessments and gameplay**

Students in the Introduction to Soil Science course were informed about the study with a combination of emails and in-class announcements 10 days previous to the intervention, to ensure that all students (those in class and absentee students) received notifications. It was made clear in all communication with students that participation in the assessments would be anonymous, non-marked, and would in no way impact their class standing. Because the pre-test was opened a few days ahead of the intervention, students had one week to consider whether or not they wanted to participate and were invited to send any questions they had to either their instructors or myself.

One lecture period (i.e., 2 days) before the intervention, students were invited to fill out the pre-test online. On the day of the intervention, students were also given the opportunity to fill out the paper version of the pre-test. The web-
version of the game was deactivated prior to the course and reactivated immediately beforehand and afterwards (until the post-test was closed) – preventing easy access to the game beyond in-class play.

When students were contacted about taking the pre-test, they also were given instructions to download the Shroomroot game files, and asked to bring their laptops to class the next lecture period. Students sat in groups of 2-3 around each laptop, taking turns to play the game. At least twice during the intervention, students were reminded to switch players (i.e., students who were actively playing instead of watching). Students were also advised that if they greatly preferred to watch their fellow students play instead of play themselves, they could (their decision was self-reported on the post-test).

In both lecture sections, a designated helper was available to assist the students with any technological issues related to the game, but no major content or game-play discussions were had between the students and the helper or instructors. Short tutorials included in Shroomroot led the students through the mechanics and goals of game-play and in some cases also presented content while explaining game-play. For example, tutorials explaining how to become a mycorrhizal root introduced the concept that mycorrhizal fungi can help the plant access organic nutrients.

The post-test was completed by students either immediately after game-play in class or online within a 2-day period after the intervention.
Assessments

In discussing the pre- and post-tests, individual units of each assessment will be called “items” to provide a catch-all term for short answer questions, multiple choice questions or Likert-style statements with rating scales. Pre- and post-tests were linked by non-identifying usernames that the students wrote on the assessments themselves. Only pre- and post-tests that had usernames that were unambiguously the same were considered “matched” and used in the final analyses.

Objective 2a (and 2c)

To evaluate whether Shroomroot (SR) improved students’ ability to achieve the target learning objectives within Introduction to Soil Science course, the pre- and post-test included nine multi-part content-oriented questions, for a total of 24 individual items about mycorrhizal content (e.g., how the mycorrhizal symbiosis benefits the fungus in various ways). To better isolate how effectively Shroomroot delivers content specifically, seven of these items (29% of the assessment) covered mycorrhizal content not expected to be addressed by Shroomroot (i.e., not taught via the game-play nor via rewards content, such as facts about mycorrhizal fungi’s impact on plant defense systems). The content knowledge portion of the assessment was therefore grouped into items that are “SR-addressed” or “not SR-addressed.” Content-based items were structured as multiple choice questions.
**Objective 2b (and 2c)**

To evaluate whether Shroomroot enhances student engagement with the content, a set of four items (on a Likert 5-point scale) were included on both the pre- and post-test. Two of these items, related to the mycorrhizal content addressed by Shroomroot (e.g., “Mycorrhizae and mycorrhizal networks are interesting” and “I would like to learn more about mycorrhiza and mycorrhizal networks”). Two additional items were related to non-target plant nutrition-related content that is presented in the early levels of Shroomroot. These statements were “Plant nutrition is interesting” and “I would like to learn more about plant nutrition”.

**Objective 2d**

To investigate the interactions among game skill, game engagement, content knowledge, and content engagement, the post-test included items related to participants’ skill playing Shroomroot and their attitudes towards playing Shroomroot (game engagement). Exact text of all of the items described below can be found on the post-test in the Appendix, section A3.4. For game skill, four of the items involved self-reported metrics of how far and how well the students performed in Shroomroot (such as how many levels they completed and how many fir cones were collected), while three other items were based on the 5-point Likert scale and were about whether participants felt they understood how to play, found Shroomroot easy, or found Shroomroot frustrating to play. For game engagement, items involved three self-reported metrics of how deep into the game the students explored (such as how many rewards they read), and two Likert-style items about
whether they enjoyed the game, both in terms of game play and in terms of reading the rewards.

Finally, the post-test also included four Likert-style items that directly addressed whether students felt they learned information from playing Shroomroot and whether they felt their interest in the target topics areas increased. I will refer to these four items as the “meta” items, as they refer to students’ metacognitive abilities to evaluate whether there was a change in their own learning and attitudes towards the subject.

The pre-test also requested that participants fill out some demographic information (such as gender and UBC Faculty) and information related to previous game experience and content experience (pre-test shown in Appendix section A3.3).

All assessments were written specifically for this study, and were reviewed for content validity and conceptual clarity by three professors (a mycorrhizal expert, and the two soil scientists teaching each section of the Introduction to Soil Science course) and one graduate student in soil science. All online assessments were done through Fluid Surveys (Ottawa, ON, Canada).

3.2.3: Ethics

This study was approved by the UBC’s Behaviour Research Ethics Board (BREB) under the code H14-02382. Course instructors and participating students gave consent to participate in this study. All results were anonymous (pre- and post-tests were linked by a non-identifying username) and physical and electronic forms of data were kept secure.
3.2.4: Bias of Researcher

The primary researcher carrying out the analysis for this study was myself, and am not directly associated with the Introduction to Soil Science course. However, I did run the exercise with Shroomroot in one of the two lecture sections, and have my own perceptions about the implementation of the exercise and perspectives about aspects that contributed to successes or failures of Shroomroot as an educational tool. As one of the main designers of Shroomroot, I also have some underlying hope that Shroomroot could be an effective educational tool in association with a desire to personally learn more about best practices in DGBL.

3.2.5: Data analysis

Analysis of Objectives 2a: content knowledge

The difference in overall pre- and post-test content scores was assessed using the nonparametric Kolmogorov-Smirnov (KS) test due to non-normality of the data. The two-sample KS test tests the null hypothesis that two distributions of data (ordinal or continuous) share the same underlying continuous distribution. The test generates the “D” statistic (reported in this thesis where the KS test has been used). Differences between cumulative pre- and post-test scores were calculated for each item and compared to how well Shroomroot was expected to address each item based on the concepts presented (Tables 3.4 and A3.3). After observing during the intervention that students seemed to put a limited time into reading content in the Rewards section of the Shroomroot, a second analysis was performed in which
questions were further subdivided into three groups: (1) “not SR-addressed” for content not included in Shroomroot at all, (2) “rewards only” for content only presented in Rewards section and not through game play, and (3) “gameplay and rewards” for content presented in both Gameplay and Rewards sections. The change in content knowledge within each group was compared using a Kruskal-Wallis test (due to non-normality of data).

**Analysis of Objectives 2b and 2c: content engagement and comparison**

All 5-point Likert items were scored from 1 to 5, with 5 being “Strongly agree” and 1 being “Strongly disagree,” except in negative cases for which reverse coding was used (e.g. “I found Shroomroot frustrating to play”). Scores on content engagement items were compared between the pre- and post-test individually using a KS test, the data being ordinal in nature. Content knowledge was compared to individual content engagement items using ANOVA, with content engagement items treated as factors (due to being ordinal Likert-style items).

**Analysis of Objective 2d: impact of game skill and engagement**

Individual game skill and game engagement metrics were summed into aggregate “game skill” and “game engagement” values. Before aggregation of item scores, individual items were normalized to contribute to aggregate scores evenly.

Not all items were included in the final aggregate scores. A total of 62% of participants either did not record or improperly recorded at least one of the game
skill and game engagement metrics. For example, within the “time spent playing” metric, 38% of the answers were unusable. Because of the high amount of missing data, items such as “time spent playing” and “fir cones gathered” were excluded from the calculation of overall game skill. Each game metric was constructed from four items, and the aggregate scores for game engagement and game skill were treated as continuous variables in comparisons with content metrics. Determining interrater reliability to validate these items was outside the scope of this exploratory study, and therefore analyses based on game metrics must be interpreted with caution.

Other potential covariates, such as previous game experience, gender, and class section were evaluated for their relationship to changes in knowledge or attitudes (in separate analyses) using ANOVA where assumptions were met, or a Kruskal–Wallis test where a non-parametric test was more appropriate. When change in scores are reported it is in mean raw difference between the pre- and post-test, and all means are reported ± standard deviation. All scores were normalized before comparison and all analysis was done in R Studio, version 3.2.2 (R core team, 2016).

3.3: Results and Discussion

3.3.1: Summary statistics of participants

About 60% of students were in attendance on the day of the intervention and played Shroomroot. Based on the number of definitively unique usernames, at least 57% of students in the Introduction to Soil Science course participated in at least
one of the assessments, filling out consent forms and providing responses to the pre- and/or post-tests (i.e., 142 pre-tests and 87 post-tests were recorded). Some students seemed to take one test but not the other, resulting in un-matched usernames. Of the tests recorded, 52 pre- and post-tests (22% of the class) could be unambiguously paired by the username students provided; hence, only these 52 tests were included in the final analyses of this study as a matched pre/post design.

A summary of demographics, previous game experience, and previous content experience can be found in Table 3.3. Of 52 participants, 35 (67%) were female, 16 (31%) were male, and 1 (2%) entry was left blank. The majority (44 students, 85%) were in the faculty of Forestry, with 5 (10%) in Land and Food Systems, 2 (4%) in Science, and 1 unknown.

The majority of students, 32 (61%), had some degree of experience with digital games, while 19 (37%) reported that they had no experience with digital games. Students reported having previous experience in playing games on mobile devices (22, or 42%), computers (24, or 46%), and consoles (21, or 40%) in approximately similar numbers (or proportions). In terms of previous content experience, the majority of students had no previous experience with Shroomroot, 43 (83%), nor previous mycorrhizal knowledge 46 (88%). However, five students (10%) had heard of Shroomroot, two students (4%) reported playing Shroomroot before the pre-test, and two other students reported playing in-between tests. Two students (4%) reported having mycorrhizal knowledge from a previous course, while four students (8%) reported looking up mycorrhizal content in between taking the pre- and post-test. One of the students who played before the pre-test
also had mycorrhizal knowledge from a previous course, while the other student had no previous mycorrhizal knowledge. Neither of the students who played in between the pre- and post-test reported having previous mycorrhizal knowledge. All but two students (4%) reported at least playing the game equally as much as watching their team-mate play.

3.3.2: Objective 2a: Pre-test vs Post-test Content knowledge

Shroomroot-addressed (SR-addressed) content knowledge scores significantly increased between the pre-test (6.1±3.4) and post-test (8.9±3.4) (D=0.327, p=0.008, Figure 3.3, Table 3.4), with a strong effect size (Cohen's d = 0.81). Non SR-addressed content scores did not significantly increase between the pre- and post-test (D=0.212, p=0.195). Content knowledge score change between tests (Figure 3.4) are grouped by questions addressed by SR (a), and questions not addressed by SR(b). When splitting SR-addressed questions into “game-play and rewards” and “rewards only,” content knowledge score improvement on “game-play and rewards” questions (10.1±7.5) was significantly higher than score improvement on “not-SR-addressed” questions (-1.9±7.3), while “rewards only” scores (3.3±8.5) were in-between (Table A3.2). Students also tended to self-report that Shroomroot increased their understanding of mycorrhiza and mycorrhizal networks, as well as plant nutrition, with the majority of students (78%) reporting that they “Agree” or “Strongly Agree” with the statement “I learned about mycorrhizae and mycorrhizal networks from Shroomroot” (for full distribution of responses see Table A3.4 in the Appendix).
Shroomroot was successful at increasing knowledge acquisition of targeted learning objectives, but post-test scores indicate that the scope of student knowledge acquisition was smaller than the scope of mycorrhizal content available in Shroomroot. The post-test average of SR-addressed questions was 8.9 out of a possible 17 points, meaning that on average students did not answer correctly about half of the SR-addressed questions. Within the target content, some learning objectives were better targeted by Shroomroot than others, but student scores improved on most SR-addressed questions to some degree (14 out of the 17 SR-addressed questions had a positive mean change, Table A3.3). This suggests that Shroomroot was relatively successful from the perspective of learning acquisition. However, without a traditional instruction group as control, it is impossible to know how this would compare to traditional instruction.

Although many studies demonstrate the ability of games to increase student knowledge acquisition, this is not a universal result (Boyle et al., 2016). Varying results with respect to digital games’ impact on knowledge acquisition point to the importance of context in DGBL, (i.e., specific game features and/or forms of game integration into the classroom). Shroomroot’s positive effect on student knowledge acquisition suggests that Shroomroot had certain key features, or was implemented in a specific context that allowed students to learn through the use of a digital game.

Shroomroot was situated in a fantasy world, particularly an endogenous one. Garris et al. (2002) describes the importance of endogenous fantasy versus exogenous fantasy, suggesting that games with mechanics and goals related directly to the study system (endogenous) have greater potential to positively influence
knowledge acquisition than games with mechanics and goals unrelated to the study system (exogenous), such as math games involving monsters. While fantasy has been seen to be a less important component of student engagement with games than other features such as competition and challenge (Hainey et al., 2011), the nature of the fantasy may be directly related to how effectively the game conveys concepts and allows simulated, within-system interaction. Shroomroot was an endogenous fantasy that allowed players to actively control a plant root, potentially contributing to the successful effect on knowledge acquisition.

The impact of Shroomroot on content knowledge acquisition is particularly interesting within the context of the intervention occurring during one 50-min lecture period with very limited to no content-based instructor support (such as scaffolding or debriefing). In a somewhat similar study, Barko and Sadler (2013) investigated the effect of the biotechnology game, Mission Biotech (MBt), on knowledge acquisition in undergraduate students, and found a significant improvement in content scores on both curriculum-oriented assessments and standards-oriented assessments. In contrast to the Shroomroot intervention, the intervention with MBt was carried out over 2-3 weeks, and gameplay was paired with activities from an MBt curriculum guide, namely small group discussion, lectures, and related laboratory activities. Barko and Sadler (2013) also state:

"On the basis of our experiences in the game design process, observations of teachers implementing MBt, and analyses of the student data, we believe that the success of MBt in the classroom is not a product of the game itself but of how the game
was situated in the context of the classroom and the other activities that helped students make biological sense of their experiences within the game.”

While Shroomroot did successfully improve knowledge acquisition without in-depth instructor support and over a short period of time, it is likely that the Shroomroot intervention would have benefited from greater integration in the form of more in-depth support. On average, students did not answer correctly about half of the questions focused on content that Shroomroot was expected to address. Some further gains in knowledge acquisition may have been possible with a more integrated pedagogical approach involving guided questioning about the game content and what the students were learning. Forms of integration and support (e.g., scaffolding) surrounding digital game play have enhanced the success of knowledge acquisition in multiple studies (Lim et al., 2006; Basu et al., 2011; Erhel and Jame, 2013).

In addition to the importance of support, the style of implementation can have a significant impact on a game’s impact on knowledge acquisition. Part of the aim of having Shroomroot played in the classroom was to encourage the social and competitive aspects of games, which may lead to greater student motivation (Garris et al., 2002, Hainey et al., 2013). Informal feedback from students and observations by myself and instructors suggested that students were unable to fully concentrate on the progression of the game and the information delivered sequentially during tutorials that are built in the Shroomroot. Also, the relatively lower score improvement on questions addressed only by Rewards-based content suggests students gathered more content from the gameplay than reading the Rewards
section in the Shroomroot. This was most likely due to the limited amount of time available for gameplay and also students taking turns to play every other level. It is possible that solitary or at-home playing of Shroomroot would have been more effective. De Grove et al. (2012) found that students who played a first-person adventure game found higher self-reported learning and enjoyment, and they attributed it to having enough time to play and master the game. Ability to focus and develop mastery of a game can also contribute to achieving a state of “flow,” a desirable state of high concentration and enjoyment more likely to be conducive to learning (Keller et al., 2011).

Given the somewhat chaotic and brief nature of the Shroomroot intervention, greater gains in knowledge acquisition may have been possible if students were to play at home and/or were provided with additional support such as scaffolding, small group discussions, or debriefing. The overall significant increase in knowledge acquisition; however, suggests that Shroomroot, a plant-focused action-based game, was successful at addressing certain learning objectives in mycorrhizal ecology in this lower level postsecondary course.

3.3.3: Objective 2b: Pre-test vs Post-test Content engagement

None of the content engagement items had significantly different scores between the pre- and the post-test based on the KS test (p>0.05, Table 3.5). One of these items (“Mycorrhizae and mycorrhizal networks are interesting”); however, showed a trend towards increasing between the pre- and post-test (D=0.269, p=0.046, α=0.013), with an average rating of 3.7(±0.8) on the pre-test, which is
between “Neutral” and “Agree,” and an average rating of 4.1 (±0.7) on the post-test, which is approximately at an “Agree” rating. Table A3.5 shows in more detail the distribution of responses for each item. Students also tended to self-report that Shroomroot increased their engagement with mycorrhiza and mycorrhizal networks, as well as plant nutrition, with the majority of students (78%) reporting that they “Agree” or “Strongly Agree” with the statement “Shroomroot increased my interest in mycorrhizae and mycorrhizal networks” (for full distribution of responses see Table A3.4 in the Appendix).

The trend towards increased student agreement with “Mycorrhizae and mycorrhizal networks are interesting” suggests Shroomroot may have had some impact on student attitudes towards mycorrhizal ecology, but not a large one. Hwang et al. (2012) showed positive effects on student attitudes in a botanical game that was not action-based. Hence, it may be that the action-based format is somewhat less successful at affecting engagement. Given the strong engagement in action-games seen outside of education, it is more likely that the social and fast-paced nature of the intervention disrupted students’ sense of flow (as described above) and made the experience less focused and satisfactory.

Student’s desire to learn more about mycorrhizal ecology was not affected. This could be connected to the structure of Shroomroot as a simple, action-based game that utilizes a behavioural, drill and repeat learning model as opposed to a more cognitive-based, intrinsically motivating style (Connolly et al., 2014). Perhaps the mechanics around mycorrhizae were not so engaging and/or mysterious that students were prompted to learn more. However, pre-test scores for the “I want to
learn more about mycorrhiza and mycorrhizal networks” item were already close to an “Agree” average at 3.87, and the impact of Shroomroot may not have been enough to move more students towards agreeing or strongly agreeing with the statement.

The slight trend of an increase in agreement with statements about plant nutrition (from an average score of 3.8 on the pre-test to 4.0 on the post-test) may suggest that Shroomroot had a mild, non-significant impact on attitudes towards plant nutrition. Early levels in Shroomroot do introduce a few elements of plant nutrition, so this may be a reason for this outcome. This may also be the result of random variation and not of Shroomroot impacting attitudes about plant nutrition.

3.3.4: Objective 2c: Relating content engagement to content knowledge acquisition

Of all comparisons made between content engagement and content knowledge scores, only pre-test scores for the content engagement item, “Mycorrhizae and mycorrhizal networks are interesting,” showed a significant relationship with pre-test SR-addressed content knowledge scores (p=0.001 for pre-test, Figure 3.5, Table A3.2), but not with the change in scores between pre- and post-test. This suggests that student engagement with the topic of mycorrhizae and mycorrhizal networks was related to their overall understanding before playing Shroomroot, but did not have a distinct relationship with their actual learning as a result of playing the game (i.e., students with higher engagement did not tend to show a greater change in knowledge scores between the pre- and post-tests). This is
in contrast to studies demonstrating a strong relationship between engagement with content and learning of content in DGBL contexts (e.g., Iten et al., 2016). My findings may be because the change in the knowledge scores was not substantial enough to distinguish student learning by level of engagement, or because of a disconnect between engagement and learning in this context.

3.3.5: Objective 2d: Game engagement and game skill

Engagement with Shroomroot was generally positive, with averages on Likert items falling between "Neutral" and "Strongly agree" responses. “I enjoyed playing and/or watching my teammates play Shroomroot” had an average score of 4.0 (±0.8), and “I enjoyed reading the Reward boxes in Shroomroot” had an average score of 3.4 (±0.8). The “quantity of Reward boxes read” item, in which students reported approximately how many reward boxes they read, had an average of 2.9±1.2 on a scale of 1 – 5 indicating that the students tended to read about half of the 20 reward boxes (56% of students reported reading half of the rewards or fewer). Items within the game skill metric included the “ease of play” and “frustration of play” (reverse-coded) items which were between “Neutral” and “Agree” (3.7±0.9 and 3.1±1.0, respectively), and the “understand instructions” item which was between “Agree” and “Strongly agree” (4.2±0.8). The majority of students (69%) reported that they completed all 12 levels in Shroomroot.

Out of a cumulative 20 points for the aggregate game skill and game engagement scores, the average was 15.6±1.8 for game skill and 13.8±2.4 for game engagement. While exploratory, this generally positive view of Shroomroot and
competence at the game suggests this plant-centric, action-based game was successfully engaging, and parallels other findings that today's postsecondary students enjoy and are comfortable with incorporating digital games in the classroom (Prensky, 2001). Details on game skill and engagement items can be seen in Table 3.6.

No relationships between aggregate game skill or game engagement scores and SR-addressed content knowledge or any content engagement scores were found. All possible comparisons between pre-test, post-test, and score change values were tested (data not shown). This suggests that knowledge acquisition and changes in content engagement are neither limited nor bolstered by student skill or engagement with the game. While somewhat unexpected given other studies have found a relationship between game enjoyment and increase in content engagement or learning (Allen et al., 2014, Iten et al., 2016), this is not necessarily a negative outcome. From the perspective of using Shroomroot as an educational tool, and knowing that different students can be more or less engaged by games (Kebritchi et al., 2008; Allen et al., 2014), it is encouraging to note that individual differences in game engagement and skill were not connected to learning and engagement outcomes. It is also possible that this outcome was due to the assessments used, as these metrics were limited and exploratory in nature.

3.3.6: Other factors

Previous Shroomroot experience and/or previous mycorrhizal knowledge was self-reported by only a small number of students (Table 3.3), but this seemed to
have an effect on their knowledge and engagement scores. I could not test the significance of this effect because of the small sample size of students with prior knowledge (see below for item-specific sample size), but I discuss differences in means below.

The mean improvement in content scores for students who had previous mycorrhizal knowledge or looked information up in between tests (4 students, 2.3±3.3) was lower than the mean improvement of the rest of students (2.8±3.5). Pre-test scores were higher in those with previous knowledge (2 students, 12±1.4) than those without (5.9±3.3), and final post-test scores were also higher in those with previous knowledge (12.2±1.6, 4 students, including those who self-reported looking up information in between tests) than those without previous knowledge (8.4±3.4). Removing students with any previous mycorrhizal knowledge from the analysis makes the mean difference between pre and post-test content scores slightly larger (2.9±3.6 as opposed to 2.8±3.5).

Students with previous mycorrhizal knowledge had slightly higher incoming content engagement scores on the “Mycorrhiza and mycorrhizal networks are interesting” item than those without (2 students, 3.8±0.75 versus the other 50, 3.7±0.79). Removing these students from the study does cause this content engagement item to result in a slightly smaller increase between the pre- and post-test (0.35±0.55 vs 0.37±0.74). This suggests that the results were somewhat impacted by the presence of students with previous mycorrizhæ-related experience, but not to a strong degree.
Neither gender, previous game experience, nor course section were significantly related to any content knowledge, content engagement, game engagement, or game skill metric (data not shown).

3.4: Limitations

There were some limitations to this exploratory study. First, as a quasi-experimental pre/post design, there was no comparison to a control group. This means it is unknown how Shroomroot would compare to traditional instruction at meeting the same learning objectives. This study also did not explore the impact of including instructor support or class discussion in the Shroomroot intervention, which would perhaps be a better format for future use of Shroomroot in classrooms, in light of the significant but relatively small content knowledge acquisition.

The assessments were exploratory, and therefore limited in scope and sensitivity. While this was done in order to create a relatively brief but broad-reaching assessment appropriate for the time constraints of the intervention, it limits the sensitivity of the results.

3.5: Conclusions

Intervention with Shroomroot was successful at increasing 2nd year undergraduate students’ knowledge acquisition to meet mycorrhizal ecology learning objectives (with an average increase in score of 46%), and tended to have a positive impact on student attitudes towards mycorrhizae and mycorrhizal ecology (content engagement item “Mycorrhizae and mycorrhizal networks are interesting”
saw 56% of students reporting Agree or Strongly Agree on the pre-test versus 83% reporting Agree or Strongly Agree on the post-test). Students also tended to self-report that the game increased their learning (78% Agreed or Strongly Agreed) and interest in mycorrhiza and mycorrhizal networks (also 78% Agreed or Strongly Agreed). The potential for digital games to have positive learning and affective outcomes is well-documented, but it is unique to see significant knowledge acquisition for a plant-focused action-based game. Both learning and affective outcomes may have been even more pronounced had more explicit instructor support (e.g., scaffolding) been included in the intervention format.

There was no relationship between game metrics and content metrics, which was unexpected but also promising because it indicates that knowledge acquisition and attitude shifts because of Shroomroot were not strongly related to variation in individual experiences with the game.

This game has the potential to be useful for other soil science, forest ecology, and even biology courses that want to encourage students to think about parts of biological systems which are unseen and hard to visualize. Further work is needed to evaluate the appropriateness and best implementation practices of Shroomroot for these contexts.
Figure 3.1. Screenshots of Shroomroot showing progression through levels. (a) Level 1: Player must control root to collect water. (b) Level 4: Player must collect water, nitrogen, and phosphorus. (c) Level 8: Player can be mycorrhizal and link with other plants.
Figure 3.2: Shroomroot screenshot showing 5 seedling links reward and explanation of source-sink dynamic of resource transfer via mycorrhizal networks.
Figure 3.3: Students’ mycorrhizal content knowledge pre- vs post-test for (a) Shroomroot (SR)-addressed questions (scores shown as percentages of 17 questions) and (b) non-SR-addressed questions (scores shown as percentages of 7 questions). Asterisk denotes significant difference in scores based on a Kolmogorov-Smirnov test (p=0.008). Error bars are standard error of the mean.
Figure 3.4: Students’ content knowledge improvement (raw difference in post-test vs pre-test scores) by question category. “Not-SR-addressed” refers to scores on questions categorized as not addressed by Shroomroot, “rewards only” are scores on questions addressing content only covered in Rewards section of Shroomroot, and “play and rewards” are scores on questions addressing content covered in both the Gameplay and Rewards sections of Shroomroot. Letters denote significantly different groups based on a Kruskal-Wallis test (p=0.01). Error bars are standard error of the mean.
Figure 3.5: Pre-test scores for content engagement item “Mycorrhizae and mycorrhizal networks are interesting” versus pre-test content knowledge score (percent out of 17 of Shroomroot (SR)-addressed correct answers). Round point is a singular “Disagree” response. Letters denote significantly different groups based on an ANOVA (p=0.001). Error bars are standard error of the mean.
Table 3.1: Shroomroot gameplay by level and associated concepts presented through level gameplay. In all levels the player is controlling the growth of the Douglas-fir root to achieve the listed Gameplay Goals. Each level offers practice of “associated new concepts” as well as concepts from previous levels. E.g. players must consider the importance of seeking out the most limiting resource from Level 4 onwards.

<table>
<thead>
<tr>
<th>Level</th>
<th>Gameplay Goals</th>
<th>Associated New Concept(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Avoid obstacles (e.g. rocks, sticks), collect water (W)</td>
<td>Plants need W, Roots grow towards water as needed</td>
</tr>
<tr>
<td>2</td>
<td>Avoid obstacles, collect W and nitrogen (N)</td>
<td>Plants need N, have to balance time spent seeking N and W, N affects collection radius</td>
</tr>
<tr>
<td>3</td>
<td>Avoid obstacles, collect W and phosphorus (P)</td>
<td>Plants need P, have to balance time spent seeking P and W, P affects energy (speed)</td>
</tr>
<tr>
<td>4</td>
<td>Avoid obstacles, collect W, P, and N</td>
<td>Importance of seeking out most limiting resource, cannot access organic P or N</td>
</tr>
<tr>
<td>5</td>
<td>Avoid obstacles, collect W, P, and N</td>
<td>Continued importance of seeking out most limiting resource, cannot access organic P or N</td>
</tr>
<tr>
<td>6</td>
<td>Avoid obstacles, collect W, P or organic P (OP), and N or organic N (ON), can choose to be mycorrhizal</td>
<td>Being mycorrhizal allows access of organic P and N</td>
</tr>
<tr>
<td>7</td>
<td>Avoid obstacles, collect W, P or OP, and N or ON, can be mycorrhizal</td>
<td>Being mycorrhizal makes access of W in between rocks easier</td>
</tr>
<tr>
<td>8</td>
<td>Avoid obstacles, collect W, P or OP, and N or ON, can be mycorrhizal, if mycorrhizal can link to mature roots</td>
<td>Being mycorrhizal when growing near a mature root allows formation of a mycorrhizal network and resource gain</td>
</tr>
<tr>
<td>9</td>
<td>Avoid obstacles, collect W, P or OP, and N or ON, can be mycorrhizal, if mycorrhizal can link to seedling roots</td>
<td>Being mycorrhizal when growing near a seedling root allows formation of a mycorrhizal network and resource gain OR LOSS if seedling has less of a given resource than player</td>
</tr>
<tr>
<td>10</td>
<td>Avoid obstacles, collect W, P or OP, and N or ON, can be mycorrhizal, can link to seedling and/or mature roots</td>
<td>Sometimes it is better for a plant to be mycorrhizal, other times it is not</td>
</tr>
<tr>
<td>11</td>
<td>Avoid obstacles, collect W, P or OP, and N or ON, can be mycorrhizal, can link to seedling and/or mature roots</td>
<td>Continued practice of all concepts</td>
</tr>
<tr>
<td>12</td>
<td>Avoid obstacles, collect W, P or OP, and N or ON, can be mycorrhizal, can link to seedling and/or mature roots</td>
<td>Continued practice of all concepts</td>
</tr>
</tbody>
</table>
Table 3.2: Examples of text shown in the “Reward” section of the Shroomroot game.

*Text of all “Rewards” can be found in the Appendix, Table A3.1.*

<table>
<thead>
<tr>
<th>Reward title</th>
<th>Associated text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collected 1st Phosphorus!</td>
<td>Phosphorus is also used for many things, including ATP and providing the backbone for DNA. In Shroomroot phosphorus causes you to go faster, representing its role in ATP (adenosine tri-phosphate).</td>
</tr>
<tr>
<td>Collected 20 Phosphoruses!</td>
<td>Phosphorus in Shroomroot can also be inorganic or organic. Forms of inorganic phosphorus in soil are commonly insoluble, which makes them unavailable for plant uptake. This means it can often be a limiting resource.</td>
</tr>
<tr>
<td>Collected 1st Organic Phosphorus!</td>
<td>Organic phosphorus is harder for plants to directly digest, but mycorrhizal fungi can help. In Shroomroot, the mycorrhizal association is the only way to get OP*, but in reality plant roots can access some OP even when non-mycorrhizal.</td>
</tr>
<tr>
<td>Made 1st Mycorrhizal Network Link!</td>
<td>Mycorrhizal networks refer to communities of plants connected by fungal hyphae in the soil, or in other words communities of fungi connected by plants roots in the soil. &quot;Myco” for fungus, &quot;rrhizal” for root. Shroomroot is a very tree-centric game, but there are also complex decisions and behaviours going on from the fungal perspective.</td>
</tr>
<tr>
<td>Made 5 Mycorrhizal Network Links!</td>
<td>In Shroomroot, the resources you receive from or lose to seedlings is based on the source-sink concept. If you have more of a nutrient than a seedling, you lose nutrients to that seedling (you are a source, it’s a sink), and vice versa.</td>
</tr>
<tr>
<td>Made 1 Pinecone!</td>
<td>One theory for plant growth is that they will put energy into reproduction only if they already have more than enough resources to grow and survive. If they have few resources they only grow and survive. If they have even fewer, they focus only on survival.</td>
</tr>
<tr>
<td>Collected 100 waters!</td>
<td>You'll find that playing with myco mode on helps you get water from harder to reach areas, because the collection radius is much bigger with mycorrhizae than without. This represents the way mycorrhizal fungi grow their mycelium throughout the soil and do help plants access hard-to-get water.</td>
</tr>
</tbody>
</table>
Table 3.3: Summary statistics of participants who completed matched pre- and post-tests (n=52).

<table>
<thead>
<tr>
<th>Value</th>
<th>Number (percent) of participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of paired tests</td>
<td>Total: 52 (100%)</td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Male: 16(31%) Female: 35(67%) Blank: 1(2%)</td>
</tr>
<tr>
<td>Faculty</td>
<td>Forestry: 44(85%) LFS: 5(10%) Science: 2(4%) Blank: 1(2%)</td>
</tr>
<tr>
<td>Had taken Forest Ecology course</td>
<td>Yes: 3(6%) No: 49(94%)</td>
</tr>
<tr>
<td>Section</td>
<td>Section 1: 18(35%) Section 2: 33(63%) Blank: 1(2%)</td>
</tr>
<tr>
<td><strong>Previous game experience</strong></td>
<td></td>
</tr>
<tr>
<td>Previous video game experience</td>
<td>Not at all: 19(37%) A bit: 12(23%) A moderate amount: 12(23%) Quite a lot: 8(15%) Other: 1 (2%)</td>
</tr>
<tr>
<td>Preferred game devices</td>
<td>Mobile: 22 (42%) Computer: 24 (46%) Console: 21 (40%) Other: 2 (4%)</td>
</tr>
<tr>
<td>Device used on game day</td>
<td>Computer: 44(85%) Android: 7(13%) Both: 1(2%)</td>
</tr>
<tr>
<td><strong>Previous content knowledge</strong></td>
<td></td>
</tr>
<tr>
<td>Previous Shroomroot experience</td>
<td>None: 43(83%) Heard of: 5(10%) Played before pre-test: 2(4%) Played in-between tests: 2(4%)</td>
</tr>
<tr>
<td>Previous mycorrhizal knowledge</td>
<td>None in-between tests: 46(88%) Previous course: 2(4%) Some in-between tests: 4(8%)</td>
</tr>
</tbody>
</table>

*LFS = Faculty of Land and Food Systems*
Table 3.4: Effect of Shroomroot on students’ content knowledge about mychorriza (n=52). SR-Addressed="Shroomroot-addressed". α=0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Points possible</th>
<th>Pre-test mean</th>
<th>Post-test mean</th>
<th>Mean change</th>
<th>df</th>
<th>D*</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>24</td>
<td>9.7±3.5</td>
<td>12.3±3.5</td>
<td>2.6±3.5</td>
<td>51</td>
<td>0.289</td>
<td>0.026</td>
</tr>
<tr>
<td>SR-Addressed</td>
<td>17</td>
<td>6.1±3.4</td>
<td>8.9±3.4</td>
<td>2.8±3.5</td>
<td>51</td>
<td>0.327</td>
<td>0.008</td>
</tr>
<tr>
<td>Not SR-addressed</td>
<td>7</td>
<td>3.6±1.0</td>
<td>3.4±1.1</td>
<td>-0.3±1.48</td>
<td>51</td>
<td>0.212</td>
<td>0.195</td>
</tr>
</tbody>
</table>

*D is the test statistic for the Kolmogorov-Smirnov test.
Table 3.5: Effect of Shroomroot on students’ mycorrhizal content engagement (n=52).

<table>
<thead>
<tr>
<th>Item</th>
<th>Maximum score</th>
<th>Pre-test mean</th>
<th>Post-test mean</th>
<th>Mean change</th>
<th>D*</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Plant nutrition is interesting.”</td>
<td>5</td>
<td>3.8±0.7</td>
<td>4.0±0.6</td>
<td>0.2±0.6</td>
<td>0.058</td>
<td>1</td>
</tr>
<tr>
<td>“I would like to learn more about plant nutrition.”</td>
<td>5</td>
<td>3.8±0.9</td>
<td>4.0±0.8</td>
<td>0.2±0.6</td>
<td>0.096</td>
<td>0.970</td>
</tr>
<tr>
<td>“Mycorrhizae and mycorrhizal networks are interesting.”</td>
<td>5</td>
<td>3.7±0.8</td>
<td>4.0±0.7</td>
<td>0.4±0.7</td>
<td>0.269</td>
<td>0.046*</td>
</tr>
<tr>
<td>“I would like to learn more about mycorrhiza and mycorrhizal networks.”</td>
<td>5</td>
<td>3.9±0.7</td>
<td>3.9±0.8</td>
<td>0.1±0.8</td>
<td>0.077</td>
<td>0.998</td>
</tr>
</tbody>
</table>

*D is the test statistic for the Kolmogorov-Smirnov test.
Table 3.6: Summary statistics of students’ game skill and engagement (n=52).

<table>
<thead>
<tr>
<th>Overall and component of score</th>
<th>Style of question</th>
<th>Maximum score/norm-</th>
<th>Absolute mean/normalized mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>alized score</td>
<td></td>
</tr>
<tr>
<td><strong>Game skill</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>level reached</td>
<td>N/A</td>
<td>27/20</td>
<td>22.0±2.5/15.6±1.8</td>
</tr>
<tr>
<td>ease of play</td>
<td>Numbered list</td>
<td>12/5</td>
<td>11.0±1.7/4.6±0.7</td>
</tr>
<tr>
<td>frustration of play (scored in reverse)</td>
<td>Likert</td>
<td>5</td>
<td>3.7±0.9</td>
</tr>
<tr>
<td>understand instructions</td>
<td>Likert</td>
<td>5</td>
<td>3.1±1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Game engagement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>quantity of reward boxes read</td>
<td>N/A</td>
<td>20/20</td>
<td>13.3±2.2/13.8±2.4</td>
</tr>
<tr>
<td>amount of time playing vs watching</td>
<td>Likert-esque</td>
<td>5</td>
<td>2.9±1.2</td>
</tr>
<tr>
<td>enjoyment of playing/watching</td>
<td>Likert</td>
<td>5</td>
<td>3.9±1.0</td>
</tr>
<tr>
<td>enjoyment of reading reward boxes</td>
<td>Likert</td>
<td>5</td>
<td>4.0±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Chapter 4: Conclusion

Mycorrhizae and mycorrhizal networks are important components of complex ecosystems. I did not find support for defense signal transfer via MNs, but my research supported the correlation of jasmonate pathway response (as measured by AOC expression) between donors and receivers in the pot system. With respect to communicating some of the basic concepts of these systems, the action-based game Shroomroot succeeded in increasing students’ knowledge and tended to have a positive impact on attitudes towards mycorrhizas and MNs. These results suggest promising avenues for future work using action-based game features to communicate content about plant and fungal systems.

4.1: Greenhouse experiment

In the greenhouse experiment I did not find evidence for signal transfer via MNs. MeJA successfully stimulated both AOC and ACS2 in donors, but either failed to stimulate an appropriate magnitude or type of defense response to cause signal transfer, or signal transfer occurred but measurement was obscured by receiver seedlings experiencing ongoing stress response (perhaps due to the unwanted infestation of the experimental pots by spider mites). There were indications that the jasmonate pathway was responding similarly in donors and receivers across treatments, suggesting some kind of relationship between donors and receivers. This may be because of positive feedback of jasmonate signals going back-and-forth
between donor and receiver seedlings, or because of joint response to spider mites or other environmental stressors. In the presence of other stressors, signal transfer may have been moving in both directions between donor and receiver, which has the potential to cause defense responses to synchronize to some extent.

Measuring donor response is an important aspect of understanding both sides of these signal transfer systems, and can aid in interpretation of receiver responses. Including baseline sampling is also important and would have improved interpretation of my results in light of the ongoing stress response observed across seedlings in both control and MeJA treatments. Investigating thresholds for successful MN-mediated signal transfer may be an interesting future avenue for this field – especially given the importance of thresholds and non-linear behaviour in complex adaptive systems such as this (Gorzelak et al., 2015).

My unexpected results highlight the complexity of these systems. Mesocosms like the ones used in this study can lead to unexpected factors such as the presence of spider mites, and are complex in the unseen and capricious mycorrhizal network. However, it is a fine balance between reducing study systems in order to control them versus reducing them beyond relevance to their corresponding natural systems, especially in complex systems that have emergent properties (the whole is greater than the sum of its parts). New methods to study complex systems are being developed (Holland, 2006; Parrott, 2010), but applying these methods to experimental studies is challenging in practice. In this experiment, I attempted to represent natural complexity in the selection of wild soil for mycorrhizal inoculum,
but conditions of the greenhouse, MeJA-stimulus, mesh treatments, and many other elements were artificial.

Further work is needed to elucidate the involvement of defense pathways, and particularly JA, in ectomycorrhizal-mediated interplant signal transfer. In future studies, care should be taken to induce a substantial donor response; for example, it may be beneficial to use more than one mode of stimulus where possible (having both natural and artificial induction treatments). Continued work is also needed to understand the details of potential mechanisms involved in all forms of mycorrhizal networks. Song et al. (2014) show that the jasmonate pathway is involved in defense signaling via MNs, but due to the specific nature of the transgenic tomato mutations, jasmonic acid itself was not fully isolated as the signal molecule (spr2 mutant has loss-of-function a few steps upstream of JA, allowing the small potential for in-between steps in the pathway to be responsible for signal transfer, such as \( \alpha \)-linolenic acid). In addition, positive feedbacks in the jasmonate response (jasmonic acid can stimulate the biosynthesis of jasmonic acid) generally complicate interpretation of jasmonate responses in receivers. Methods such as using \( \text{C}_{13} \)-labeled jasmonic acid, and isolation of compounds in the hyphal tissues in between plants, may prove the most definitive way to test whether JA is indeed responsible for carrying the defense-related information from donor to receiver.

**Another potential mechanism**

Johnson and Gilbert suggest signal transfer could occur in multiple forms – as chemical or electric signals (2015). All currently proposed mechanisms involve a
compound or electrical potential moving between plants as a “messenger” of information. As a final comment, I would like to propose an alternative explanation for the MN-mediated signal transfer observed in other studies – I don’t argue that it is necessarily more likely, but perhaps worth consideration.

Song et al. (2015) showed that carbon transfer on the ectomycorrhizal network increases from donor plants to receiver plants in the presence of a defensive threat. Other studies have shown similar patterns of carbon transfer within MNs in the face of donor stress (Simard et al., 2002; Pietikäinen and Kytöviita, 2007).

In the face of an influx of carbon from a stressed host plant, associated mycorrhizal fungi seem to respond by flushing some of this additional carbon to neighbouring plant partners. This carbon transfer in both exchanges – plant to fungus and fungus to plant - could of course take the form of various compounds in different proportions.

Is it conceivable that the mycorrhizal fungi would change their behaviour when the amount and type of compounds received in resource transfer suddenly shifted, and that the neighboring plant receiving a flush of carbon-containing compounds would do the same? If the sudden changes in amount and type of resource exchange along the network were historically associated with stress occurring somewhere on the network, then the magnitude and type of resources being exchanged could provide information without there needing to be a single actual signal molecule or electrical impulse.
The puzzling aspect of this mechanism is whether fungi or plants can “interpret” this information, and this may be an argument for why a signal molecule is more likely. However, if these changes in resource exchange dynamics were consistent enough in their indication of some form of nearby defensive threat, it may have been evolutionarily advantageous for plants and fungi to respond to sudden changes in resource exchange dynamics in particular ways, such as mounting a defense response. This is an explanation for how information might flow based on changes in relationship as opposed to via the use of a “messenger,” and offers an alternative mechanism for MN-mediated interplant signal transfer.

The study performed in this thesis does not separate between potential mechanisms. However, the negative results found here should not be interpreted to mean that interplant signal transfer never occurs via MNs. Previous studies show strong evidence for this phenomenon, and future studies must account for the complexity of these systems. That there are limits on when and how signal transfer occurs is not entirely surprising, and one next step is to clarify the context of MN-mediated signal transfer. To account for complexity, I recommend that future studies take care to incorporate (where possible) the numerous natural factors that may be important to stimulating signal transfer (for example, using a natural defense stimuli instead of or in addition to MeJA). Elucidation of these signal transfer patterns via mycorrhizal networks will allow deeper understanding of community-level responses to defensive threats, as well as providing insight into the balance of facilitation and competition in forest ecosystems.
4.2: Shroomroot development and intervention

As a part of this thesis, I successfully developed the game Shroomroot, and performed an exploratory evaluation. I discuss development in more detail in the Appendix, and will only present conclusions related to the evaluation here.

Intervention with Shroomroot was successful at increasing 2nd year undergraduate students’ knowledge acquisition of mycorrhizal ecology learning objectives, and had a trending positive impact on their attitudes towards mycorrhizae and mycorrhizal ecology. This change in learning was significant in the context of a fairly limited intervention taking place over 50 mins with little to no content-based instructor support. Results from this study suggest that this approach is potentially fruitful for communicating basic principles of mycorrhizal ecology to 2nd year undergraduates.

To my knowledge this is the first study demonstrating the potential for an action-based game to increase knowledge towards concepts of plant and fungal ecology in the form of the mycorrhizal symbiosis. Other ecological game-based interventions have focused on animal and human-oriented systems (Cross et al., 1993; Sarab et al., 2009; Schaller et al., 2009; Wrzesien and Alcañiz Raya, 2010), while other botanical game- or technology-based interventions have not been in the format of action games (Moreno et al., 2002; Mehrer et al., 2012; Culp et al., 2015).

Recent work in the science of plant behaviour has emphasized the animal-like abilities of plant roots to seek nutrients and water (Hodge, 2004, Prieto et al., 2012; Cahill et al., 2010), and potentially perceive and react to electrical or sonic underground signals (Balüska and Mancuso, 2009; Gagliano et al., 2012).
Shroomroot represents this animal-like behaviour of plants to provide an action-oriented window into mycorrhizal ecology. The assessments used here were exploratory and did not measure whether students’ empathy with plants or fungi was affected by the game, but the speculation is that the more tangible, experience-oriented style of game may have impacted Shroomroot’s success at affecting learning and potentially attitudes. Future work with more detailed assessments would be needed to untangle the mechanisms behind Shroomroot’s effects on student learning and attitudes.

In addition to the importance of understanding mycorrhizal ecology for forest managers, forest ecologists, soil scientists, and other natural resource professions, there is currently a gap in the general public’s understanding between the popularized information about MN-mediated plant communication and the fundamental processes and principles of mycorrhizal ecology and MNs. Shroomroot was developed to build and support this excitement around MN-mediated plant communication, while presenting some of the key basic background about mycorrhizal ecology. Shroomroot also has the potential to be useful for other soil science, forest ecology, or even biology courses that aim to encourage students to think about components of biological systems that are unseen and perhaps harder to visualize. However, this is an exploratory study and like any intervention, care would need to be taken to explore whether the results found here are applicable to other contexts.
Limitations of study

There were some limitations to this study. As described in section 3.4 there was no comparison to traditional instruction, making it impossible to know whether Shroomroot would improve on previous techniques. Because any change in teaching method can involve investment of time and effort, it can be useful to show evidence for a new method being worth this investment. This study shows Shroomroot has the potential to increase student knowledge of and engagement with mycorrhiza without instructional support, suggesting minimal effort may be required to see some degree of positive outcome. Still, future use of Shroomroot as an in-class activity would have to be considered for its comparative efficiency – is the amount of content delivered balanced appropriately by the fact that playing the game may require an entire lecture session? An integration structure in which Shroomroot is more of a supporting tool played at home may be more appropriate and efficient, and would be useful to study further.

Finally, as mentioned in section 3.4, the assessments were exploratory, and therefore limited in scope and sensitivity. In particular, the aggregate variables of game skill and game engagement were calculated from very few items, making it impossible to properly carry out any sort of factor analysis. No in-depth interviews or qualitative analysis was performed. While this exploratory, purely quantitative format was used in order to create a relatively brief but broad-reaching assessment appropriate for the time constraints of the intervention, it limits the sensitivity of the results.
Future directions

Perceptions of myself and the instructors involved suggest that while Shroomroot had a significant impact on student learning, the intervention may have been even more successful with support. Scaffolding and other forms of support have been seen to greatly improve integration of games into the classroom (Connolly et al., 2012).

One relatively simple format of support would be to include guided questions with Shroomroot gameplay. Instead of having students take anonymous tests before and after Shroomroot (as done in this study), questions could be provided that were intended to be answered alongside gameplay. These questions could then be collected and returned to students after marking (in this study students did not receive feedback on assessments performed). Content items from the assessments in this study are potential candidates for guiding questions. Note that in the following discussion I will refer to the overarching content-related “questions” as they are numbered on the assessments (see Appendix A3.3 and A3.4), some of which contain multiple correct responses (generating the 24 content items used in the analysis).

In particular, one could use a mixture of basic questions regarding the mycorrhizal symbiosis (most basic: content questions 1 and 2, slightly more challenging: 3, 4, 5 and 6) and questions that require more synthesis of information and prediction of system behaviour (questions 7, 8, and 9). To aide selection of potential questions, Figure 4.1 is provided to show which of the specific 24 content items were best addressed by Shroomroot in this study. One consideration is that
the most fruitful guided questions may be those specific items that are SR-addressed (labeled in black text, Figure 4.1), but showed little to no increase in student content knowledge from unfacilitated gameplay in this study (e.g. 3b, 4a, 7b, 8a, 8b). More focused student attention, in addition to post-gameplay correction of responses to these guided questions by instructors, may serve to fill the knowledge gap that unfacilitated gameplay could not. Particular emphasis on the more complex questions (7, 8, 9) would also allow more material for in-depth discussion, but this may go beyond learning objectives for Introduction to Soil Science relative to mycorrhizae and mycorrhizal networks. While going beyond learning objectives is not necessarily a concern, taking the time to perform the discussion may not be desirable in light of the many other learning objectives in Introduction to Soil Science beyond mycorrhiza and mycorrhizal networks.

If more extensive discussion is desired, the Generate-Evaluate-Modify (GEM) model of support is one promising framework for facilitating integration of games into the classroom. GEM is a form of inquiry-based learning, in which students are directed to address content in ways similar to scientific thinking – observing evidence, generating hypotheses, testing these hypotheses, and adjusting understanding accordingly (Khan, 2007). GEM specifically involves having students generate predictions, evaluate these predictions, and then modify them based on observations and discussions (for a nice example of technology-oriented GEM, see Khan, 2012). Discussion surrounding content questions 7, 8, and 9 from the assessment could potentially be led using a GEM framework – by having students give a short answer response to each question (with minor modification to current
question wording) before playing the game (“generate”), having them play the game and watch for gameplay and decisions related to the question (“evaluate”), and then having them supply a post-gameplay response that describes how their prediction matches or does not match what they saw and chose to do within the game (“modify”).

In terms of further research, this study solely focuses on short-term retention, leaving Shroomroot’s effect on long-term retention of both content knowledge and content engagement unknown. Rondon et al. found that students who used the anatomy and physiology-related software Anatesse 2.0 (which contains a selection of game-like activities) experienced equivalent learning gains to students who received traditional instruction when measuring short-term outcomes, but experienced significantly lower gains than the traditional instruction group when measuring long-term retention (2013). Measurement of long-term retention effects of DGBL is limited (Rondon et al. 2013), but at least one other study found an opposite effect. Egenfeldt-Nielsen found that students who played a history-oriented simulation game showed no difference in short-term content knowledge gains than a traditional instruction group, but had higher long-term retention (2005). Identifying how Shroomroot affects long-term retention of mycorrhizal content could be an interesting future research direction.

Lastly, it is also possible that more games could be developed in this area of plant-centric action-games. There is much content that Shroomroot does not address that may be well-suited to a similar style game that animates non-animals at macroscopic scales, including aspects of plant nutrition, plant behaviour and
fungal ecology. Of course, it would be important to evaluate the educational efficacy of these games as well, and not assume the automatic extension of similar positive outcomes. At a time when plant behaviour and MN-mediated plant communication are changing our perspectives on plants and fungi, there are exciting opportunities to incorporate these new perspectives into educational games.

4.3: Final conclusions

This thesis is unified by a drive to deepen our understanding of the mycorrhizal symbiosis and mycorrhizal networks, both in terms of their internal mechanisms and processes and in terms of the challenges presented by teaching and learning about them. Mycorrhizae and mycorrhizal networks are a fruitful part of complex ecological systems, presenting challenges for both researchers and students – it can be hard to capture their dynamics in a reductionist greenhouse experiment, and hard to visualize and build intuition about them (but creative tools like Shroomroot can help!).

In the first study, I explored the facilitation of plant communication via mycorrhizal networks and in the second, the communication of mycorrhizal ecology via the use of a digital game. Communication as an abstract concept is at the heart of many complex systems – at its most fundamental it is the movement of information between parts of a system. Without interactions, any system is just a set of parts, and no more than the sum of them. Interactions like communication are what cause complex systems to have emergent properties, and understanding these interactions and emergent properties is crucial to adaptive management.
Communication as a more emotional, human-oriented concept, as a mechanism for vital and grounding connection between individuals, is something we are both excited and hesitant to recognize in systems very different from our own (such as plants and fungi). Time will tell whether some of the more human-seeming aspects of communication are present – intent, for example – but in the meantime, I believe that opening our hearts and minds to the potential of plant and fungal communication is a valuable exploration in science and in our emotional relationship with nature. Deepening our understanding of mycorrhizal systems has the potential to improve the adaptability and sustainability of our ecosystem management, as well as impact our social and emotional relationships with forests and other ecosystems.
Figure 4.1: Raw score improvement across students between pre-test and post-test by individual content knowledge items. Items labeled in red are non-SR-addressed items. Values above zero indicate that more students gave correct answers to that specific item on the post-test than on the pre-test.
References


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Smith SE., Read DJ. 2008. Mycorrhizal Symbiosis. Copyright © 2008. Elsevier Ltd. All rights reserved.


Appendix

Chapter 2: Greenhouse study

Table A2.1: ANOVA results of Donor seedling relative expression. AOC = allene oxide cyclase (jasmonate pathway gene); ACS = aminocyclopropane carboxylic acid (ethylene pathway gene). Relative expression was calculated using the ddCt method after calibrating for amplicon-specific amplification efficiencies in LinRegPCR (2009). α=0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Factor</th>
<th>SS</th>
<th>df</th>
<th>F value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOC</td>
<td>Time step</td>
<td>3.785</td>
<td>1</td>
<td>0.4108</td>
<td>0.536</td>
</tr>
<tr>
<td></td>
<td>Residuals</td>
<td>92.145</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS2</td>
<td>Time step</td>
<td>14.838</td>
<td>1</td>
<td>2.2956</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>Residuals</td>
<td>58.17</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A2.2: ANOVA results of Receiver seedling relative expression. AOC = allene oxide cyclase (jasmonate pathway gene); ACS = aminocyclopropane carboxylic acid (ethylene pathway gene). Relative expression was calculated using the ddCt method after calibrating for amplicon-specific amplification efficiencies in LinRegPCR (2009). Results displayed for AOC are with one major outlier removed (Cook’s distance >0.5). α=0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Factor</th>
<th>SS</th>
<th>df</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOC</td>
<td>Time step</td>
<td>5.5887</td>
<td>1</td>
<td>5.7011</td>
<td>0.04071*</td>
</tr>
<tr>
<td></td>
<td>Mesh</td>
<td>1.6036</td>
<td>2</td>
<td>0.8179</td>
<td>0.47165</td>
</tr>
<tr>
<td></td>
<td>Time step*Mesh</td>
<td>5.0112</td>
<td>2</td>
<td>2.556</td>
<td>0.13211</td>
</tr>
<tr>
<td></td>
<td>Residuals</td>
<td>8.8225</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS2</td>
<td>Time step</td>
<td>3.422</td>
<td>1</td>
<td>0.3978</td>
<td>0.5424</td>
</tr>
<tr>
<td></td>
<td>Mesh</td>
<td>3.156</td>
<td>2</td>
<td>0.1835</td>
<td>0.8351</td>
</tr>
<tr>
<td></td>
<td>Time step*Mesh</td>
<td>6.889</td>
<td>2</td>
<td>0.4004</td>
<td>0.6803</td>
</tr>
<tr>
<td></td>
<td>Residuals</td>
<td>86.029</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A2.2: Foliar nutrients

Mesh size had a trending impact on Ca ($p=0.0074$, $\alpha=0.003$ for 15 comparisons). Seedlings in 35µm mesh had significantly higher foliar calcium (77±8.3%) than seedlings in both 250µm mesh (63±13%, $p=0.04$) and 0.5µm mesh (58±6.0%, $p=0.007$). No other nutrients were trending or significantly different across mesh sizes. When overlaying foliar nutrients onto an NMDS of the morphotype data from the same subset of samples, potassium (K) alone was a significant "environmental" variable that was significantly correlated to the distribution of morphotypes ($p=0.028$). Effects on Ca or K are not a common response to mesh bags and/or mycorrhizal network connectivity (Asay, 2013; Luow, 2015) and are difficult to interpret.
Figure A2.1: Foliar macronutrient concentration (as percent) across mesh sizes. Error bars are standard error of the mean.
Figure A2.2: Foliar micronutrient concentration (mg/Kg) across mesh sizes. Error bars are standard error of the mean.
Chapter 3: Shroomroot study

A3.1: Additional notes about ethics:

If students retracted consent on either test, results for the paired test for that username were also removed from the study (this occurred twice). Consent forms used can be viewed in the Appendix. Participation was anonymous, with students using a non-identifying username to link their pre-test and post-test results. No class marks are associated with this study nor can marks be linked to individual assessments. The data were and will only be used as part of this study.

All paper versions of assessments were kept in a locked cabinet to which only the main graduate student researcher, JAM, had access. Digital data was kept on a pass-word protected laptop also only accessible to JAM. Student participation was anonymous and therefore neither JAM nor course professors were aware of which particular students filled out the pre and/or post-test.

A3.2: Additional Details on Development of Shroomroot

A3.2.1: Process of development

In the early stages of Shroomroot development, I worked alone, having basically no previous programming experience. I attempted to build the game in a somewhat user-friendly gaming engine called Stencyl (Stencyl LLC, California, USA), but this proved extremely challenging and I made little-to-no real progress. I mention this early stage because it informed my views about the efficiency and power of collaboration, and so to anyone else embarking on a digital project
involving programming, graphic design, gameplay design, science (or any other content area), and education, I join my voice to the many out there who strongly recommend collaboration as a way forward in creating educational games (Connolly et al., 2014).

Following this initial solo attempt, I partnered with Area Denial Games (Vancouver, BC) to build Shroomroot. I primarily worked with Christopher Adderley and Alex Catamo. Construction of the game occurred from June to November 2014, and took approximately 750 man hours. Chris was responsible for the programming, user interface, and game design, Alex was responsible for the sounds, and I was responsible for the graphic design, in-level gameplay, and game design. At each step, decisions were made that had to balance programming limitations, entertainment value, educational goals, and scientific accuracy. To address a perceived tendency in educational games to be overly serious, I tended to make decisions with a bias towards entertainment value.

Beta testing of the game began in November 2014. Several graduate students with a wide range of gaming experience and I played Shroomroot, reviewed the Rewards section text, and generally clicked through the various screens to test features and functionality of the game. As a result of this beta-testing, the game was made a bit easier for non-gaming experienced players and the wording of tutorials was clarified.
A3.2.2: Enriching self-competition aspect in Shroomroot

In every level, if the player finishes the level with all resources above a certain threshold, their tree is said to have “grown.” If they finish with all resources above an even higher threshold, their tree is said to have “reproduced,” and they are recorded to have produced one fir cone for that level. Shroomroot has a level-selection page from which players can go directly to any level they have previously unlocked (by beating it once), and there is a young Douglas-fir pictured on the level-selection page that gets taller for every level in which the player “grows” (if they “reproduce”, that counts as also “growing”). This capacity to finish progressively higher within the same levels encourages mastery of the game and aims to create a sense of self-competition.

A3.2.3: Features left out of Shroomroot

Initial game design planned for some features that were not included in the finished game. One of these features was to incorporate in-game tracking of how quickly the player achieved certain milestones, such as moving between levels or unlocking certain rewards. Another unrealized feature was for each end-of-level screen to show icons of rewards that had been unlocked by the player during the play of that level. One other desired feature was to allow players to select a level of difficulty, allowing experienced gamers to move through Shroomroot at a faster root-growing pace and with more limited resources, while less experienced gamers could grow their root slower with more abundant resources.
Shroomroot also has some minor issues with visual presentation. The resource bars at the top left of the screen have the potential to obscure the view of the root growing, and are particularly disproportioned in the Android version of the game. Text in the end-of-level screen and a few of the rewards overextends beyond the graphic button or box behind it.
Table A3.1: Text shown for all “Rewards” of the Shroomroot game, showcasing concepts presented via rewards.

<table>
<thead>
<tr>
<th>Reward title</th>
<th>Associated text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collected 1st Water!</td>
<td>Water helps plants transport nutrients within their bodies and keeps their leaves or needles turgid.</td>
</tr>
<tr>
<td>Collected 1st Nitrogen!</td>
<td>Nitrogen is used by plants for many things, including enzymes and proteins. In Shroomroot Nitrogen gives you a wider collection radius, to represent its impact on a plant’s ability to grow root hairs and produce enzymatic exudates.</td>
</tr>
<tr>
<td>Collected 1st Phosphorus!</td>
<td>Phosphorus is also used for many things, including ATP (adenosine tri-phosphate) and providing the backbone for DNA. In Shroomroot phosphorus causes you to go faster, representing its role in ATP.</td>
</tr>
<tr>
<td>Collected 30 Waters!</td>
<td>Plants start to wilt even when there is technically still water in the soil. This is because the soil can contain water that is too tightly bound to soil particles for roots to access.</td>
</tr>
<tr>
<td>Collected 20 Nitrogens!</td>
<td>Nitrogen in Shroomroot takes two forms - inorganic and organic. Inorganic nitrogen that is usable by plants includes nitrate, nitrite, and ammonium.</td>
</tr>
<tr>
<td>Collected 20 Phosphoruses!</td>
<td>Phosphorus in Shroomroot can also be inorganic or organic. Forms of inorganic phosphorus in soil are commonly insoluble, which makes them unavailable for plant uptake. This means it can often be a limiting resource.</td>
</tr>
<tr>
<td>Grew your roots 1 meter!</td>
<td>In Shroomroot you grow entirely sideways, but the first thing Douglas-fir trees do is send out a vertical taproot. You can just see the taproot of your seedling in the Level Selection Screen underneath the Level 2 button.</td>
</tr>
<tr>
<td>Grew your roots 5 meters!</td>
<td>While we can’t easily see into the world below our feet, it’s cool to realize that oftentimes more than 50% of an ecosystem’s biomass exists below the surface.</td>
</tr>
<tr>
<td>Collected 1st Organic Phosphorus!</td>
<td>Organic phosphorus is harder for plants to directly digest, but mycorrhizal fungi can help. In Shroomroot, the mycorrhizal association is the only way to get OP, but in reality plant roots can access some OP even when non-mycorrhizal.</td>
</tr>
<tr>
<td>Collected 1st Organic Nitrogen!</td>
<td>Like with organic phosphorus, mycorrhizal associations help plants access organic nitrogen. This is because fungi can produce digestive enzymes that breakdown organic matter. Again, in reality plants can access some small quantities of ON even when non-mycorrhizal.</td>
</tr>
<tr>
<td>Made 1st Mycorrhizal Network Link!</td>
<td>Mycorrhizal networks refer to communities of plants connected by fungal hyphae in the soil, or in other words communities of fungi connected by plants roots in the soil. &quot;Myco&quot; for fungus, &quot;rrhizal&quot; for root. Shroomroot is a very tree-centric game, but there are also complex decisions and behaviours going on from the fungal perspective.</td>
</tr>
<tr>
<td>Made 1st Link with a Mother tree!</td>
<td>&quot;Mother trees&quot; is the name given to old mature trees that have established many connections on a mycorrhizal network. Research has found that resources often move from these trees into nearby seedlings. In Shroomroot this always happens, but in nature it is of course more complex, and resources can sometimes move the other way (seedling to mature tree).</td>
</tr>
</tbody>
</table>
Table A3.1: Text shown for all “Rewards” of the Shroomroot game, showcasing concepts presented via rewards (cont’d).

<table>
<thead>
<tr>
<th>Reward title</th>
<th>Associated text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Made 1st Link with a Seedling!</td>
<td>Seedlings can also be connected on the network, and can gain or lose nutrients. One of the main theories for whether a seedling &quot;gains&quot; or &quot;loses&quot; is called the source-sink concept. The basic idea is that specific nutrients flow down concentration gradients from sources to sinks.</td>
</tr>
<tr>
<td>Made 5 Mycorrhizal Network Links!</td>
<td>In Shroomroot, the resources you receive from or lose to seedlings is based on the source-sink concept. If you have more of a nutrient than a seedling, you lose nutrients to that seedling (you are a source, it’s a sink), and vice versa.</td>
</tr>
<tr>
<td>Made 10 Mycorrhizal Network Links!</td>
<td>Carbon also has been seen to travel between plants on mycorrhizal networks. This is particularly surprising because it represents a reversal to the normal direction of resources - normally mycorrhizal fungi give plants nutrients and plants give fungi carbon. When carbon travels from one plant to another on a mycorrhizal network, this means the fungi are giving the plants carbon.</td>
</tr>
<tr>
<td>Made 20 Mycorrhizal Network Links!</td>
<td>In Shroomroot, mycorrhizal networks appear to occur only between very nearby roots. This is a gameplay element, and in reality trees can be connected by much longer lengths of hyphae. Roots that are connected on the mycorrhizal network can actually be meters away from each other.</td>
</tr>
<tr>
<td>Made 1 Pinecone!</td>
<td>One theory for plant growth is that they will put energy into reproduction only if they already have more than enough resources to grow and survive. If they have few resources they only grow and survive. If they have even fewer, they focus only on survival.</td>
</tr>
<tr>
<td>Made 5 Pinecones!</td>
<td>Shroomroot has a very simple system in which you have to have all of your resources above a certain level to &quot;grow&quot; and then above a higher level to &quot;reproduce.&quot; In reality this can be more complex, as the resource thresholds can depend on each other and on the specific plant.</td>
</tr>
<tr>
<td>Made 10 Pinecones!</td>
<td>Congratulations for getting one of the hardest rewards in the game! You have done well growing as a plant, but you might not want to consider it as a career unless you can figure out how to be photosynthetic.</td>
</tr>
<tr>
<td>Collected 100 waters!</td>
<td>You’ll find that playing with myco mode on helps you get water from harder to reach areas, because the collection radius is much bigger with mycorrhizae than without. This represents the way mycorrhizal fungi grow their mycelium throughout the soil and do help plants access hard-to-get water.</td>
</tr>
</tbody>
</table>

*OP = organic phosphorus. ATP (adenosine tri-phosphate) is a molecule used by cells to transport and provide energy for cellular processes.
Table A3.2: Kruskal-Wallis test of content knowledge score improvement vs whether question content was expected to be addressed by Shroomroot. Question categories were “not-SR-addressed”, “Rewards only”, and “Gameplay and Rewards.” Asterisk denotes significant p-value. $\alpha=0.05$.

<table>
<thead>
<tr>
<th>Question Category</th>
<th>$\chi^2$</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Question Category</td>
<td>8.4051</td>
<td>2</td>
<td>0.015*</td>
</tr>
</tbody>
</table>
Table A3.3: Effect of Shroomroot on students' content knowledge, overall and by specific question. Exact question text can be found in section A3.3 where the pre-test is shown. "SR-add" = SR-addressed.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pretest mean</th>
<th>Posttest mean</th>
<th>Mean change</th>
<th>df</th>
<th>D</th>
<th>D- value</th>
<th>Question content</th>
<th>Addressed by Shroomroot?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (n=24)</td>
<td>9.7± 3.5</td>
<td>12.3± 3.5</td>
<td>2.6±3.5</td>
<td>51</td>
<td>0.289</td>
<td>0.026</td>
<td>All</td>
<td>Mix</td>
</tr>
<tr>
<td>SR-add (n=17)</td>
<td>6.1±3.4</td>
<td>8.9±3.4</td>
<td>2.8±3.5</td>
<td>51</td>
<td>0.327</td>
<td>0.008</td>
<td>Only content addressed by Shroomroot</td>
<td>Yes</td>
</tr>
<tr>
<td>non-SR-add (n=7)</td>
<td>3.6±1.0</td>
<td>3.4±1.1</td>
<td>-0.3±1.5</td>
<td>51</td>
<td>0.212</td>
<td>0.195</td>
<td>Only content not addressed by Shroomroot</td>
<td>No</td>
</tr>
<tr>
<td>Question 1</td>
<td>0.52</td>
<td>0.77</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>Describe mycorrhiza</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 2</td>
<td>0.56</td>
<td>0.77</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td>Describe mycorrhizal network</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 3a</td>
<td>0.54</td>
<td>0.79</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>Plant benefits: P or N</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 3b</td>
<td>0.33</td>
<td>0.42</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
<td>Plant benefits: water</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 3c</td>
<td>0.37</td>
<td>0.73</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
<td>Plant benefits: resources from neighbours</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 3d</td>
<td>0.31</td>
<td>0.15</td>
<td>-0.15</td>
<td></td>
<td></td>
<td></td>
<td>Plant benefits: increased defense</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 3e</td>
<td>0.54</td>
<td>0.75</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td>Plant benefits: increase resource access</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 4a</td>
<td>0.33</td>
<td>0.40</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td>Plant costs: C to fungus</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 4b</td>
<td>0.19</td>
<td>0.62</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
<td>Plant costs: lose N to fungus</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 4c</td>
<td>0.19</td>
<td>0.33</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td>Plant costs: use defense resources</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 4d</td>
<td>0.77</td>
<td>0.85</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td>Plant costs: increased risk of infection</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 5a</td>
<td>0.40</td>
<td>0.52</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td>Fungi benefits: C from plant</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 5b</td>
<td>0.19</td>
<td>0.19</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td>Fungi benefits: increased defense</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 5c</td>
<td>0.73</td>
<td>0.56</td>
<td>-0.17</td>
<td></td>
<td></td>
<td></td>
<td>Fungi benefits: increased digestive ability</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 6a</td>
<td>0.38</td>
<td>0.67</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td>Fungi costs: P and N to plant</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 6b</td>
<td>0.06</td>
<td>0.17</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td>Fungi costs: lose C to plant</td>
<td>Rewards only</td>
</tr>
<tr>
<td>Question 6c</td>
<td>0.60</td>
<td>0.38</td>
<td>-0.21</td>
<td></td>
<td></td>
<td></td>
<td>Fungi costs: dependent on the plant for water</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 6d</td>
<td>0.81</td>
<td>0.88</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td>Fungi costs: increased risk of attack</td>
<td>Not addressed</td>
</tr>
<tr>
<td>Question 7a</td>
<td>0.29</td>
<td>0.56</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
<td>Non mycorrhizal - rich in inorganic resources</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 7b</td>
<td>0.69</td>
<td>0.56</td>
<td>-0.13</td>
<td></td>
<td></td>
<td></td>
<td>Non mycorrhizal - rich in organic resources</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 7c</td>
<td>0.12</td>
<td>0.21</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
<td>Non mycorrhizal - relatively moist</td>
<td>Play and rewards</td>
</tr>
<tr>
<td>Question 8a</td>
<td>0.23</td>
<td>0.19</td>
<td>-0.04</td>
<td></td>
<td></td>
<td></td>
<td>Most likely to get resources on network</td>
<td>Rewards only</td>
</tr>
<tr>
<td>Question 8b</td>
<td>0.15</td>
<td>0.06</td>
<td>-0.10</td>
<td></td>
<td></td>
<td></td>
<td>Which resource would it be?</td>
<td>Rewards only</td>
</tr>
<tr>
<td>Question 9</td>
<td>0.40</td>
<td>0.67</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
<td>What's a mother tree</td>
<td>Rewards only</td>
</tr>
</tbody>
</table>

*D is the test statistic for the Kolmogorov-Smirnov test.
Table A3.4: “Meta” (M) statements about whether students thought their learning or engagement increased as a result of Shroomroot. Myco=mycorrhiza and mycorrhizal networks, PN=plant nutrition. Exact text for each meta statement can be found in section A3.4 where the post-test is shown.

<table>
<thead>
<tr>
<th>Direct analysis statement short form</th>
<th>Mean</th>
<th>Strongly Disagree</th>
<th>Disagree</th>
<th>Neutral</th>
<th>Agree</th>
<th>Strongly Agree</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1: understanding, myco</td>
<td>3.9±0.6</td>
<td>0</td>
<td>0</td>
<td>11(21%)</td>
<td>33 (63%)</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>M2: engagement, myco</td>
<td>3.9±0.6</td>
<td>0</td>
<td>0</td>
<td>11(21%)</td>
<td>34 (65%)</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>M3: understanding, PN</td>
<td>3.8±0.6</td>
<td>0</td>
<td>2 (4%)</td>
<td>11(21%)</td>
<td>35 (67%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>M4: engagement, PN</td>
<td>3.7±0.7</td>
<td>0</td>
<td>2 (4%)</td>
<td>18 (35%)</td>
<td>28 (54%)</td>
<td>4 (8%)</td>
</tr>
</tbody>
</table>
Table A3.5: Distribution of students’ responses for mycorrhizal content engagement (CE) items. CE1 = “Plant nutrition is interesting,” CE2 = “I want to learn more about plant nutrition,” CE3 = “Mycorrhizae and mycorrhizal networks are interesting,” CE4 = “I want to learn more about mycorrhiza and mycorrhizal networks.” Values shown are percentages of responses for each item.

<table>
<thead>
<tr>
<th>Item</th>
<th>Strongly Disagree</th>
<th>Disagree</th>
<th>Neutral</th>
<th>Agree</th>
<th>Strongly Agree</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE1 pre</td>
<td>0</td>
<td>8</td>
<td>15</td>
<td>65</td>
<td>12</td>
</tr>
<tr>
<td>CE1 post</td>
<td>0</td>
<td>2</td>
<td>15</td>
<td>65</td>
<td>17</td>
</tr>
<tr>
<td>CE2 pre</td>
<td>2</td>
<td>6</td>
<td>21</td>
<td>56</td>
<td>15</td>
</tr>
<tr>
<td>CE2 post</td>
<td>0</td>
<td>2</td>
<td>25</td>
<td>48</td>
<td>25</td>
</tr>
<tr>
<td>CE3 pre</td>
<td>0</td>
<td>2</td>
<td>42</td>
<td>39</td>
<td>17</td>
</tr>
<tr>
<td>CE3 post</td>
<td>0</td>
<td>2</td>
<td>15</td>
<td>56</td>
<td>27</td>
</tr>
<tr>
<td>CE4 pre</td>
<td>0</td>
<td>2</td>
<td>27</td>
<td>54</td>
<td>17</td>
</tr>
<tr>
<td>CE4 post</td>
<td>0</td>
<td>4</td>
<td>27</td>
<td>44</td>
<td>25</td>
</tr>
</tbody>
</table>
Table A3.6: ANOVAs comparing students’ mycorrhizal content engagement item (CE) with SR-addressed students’ content knowledge. (α = 0.004, comparisons=12). CE1 = “Plant nutrition is interesting,” CE2 = “I want to learn more about plant nutrition,” CE3 = “Mycorrhizae and mycorrhizal networks are interesting,” CE4 = “I want to learn more about mycorrhiza and mycorrhizal networks.”

<table>
<thead>
<tr>
<th>Knowledge</th>
<th>Dep. variable</th>
<th>SS</th>
<th>Df</th>
<th>F value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-test</td>
<td>CE1pre</td>
<td>70.068</td>
<td>3</td>
<td>2.106</td>
<td>0.112</td>
</tr>
<tr>
<td>Post-test</td>
<td>CE1post</td>
<td>18.918</td>
<td>3</td>
<td>0.528</td>
<td>0.665</td>
</tr>
<tr>
<td>Difference</td>
<td>CE1diff</td>
<td>56.160</td>
<td>3</td>
<td>1.613</td>
<td>0.199</td>
</tr>
<tr>
<td>Pre-test</td>
<td>CE2pre</td>
<td>38.755</td>
<td>4</td>
<td>0.808</td>
<td>0.527</td>
</tr>
<tr>
<td>Post-test</td>
<td>CE2post</td>
<td>9.405</td>
<td>3</td>
<td>0.258</td>
<td>0.855</td>
</tr>
<tr>
<td>Difference</td>
<td>CE2diff</td>
<td>56.425</td>
<td>3</td>
<td>1.621</td>
<td>0.197</td>
</tr>
<tr>
<td>Pre-test</td>
<td>CE3pre</td>
<td>166.679</td>
<td>3</td>
<td>6.119</td>
<td>0.001*</td>
</tr>
<tr>
<td>Post-test</td>
<td>CE3post</td>
<td>98.895</td>
<td>3</td>
<td>3.209</td>
<td>0.031</td>
</tr>
<tr>
<td>Difference</td>
<td>CE3diff</td>
<td>60.132</td>
<td>3</td>
<td>1.740</td>
<td>0.171</td>
</tr>
<tr>
<td>Pre-test</td>
<td>CE4pre</td>
<td>15.523</td>
<td>3</td>
<td>0.423</td>
<td>0.737</td>
</tr>
<tr>
<td>Post-test</td>
<td>CE4post</td>
<td>16.698</td>
<td>3</td>
<td>0.464</td>
<td>0.709</td>
</tr>
<tr>
<td>Difference</td>
<td>CE4diff</td>
<td>8.397</td>
<td>4</td>
<td>0.163</td>
<td>0.956</td>
</tr>
</tbody>
</table>
A3.3: APBI 200 Educational Game Pre-test

This survey information will be used to improve and develop the soil biology section of APBI 200. If you give your consent, the information will also be included in a graduate thesis, and potentially a publication about the use of games in higher education. Your information is collected anonymously, and would only be published as aggregate values (i.e. mean, variance, distribution). Saying “yes” will indicate your permission to include your data in the graduate thesis research and potential publication. Saying “no” will exclude your data from the research and potential publication. Your answer is anonymous and your choice will not affect your grade or class standing in any way.

Do you give your permission to have your anonymous responses included in a graduate research thesis and a potential publication?

☐ Yes ☐ No

For more information about the graduate research aspect of this survey, please contact Julia Amerongen Maddison at joules53@gmail.com. She is supervised by Dr. Suzanne Simard in UBC Forestry. If you have any concerns or complaints about your rights as a research participant and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the UBC Office of Research Ethics at 604-822-8598 or if long distance e-mail RSIL@ors.ubc.ca or call toll free 1-877-822-8598.
On the line below, please write a unique username that you can remember for the post-test. (Please do not use an obvious identifier such as your real name, nor something common such as the course name.)

YOU WILL NEED THIS USERNAME ON THE POST-TEST ON MARCH 4th.

PLEASE WRITE IT DOWN SOMEWHERE and/or REMEMBER IT.

Username:_______________________________

Demographic Questions

Please only answer what you feel comfortable answering. Responses are anonymous.

Gender: ____________

Faculty: (LFS, Forestry, Science, etc) ____________

How much do you play video games?
☐ Not at all
☐ A bit
☐ A moderate amount
☐ Quite a lot
☐ Other, please specify... ___________________________

If you play video games, what do you use to play them?
Check all that apply:
☐ Mobile
☐ Computer
☐ Console
☐ Other, please specify... ___________________________

Have you taken FRST 201 (Forest Ecology)?
☐ Yes
☐ No

Content questions

1. Which of the following best describes mycorrhizae?
Choose only one:
They are symbiotic associations that occur between a fungus and a plant root
They are specific plant organs in roots that collect nitrogen and phosphorus
They are symbiotic associations that occur between a decomposer fungus and a soil algae
I don’t know

2. Which of the following best describes a mycorrhizal network?
Choose only one:
☐ Multiple plants and multiple mycorrhizal fungi that are connected together
☐ A single plant with its mycorrhizal fungi that are spread through the soil
☐ Many species of fungi growing in the same patch of soil
☐ I don’t know
3. For PLANTS in mycorrhizal associations, what are the potential benefits of being in a mycorrhizal association? 
Select all that apply:
☐ They may receive phosphorus and/or nitrogen from the fungus 
☐ They may receive water from the fungus 
☐ They may receive resources from neighbouring plants via the mycorrhizal network 
☐ They may have increased defense against non-mycorrhizal fungi and bacteria 
☐ They may have increased ability to access resources in the soil 
☐ I don’t know

4. For PLANTS in mycorrhizal associations, what are the potential costs of being in a mycorrhizal association? 
Select all that apply:
☐ They may give carbon to the fungus 
☐ They may lose nitrogen to the fungus 
☐ They may spend defense resources interacting with the fungus 
☐ They may have an increased risk of infection from other fungi/bacteria 
☐ I don’t know
5. For FUNGI in mycorrhizal associations, what are the potential benefits of being in a mycorrhizal association? Select all that apply:
☐ They may receive carbon from the plant
☐ They may have increased defense against external pathogens compared to decomposer fungi
☐ They may have increased ability to digest plant material in the soil compared to decomposer fungi
☐ I don’t know

6. For FUNGI in mycorrhizal associations, what are the potential costs of being in a mycorrhizal association? Select all that apply:
☐ They may give nitrogen and phosphorus to the plant
☐ They may lose carbon to the plant
☐ They may be dependent on the plant for water
☐ They may have increased risk of attack by pathogens
☐ I don’t know
7. In which of these situations might a plant “choose” not to be mycorrhizal? Select all that apply:
- ☐ When the soil is rich in inorganic resources (such as inorganic forms of nitrogen)
- ☐ When the soil is rich in organic resources (such as organic forms of nitrogen)
- ☐ When the soil is relatively moist
- ☐ I don’t know

8a. Considering a group of plants connected on a mycorrhizal network, which one of the following plants would be most likely to receive resources from neighbouring plants via the mycorrhizal network? Choose only one:
- ☐ A medium-aged tree in a local patch of organic-nutrient-rich soil
- ☐ A medium-aged tree in a local patch of inorganic-nutrient-rich soil
- ☐ A young tree in a local patch of heavy shade
- ☐ A young tree in a local patch of moist soil
- ☐ I don’t know

8b. From the question above (8a), which resource would the selected plant most likely receive? Choose only one:
- ☐ Carbon
- ☐ Water
- ☐ Inorganic nutrients
- ☐ Organic nutrients

9. Within the field of mycorrhizal network ecology, what kind of tree does the “mother tree” concept primarily refer to? Choose only one:
- ☐ An older tree from which carbon flows through the mycorrhizal network to nearby relatives more than to nearby non-relatives
- ☐ An older tree that is more greatly linked on the mycorrhizal network, from which resources tend to flow to younger seedlings
- ☐ An older tree that provides many functions in the forest for multiple organisms, especially animals
- ☐ An older tree that is a canopy dominant and has dispersed seedlings more widely than other trees
Please circle your response to the following statements:

Plant nutrition is interesting.

<table>
<thead>
<tr>
<th>Strongly Agree</th>
<th>Agree</th>
<th>Neutral</th>
<th>Disagree</th>
<th>Strongly Disagree</th>
<th>I don't know</th>
</tr>
</thead>
</table>

I would like to learn more about plant nutrition.

<table>
<thead>
<tr>
<th>Strongly Agree</th>
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<th>Neutral</th>
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Mycorrhizae and mycorrhizal networks are interesting.

<table>
<thead>
<tr>
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<th>Neutral</th>
<th>Disagree</th>
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I would like to learn more about mycorrhizae and mycorrhizal networks.

<table>
<thead>
<tr>
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Thank you for completing the pre-test!
A3.4: APBI 200 Educational Game Post-test

We would like to reaffirm your consent to participate in the graduate research aspect of this survey. Again, if you give your consent, your anonymous responses will be used in a graduate thesis and potential publication about the use of games in higher education. Your information is anonymous, will not be connected to class standing or grades, and will only be published in research as aggregate values (i.e. mean, variance, distribution). A “no” answer will remove both your pre-test and post-test responses from the research study.

Do you give your permission to have your information included in a graduate research project and potential publication?

☐ Yes ☐ No

For more information about the graduate research aspect of this survey, please contact Julia Amerongen Maddison at joules53@gmail.com. She is supervised by Dr. Suzanne Simard in UBC Forestry.

If you have any concerns or complaints about your rights as a research participant and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the UBC Office of Research Ethics at 604-822-8598 or if long distance e-mail RSIL@ors.ubc.ca or call toll free 1-877-822-8598.
Please write your username from the pre-test on the line below.
If you lost or forgot it, please write it to the best of your memory. If you did not take
the pre-test, please write “no pretest.”
Username:______________________________

What section did you play the game in on March 4th?
☐ 001, Maja Krzic’s section, MCML 166
☐ 002, Sandra Brown’s section, ESB 1012

What device did your team use to play the game?
☐ Computer
☐ Android
☐ Other, please specify... ___________________________

Before class on Wednesday, March 4th, had you ever played Shroomroot?
This question will help us interpret our results.
☐ Yes, I played Shroomroot before taking the pre-test
☐ Yes, I played Shroomroot between taking the pre-test and class on March 4th
☐ No, I had heard of Shroomroot but had not played before
☐ No, I had not heard of Shroomroot before

AFTER taking the PRE-TEST, but BEFORE playing the game in class on March
4th, did you look up anything about mycorrhizae?
This question will help us interpret our results.
☐ Yes, I read and/or watched videos about mycorrhizae after taking the pre-test
and before playing the game
☐ No, I didn’t look up anything about mycorrhizae after taking the pre-test and
before playing the game
☐ Other, please specify... ___________________________
Content questions

1. Which of the following best describes mycorrhizae?
Choose only one:
☐ They are symbiotic associations that occur between a fungus and a plant root
☐ They are specific plant organs in roots that collect nitrogen and phosphorus
☐ They are symbiotic associations that occur between a decomposer fungus and a soil algae
☐ I don’t know

2. Which of the following best describes a mycorrhizal network?
Choose only one:
☐ Multiple plants and multiple mycorrhizal fungi that are connected together
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8b. From the question above (8a), which resource would the selected plant most likely receive? Choose only one:

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☐ Water
☐ Inorganic nutrients
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9. Within the field of mycorrhizal network ecology, what kind of tree does the “mother tree” concept primarily refer to? Choose only one:

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☐ An older tree that provides many functions in the forest for multiple organisms, especially animals
☐ An older tree that is a canopy dominant tree and has dispersed seedlings more widely than other trees
I don’t know

**Please circle your response to the following statements:**

**Plant nutrition is interesting. (CE1)**

<table>
<thead>
<tr>
<th>Strongly Agree</th>
<th>Agree</th>
<th>Neutral</th>
<th>Disagree</th>
<th>Strongly Disagree</th>
<th>I don’t know</th>
</tr>
</thead>
</table>

I would like to learn more about plant nutrition. (CE2)

<table>
<thead>
<tr>
<th>Strongly Agree</th>
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<th>Neutral</th>
<th>Disagree</th>
<th>Strongly Disagree</th>
<th>I don’t know</th>
</tr>
</thead>
</table>

Mycorrhizae and mycorrhizal networks are interesting. (CE3)

<table>
<thead>
<tr>
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<th>Agree</th>
<th>Neutral</th>
<th>Disagree</th>
<th>Strongly Disagree</th>
<th>I don’t know</th>
</tr>
</thead>
</table>

I would like to learn more about mycorrhizae and mycorrhizal networks. (CE4)

<table>
<thead>
<tr>
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<th>Disagree</th>
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<th>I don’t know</th>
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</thead>
</table>
Game-related Questions

Did you play Shroomroot or did you watch your teammates play?
☐ I played the whole time
☐ I mostly played and watched a little
☐ I played and watched equally
☐ I mostly watched and played a little
☐ I watched the whole time
☐ Other, please specify... ________________

Of the 20 total reward info boxes, approximately how many did you read?
This will depend partially on how many your team unlocked.
☐ None of them (0)
☐ Less than half (1 - 9)
☐ About half (~10)
☐ More than half (11-19)
☐ All of them, including the 10 fircones one (20)

Did your team finish the game in the time allowed? If not, what level did you get to?
☐ Yes, we finished level 12
☐ No, we got to level: ________________

How much time did your team spend playing the game in class?
This is the “Total Time Played” value we asked you to record before closing the game.
Total Time Played: ________________

Out of 12 total fircones, how many fircones did your team collect?
☐ We collected: ________________
We didn’t have time to record this.

Did you read the reward info box that showed this picture?

☐ Yes
☒ No
☒ I don’t remember
Please circle your response to the following statements:

I enjoyed playing and/or watching my team play Shroomroot.

<table>
<thead>
<tr>
<th>Strongly Agree</th>
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<th>Disagree</th>
<th>Strongly Disagree</th>
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</table>

I enjoyed reading the reward information boxes in Shroomroot.

<table>
<thead>
<tr>
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<th>Disagree</th>
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</table>

I found Shroomroot frustrating to play. ***

<table>
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</table>

I found Shroomroot easy to play.

<table>
<thead>
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</table>

At each step, I understood what I needed to do to progress in the game.

<table>
<thead>
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</table>

I learned about mycorrhizae and mycorrhizal networks from Shroomroot.

<table>
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Shroomroot increased my interest in mycorrhizae and mycorrhizal networks.

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I learned about plant nutrition from Shroomroot.

<table>
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Shroomroot increased my interest in plant nutrition.

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Shroomroot may be a good way to learn for some people but not for me.

<table>
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We would appreciate any comments you may have about your Shroomroot experience.
[space for comments removed]

Thank you for completing the post-test!