Effect of Azoxystrobin and Arbuscular Mycorrhizal Fungal Colonization on Four Non-Target Plant Species

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To Teta, my savior. And to my beloved mother, father, and friends, Dumia, Michele, and Bashir. And to K, for being in my life.

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Abstract

Azoxystrobin (AZY), a systemic broad-spectrum fungicide, is applied on crops to control soil-borne pathogenic fungi. This study aimed to determine the effects of AZY on non-target plant species and *Glomus intraradices* Schenck & Smith, an arbuscular mycorrhizal fungus (AMF) associated with plants' roots. We hypothesized that AZY negatively affects AMF viability; and that, if the plants were dependent on this symbiosis, AZY exerts an indirect detrimental effect on plant growth. To test this, three mycotrophic (*Phalaris arundinacea* L.*, Solidago canadense* L.*, Geum canadense* Jacq.) and one non-mycotrophic (*Chenopodium album* L.) native plant species were subjected to five AZY doses with or without AMF. Plants were grown for 60 days in a greenhouse, in individual pots, (4 plants X 2 AMF X 5 AZY X 6 replicates), and mesocosms (1 mes. X 2 AMF X 5 AZY X 6 replicates), and harvested 30 days after spraying, and dry mass was taken. Fresh root samples were used for microscopic assessment of AMF colonization. The results from the individual pot experiment show that the effects of AZY on biomass varied across plant species. AZY led to a significant increase in shoot and root mass of *P. arundinacea,* and a decrease in shoot mass of AMF inoculated *G. canadense.* The presence of AMF resulted in a significant increase in root and shoot mass of *P. arundinacea*, and an increase in root mass of *S. canadense* and shoot mass of *C. album*. In the mesocosm experiment AZY did not have a significant effect on the measured parameters, although the presence of AMF significantly increased root, shoot, and total dry mass of *G. canadense* and *P. arundinacea*. Conversely, AMF significantly decreased shoot and total dry mass of *S. canadense*. The results suggest that both direct and indirect effects should be taken into account when assessing the impact of pesticides on non-target plant species.

Résumé

Azoxystrobine (AZY), un fongicide systémique à large spectre, est utilisé pour les cultures afin de contrôler les champignons pathogènes du sol. Cette étude visait à déterminer les effets de AZY sur des espèces de plantes non-ciblées ainsi que sur *Glomus intraradices* Schenck & Smith, un champignon mycorhizien à arbuscules (CMA) associé aux racines des plantes. Nous avons postulé que AZY affecte négativement la viabilité des CMA et que si les plantes étaient dépendantes de cette symbiose, AZY exercerait un effet indirect négatif sur la croissance des plantes. Pour tester ceci, trois espèces végétales mycotrophiques (*Phalaris arundinacea* L., *Solidago canadense* L. et *Geum canadense* Jacq.) et une espèce non-mycotrophique (*Chenopodium album* L.) ont été soumises à cinq doses de AZY avec ou sans CMA. Les plantes ont été cultivées pendant 60 jours en serre dans des pots individuels (4 plantes X 2 CMA X 5 AZY X 6 réplicats) et dans des mésocosmes (1 més. X 2 CMA X 5 AZY X 6 réplicats). Pour les deux expériences, les plantes ont été récoltées 30 jours après la pulvérisation de AZYet les masses sèches ont été mesurées. Des échantillons de racines fraîches ont été utilisées pour l'évaluation microscopique de la colonisation des CMA. Les résultats de l'expérience en pots individuels ont montré que les effets de AZY sur la masse sèche varient entre les espèces végétales. AZY a mené à une augmentation significative de la masse des pousses et des racines de *P. arundinacea*, et à une diminution de celle des pousses de *G. canadense* inoculé avec le CMA. La présence de l'AMF a mené à une augmentation significative de la masse des racines et des pousses de *P. arundinacea*, et à une augmentation de la masse des racines de *S. canadense* et de la masse des pousses de *C. album*. Dans l'expérience des mésocosmes, AZY n'a eu aucun effet alors que la présence de l'AMF a fait augmenter significativement la masse des racines, des pousses, et de la plante entière

de *G. canadense* et *P. arundinacea*. Par contre, l'AMF a significativement réduit la masse de la partie aérienne et de la plante entière de *S. canadense*. Les résultats suggèrent que les effets directs et indirects doivent être pris en compte afin d'évaluer l'impact global des pesticides sur les espèces végétales non-ciblées.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

The history of agriculture parallels, in essence, the history of the evolution of human life on Earth. Organized agricultural systems evolved in order to ensure the continuity of the food supply as the human population increased. The second part of the 20th century saw the global population double from approximately 3 billion to 6 billion, and it is projected to reach 9 billion by 2050 (UN Department of Economic and Social Affairs 2006), with nearly 95% of the estimated surge to take place in developing nations, mostly in Asia and Africa. The increased demand for food supplies will, in turn, lead to the intensification of agricultural output. This need to increase productivity per unit of land area will consequently impose increasing demands on the natural environment (Brown *et al.* 2000). Moreover, the intensification of production has occurred in tandem with, and indeed was facilitated by, a substantial increase in the use of pesticides and fertilizers, as well as advances in science such as artificial crop selection and more recently the introduction of genetically modified crops and integrated pest management methods (Merrington *et al.* 2002).

The role that chemical pesticides play in food production has been significant since the 1960s (Pesticide Industry Sales and Usage USEPA 1997). Together with mineral fertilizers and irrigation, pesticides have been largely responsible for what is known today as the 'green' revolution. However, any balanced appraisal of the role of pesticides in food production must include an assessment of risks and benefits. In many cases, the environmental damage from the use and sometimes misuse of pesticides outbalances their benefits (Thrupp 1990).

While the benefits stemming from the increased use of agrochemicals are obvious, primarily the increase in food production and efficiency of production, the risks associated with pesticide use and the full extent of the resulting damage are more opaque in nature. One aspect that contributes to this ambiguity is the way in which pesticide hazards and exposure are measured by regulating bodies as well as by the agrochemical industry and independent scientists (Vogel 2005). Pesticide tests are designed to evaluate health and ecological hazards associated with exposure to individual pesticides, which is simply not very representative of actual exposure. Humans, for instance, are seldom exposed to only one pesticide, as crops are seldom sprayed with just a single pesticide. This apparent pitfall becomes more relevant when we consider the synergistic effects that many pesticides have when found together (Racke 2000).

In addition, there exists a wide array of processes by which pesticides can impact the environment. Agricultural pollution may affect the quality of natural resources, primarily the chemical and biological characteristics of soil, water and air, and it may affect the composition, structure, and functioning of terrestrial, aquatic and marine ecosystems (OECD 1997).

An additional limitation that arises when attempting to quantify the effects of pesticides is the sheer complexity of contamination and pollution pathways. For instance, a pesticide may directly affect an organism through direct exposure, indirectly through contaminated food web, or by disruption of habitats in which an organism strives.

Another factor to consider is the multifaceted nature of pesticides. A pesticide has several breakdown products that depend on various environmental variables such as climate, soil type, and season, to name a few. The nature of pesticide properties

that are of concern to us, such as persistence and lipophilicity, (these properties change as the 'parent' pesticide undergoes degradation and metabolism), makes the task of quantifying pesticide impact on agroecosystems and on human health even more perplexing.

Finally, we have to consider the effect that pesticides have on non-target organisms as well as non-target habitats that may be located far from the point(s) of pesticide application. Significant amounts of pesticides do not reach their target organisms and can have an impact on distant ecosystems through mobility, leaching, runoff (Pimentel and Levitan 1986).

1.1 Pesticide Usage

The last decade has brought with it increased environmental awareness and improved knowledge of the consequences of our increased reliance on pesticides, irrespective of costs that come with such use. It is ironic that, in spite of this increased awareness, pesticide manufacturers and sellers continue to thrive. The agricultural sector is primarily responsible for the majority of pesticide usage in Canada. Pesticide sales in Canada were $ 1.31 billion as of 2003, comprising 26,636,793 Kg of active ingredients and over 7,000 products registered for domestic use, of which 9% are fungicides (Brimble *et al.* 2003).

1.2 Fungicides

Fungicides are designed to kill and/or inhibit the growth of pathogenic fungi. However, most fungicides in use today, like most other pesticides, belong to a category of organic chemicals that were developed and gained wide use after World War II. Early fungicides (and pesticides in general) were mainly inorganic

compounds such as elemental sulfur, copper compounds including the famous Bordeaux mixture, in addition to mercuric chloride ($HgCl_2$), mercurous chloride (Hg_2Cl_2) (Russel 2005), and cupric oxide (Somers 1956).

Pesticides interfere with critical metabolic processes in the "pests" which they target. Some insecticides, for instance, interfere with nerve impulses, while fungicides may interfere with cell devision and growth, energy production, and/or biosynthesis of essential molecules (Merrington *et al.* 2002).

In parallel with other pesticides, the balance between benefits and hazards keeps changing with every new compound as fungicides evolve and as newer compounds are introduced to the market since the benefits and hazards are related to the chemical and physical properties of these compounds. The trend has been to move towards compounds that are less acutely toxic and can be applied at lower rates (Merrington *et al.* 2002). Newly developed fungicides, such as the strobilurins, have been derived from indigenously occurring compounds in nature (Washington 2002), perhaps the most prominent example is neem seed extracts that serve as azadirachtin-free fungicides (Moline and Locke 1993).

Fungicides differ from other pesticides, though, in the method and timing of their application. Fungi are very versatile organisms and they possess the ability to inflict serious damage from a relatively small population base, in addition to the fact that they can travel long distances because their spores are small enough (10 μm) to be carried by wind (Hawker 1957). As a result, fungicides are therefore applied in a prophylactic manner in order to achieve the maximum benefit.

1.3 Plant-AMF Association

Plants and arbuscular mycorrhizal fungi (AMF) have a unique mutualistic relationship. The basic model of the host plant providing food in the form of carbohydrates to its fungal partner in return for more efficient mineral uptake evolved approximately 400-500 MY ago and is believed to have enabled plants to colonize terrestrial habitats (Remy *et al.* 1994). However, the role of AMF associations is more than to merely "supplement" what roots already provide for the plant. As a matter of fact, recent findings confirm that mycorrhizae and not roots were the primary organs of nutrient acquisition by terrestrial plants as the earliest land plants, which lacked true roots, were colonized by hyphal fungi that formed structures very similar to those found today (Smith and Read 1997).

Moreover, the AMF provide the plant with more than just minerals. In addition to increased mineral (Audet and Charest 2006) and phosphorus uptake through fungal extraradical hyphae (Schweiger and Jakobsen 1998), AMF enhance the plant's resistance to drought (Subramanian and Charest 1997, 1998, 1999), as well as to pathogens (Gange 2000). Additionally, AMF have also been shown to significantly improve soil structure through increasing water stable aggregation (Schreiner and Bethlenfalvay 1997b). Furthermore, AMF play an important role in nutrient cycling as they are prominent soil decomposers; releasing high amounts of bioavailable nutrients into soil and making them readily available for plant uptake (Wardle *et al.* 2004). This bidirectional movement of nutrients is the cornerstone of mutualism in most mycorrhizal associations.

1.4 Effects of Fungicides on Mycorrhizal Associations

With the increasing awareness of the pivotal role that AMF play in ecosystems, research has been focusing on the effects that fungicides designed to kill pathogenic fungi, have on AMF and their plant hosts.

Patterns in this research have yet to emerge as contradictory results are not uncommon. Indeed, conflicting results are the rule rather than the exception (Perrin and Plenchette 1993). Complicating factors include: type of soil, type of pesticide, method of application, plant growth stage at time of application, and interactions between the AMF and soil micro-organisms. Furthermore, there are factors that influence the type of interaction between AMF and soil micro-organisms and contribute to whether the outcome of that interaction is positive or negative, namely the type of soil micro-organism, type of AMF, and species of plant being colonized (Hodge 2000).

In a study investigating the effects of the fungicide pentachloronitrobenzene (PCNB) on the mycorrhyzal fungus *Glomus mosseae* root colonization of oats (*Avena sativa* L. cv. Alfred), PCNB was mixed with the soil at doses ranging from 0 to 50 mg kg^{-1} air dried soil in an open field experiment (Gnekow and Marschner 1989). It was observed that shoot dry mass and total root length were decreased by PCNB at 50 mg kg. The degree of mycorrhizal colonization decreased starting with the lowest dose (2 mg) and further decreased in a dose-dependent manner. Plant yield, on the other hand, was reduced only at the highest PCNB dose of 50 mg/kg.

Parvathi *et al.* (1985) tested the toxicity of the fungicides PCNB, gypsum, Captafol, Captan, and Mancozeb on *Glomus mosseae* in groundnut (*Arachis hypogaea* L.) in a greenhouse experiment. Fungicides were applied at rates of 10, 25, 50, and 100 mg a.i./kg soil by immersing the potting mixtures in aqueous solutions of

the fungicides. The doses used were close to the field application rates. For all treatments, increasing levels of fungicide progressively decreased root colonization as well as the number of spores per cm of root. Plants that were administered higher doses of gypsum, PCNB and captafol (50 – 100 mg/kg) exhibited absolutely no mycorrhizal growth. The lowest dose of PCNB (10 mg/ kg, close to the recommended dose) was able to significantly reduce percent root colonization and spore number. Interestingly, PCNB, gypsum, and Captafol treated plants had significantly lower dry mass (Parvathi *et al.* 1985).

The arbuscular mycorrhizal fungus, *Glomus mosseae*, was the subject of yet another study (Zhang *et al*. 2006), investigating the effect of the fungicide chlorothalonil on the growth of upland rice (*Oryza sativa* L.) in China. Chlorothalonil significantly inhibited root colonization in a dose-dependent manner. Furthermore, the fungicide inhibited the growth of all rice plants; however *G. mosseae* significantly increased shoot and root dry mass at all doses of chlorothalonil. Mycorrhizal dependency was higher at the lower fungicide dose (50 mg/kg), and plants were more severely affected at the higher dose (100 mg/kg); this suggests that high doses negatively affect the AMF. This study showed that chlorothalonil application reduces rice plant growth, while inoculation with *G. mosseae* provides protection against the side effects of the fungicide (Zhang *et al*. 2006).

Plant and soil responses to single and mixed AMF under fungicidal stress were examined by Schreiner and Bethlenfalvay (1997b). In a study of the effects of five different AMF treatments (no treatment, *Glomus etunicatum* (Ge), *Glomus mosseae* (Gm), *Gigaspora rosea* (Gr) and a mixture of the three (Mix)) and different fungicides (benomyl, PCNB, and captan) on pea plants, it was demonstrated that all

of the AMF treatments improve shoot dry matter, increase seed yield and seed mass, the highest effects being with Ge and Mix. In turn, fungicides decreased total colonized root length (except with Ge and Mix); PCNB and Captan decreased shoot dry mass and seed yield. Root length was also decreased by all fungicide treatments. Fungicide treatment also reduced spore density, with the Mix soil being the least affected. This study, which employed several mycorrhizal species alone and as a mixture, suggests that a mixed population of AMF is likely to respond differently to fungicide than individual species of AMF, and would likely better withstand fungicide treatment (Schreiner and Bethlenfalvay 1997b).

The effects of benomyl application and inoculation with *Glomus fasciculatus* on a tulip poplar were also studied (Verdake and Hamilton 1983). It was shown that plants inoculated with *G. fasciculatus* and drenched with water (rather than benomyl) exhibited substantial mycorrhizal colonization (10-30%), highest root and shoot dry mass, total root length, and P uptake. Conversely, inoculated plants drenched with benomyl showed the lowest levels of the above characteristics. This study demonstrated that benomyl, by preventing AMF colonization, decreases the efficiency of the root system and therefore nutrient absorption.

To evaluate the effect of the fungicide metalaxyl on root colonization by *Glomus intraradices* fumigated with methyl bromide and non-fumigated plots of onion, cotton and pepper, metalaxyl was applied at a dose of 5 mg a.i./m^2 (Afek *et al.* 1990). In non-fumigated plots, AMF plants treated with metalaxyl had a 2.4 – 3.4 fold increase in root colonization versus untreated AMF plants. In the fumigated plots, metalaxyl treatment had no significant effect on percent root colonization and overall root length. In the non-fumigated plots, it is probable that metalaxyl exerted

its greatest effect on AM root colonization by acting on the pathogenic *Pythium* fungi, thus reducing competition in the rhizosphere.

In an intriguing series of experiments, the effects of fungicides on the developmental stages of AMF were investigated (Schreiner and Bethlenfalvay 1997a). *Glomus etunicatum*, *G. mosseae* and *Gigaspora rosea* were grown in Petri dishes, and treated with either Benomyl, PCNB, or Captan at doses of 10 or 20 mg/kg substrate. All three fungicides completely inhibited spore germination at the highest dose. At the lower dose, Benomyl completely inhibited germination, PCNB decreased germination by both *Glomus* species, and Captan had no effect. In another set of experiments, the influence of burying the spores in soil was studied. The effect of Benomyl on spore germination disappeared at the 10 mg/kg dose, and was slight at the higher dose; however the opposite was observed for hyphal growth. The same was observed with PCNB, and Captan inhibited both germination and hyphal growth.

Jabaji-Hare and Kendrick (1987) showed that individual fungicides can have different effects on a given AMF species. Fosetyl-Al significantly enhanced the colonization of leek (*Allium porrum* L.) roots by *Glomus intraradices*, and in parallel the shoot dry mass of the plant. In contrast, metalaxyl had significant opposite and detrimental effects on the degree of root colonization and plant growth (Jabaji-Hare and Kendrick 1987). It is possible that the mode of application could have played a role in these contradictory results. Fosetyl-Al was applied to the plant foliage and translocated via the phloem to the roots and onto the mycorrhizae, while metalaxyl was applied directly as a soil drench, and therefore was not metabolized by the plant before coming into contact with the AMF.

Early detection of the potential effects of fungicides is desirable, however structural or physical changes are usually not detectable for weeks following

fungicide application. To that effect, biochemical measurements can be invaluable to detect changes in AM metabolic activity. In one such study, the measurement of succinate dehydrogenase (SDH), a tricarboxylic acid cycle enzyme, was employed as an indicator of the metabolic activity of fungal tissue. Onions (*Allium cepa* L . cv. Hyper) inoculated with *Glomus caledonicum* or *G. intraradices* were treated with either 100 µg a.i./g soil of Benomyl or Captan at 4 weeks of age. After four weeks of the fungicide treatment, AM plants exhibited a higher growth over non-AM plants, and after fungicide treatment, AM plants were still at an advantage over non-AM plants, although the difference between the two groups was attenuated by the fungicide treatment. The fungicide had no effect on the non-AM controls. Although structural changes were not detectable after three days of fungicide treatment, significant decreases in the amount of SDH active fungal tissue within the roots were detected; and this was true for both AMF species and fungicides (Kough *et al.* 1987).

As previously mentioned, soil composition may influence the effect of fungicides on AMF colonization. Perrin and Plenchette (1993) investigated the effects of four fungicides (Benomyl, Mepronil, Furalaxyl and Thiram) on AMF (*Glomus intraradices*) colonization and plant receptiveness in two different soils. Colonization was defined as "the ability of a soil containing native mycorrhizal propagules to initiate mycorrhizal infection on host plants", and receptiveness, as "the ability of a soil to allow the development of mycorrhizal association on host plants from an introduced AM inoculum". The two types of soil (A and B) mainly differed in their physical and chemical properties, with soil A being predominantly made of loam (60%), with the remainder divided between clay (26%) and sand (15%) and containing 1.4 mg of N and 15 µg P per gram of soil. As for soil B, the proportions of clay, loam and sand were approximately equal (*e.g.,*37%, 33%, and

30%) and contained 4 mg/g N and 2 μg/g P . All fungicides were applied directly into the soil. Of the four applied fungicides, Benomyl had consistent detrimental effects on mycorrhizal development (both native and introduced) irrespective of soil type. Although the remaining three fungicides exerted negative effects on the AMF colonization of soil A, no effect was observed for Furalaxyl on soil B, and both Mepronil and Thiram had positive effects on AMF root colonization in soil B, but all treatments exerted negative effects except for Mepronil on soil A only. Soil composition may therefore help explain the contradictory results found in the literature, as some fungicides may show no effect or even enhance mycorrhizal development under certain conditions. Characteristics such as the different AMF species present, the associated microflora, or the soils' physicochemical characteristics may influence fungicide – AMF – plant interactions (Perrin and Plenchette 1993).

1.5 Azoxystrobin

Azoxystrobin is a systemic, broad-spectrum fungicide that belongs to the strobilurin family (Bartlett *et al.* 2001). It is used in a preventive and curative capacity to control foliar and soil-borne pathogenic fungi belonging to Ascomycota (such as early blight, powdery mildew and stem rot), Basidiomycota (most notably stem and stolon canker),the heterotrophic protists Oomycota (such as late blight and downy mildew) and the Deuteromycota anamorphic fungi (namely rice blast molds and rots) groups, although its wide range of action ensures that its use is not limited to these fungi (Bartlett *et al.* 2002). In Canada, it is used on many crops including seed corn, field corn, canola, field tomato, and beans (PMRA 2000).

Strobilurin A (Fig. 1), the precursor of AZY, was discovered in the mycelium of the wood decaying fungus *Strobilurus tenacellus* (Diedhiou *et al.* 2004). AZY has a specific mode of action that works by disrupting electron transport via binding to the quinone site in complex III of the fungal mitochondrion, thus impeding the production of ATP, resulting in inhibition of spore germination and mycelial growth (PMRA 2000).

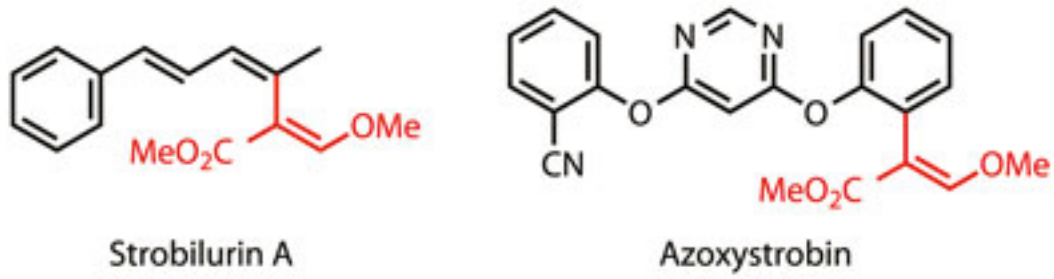

Figure 1: Chemical structure of Strobilurin A and Azoxystrobin (www.syngentacropprotection.com)

1.6 Toxicological Profile

Although the label states that it is non-toxic to bees, terrestrial animals and target plants, AZY is considered as highly toxic to other organisms. It is classified as highly toxic to marine/freshwater fish and freshwater invertebrates, with a half maximal effective concentration (EC_{50}) reaching 256 ppb for acute toxicity to waterfleas, and as highly toxic to marine invertebrates, with a lethal concentration (LC_{50}) as low as 56 ppb for acute toxicity of myrid shrimp, as well as being highly toxic to aquatic algae (Aspelin 1997).

Moreover, AZY has been found to be highly toxic to apple trees cv. MacIntosh and crabapple trees (Lange 2004). It is also possible that other plants belonging to the Rosaceae family exhibit sensitivity to AZY. In fact, research shows that residues from spray tanks that contained AZY or drift from aerial application in near-by fields can cause substantial damage to MacIntosh and crabapple trees when applied with surfactants, with concentrations as low as 100 ppt exhibiting toxicity to the above varieties (Lange 2004).

Furthermore, AZY is a compound that does not break down readily in the environment. It is resistant to hydrolysis and exhibits high persistence in soils and water, with a DT_{90} (dissipation time) value of (468 to 738 days). AZY also has a Groundwater Ubiquity Score (GUS) (Gustafson 1989) classification as a borderline leacher (PMRA 2007). GUS classifications are a simple tool that measures the predisposition of a pesticide to leach into and pollute ground water. Pesticides are classified as either leachers, non-leachers, and borderline leachers. Persistence and leaching potential, combined with its known toxicity to certain terrestrial plants, fish, aquatic algae and invertebrates, highlight the potential of soil and water body impact by AZY.

1.7 Plant Species

All plant species except *Chenopodium album* were obtained from commercial seed suppliers. *C. album* seeds were donated by Agriculture and Agri-Food Canada. All four plant species tested are considered wild species that are often found on the periphery of agricultural fields (Boutin *et al.* 2001). Each species, however, possesses different characteristics that may alter its response to AZY or the AMF, or both.

Phalaris arundinacea L. is a competitive wetland grass. It is an invasive mycotrophic perennial that is found abundantly in Canada, and is classified as facultative upland species that regularly occurs in wetlands (Oldham *et al.* 1995). *Solidago canadense* L. is a perrennial mycotrophic plant that is mostly found in upland areas, but can also occur in wetlands. According to Oldham *et al.* (1995) it is a facultative upland species. Both *P. arundinacea and S. canadense* possess an extensive underground root network as well as rhizomes that make them efficient in nutrient acquisition. *Geum canadense* Jacq., a perennial mycotrophic plant was chosen because, like Macintosh apple trees, it belongs to the Rosaceae family and may be especially susceptible to AZY. It is classified as a facultative upland/wetland plant, that be found in both upland and wetland areas. *Chenopodium album* L. is a facultative upland species rarely found in wetland areas. It is an annual non-mycotrophic plant that was added in order to better evaluate the direct effect of AZY on plant growth (Table 1).

Table 1: Plant species used and their description

Plant species	English Common Name	Family	Life Cycle
Phalaris arundinacea L.	Reed canary grass	Poaceae	Perennial
Solidago canadense L.	Canada goldenrod	Asteraceae	Perennial
Geum canadense Jack.	White avens	Rosaceae	Perennial
Chenopodium album L.	Lambsquarters	Chenopodiaceae	Annual

1.8 Rationale, Objectives, Hypotheses and Predictions

The effects of fungicides on the development of plant-AMF symbiosis are difficult to generalize due to both adverse and positive effects having been reported (Kjoller and Rosendahl 2000). The present study aimed at investigating the effects of the fungicide AZY on four specific plants and their interactions with AMF.

Specific objectives

1. To examine the effect of the fungicide on plant health when plants are grown individually or in a mesocosm, with or without AMF colonization.
2. To examine the effect of the fungicide on the AMF root colonization *per se*.

Hypotheses

The following hypotheses were tested:

1. If the plant full growth potential is dependent on the viability and symbiotic capacity of the AMF, then there will be detrimental (indirect) effect on its growth (in terms of dried biomass) with AZY applications.
2. The viability and vigour of the AMF will decrease under the effect of the fungicide.

Predictions

The following statistically testable predictions based on the hypotheses were made:

a- Single species experiment:

   1- AZY will exert a direct effect on the dry biomass of *G. canadense,* and there will be an indirect effect on AMF.

   2- There will be neither direct nor indirect effect(s) on the dry biomass of *C. album*.

3- AZY will not have an effect on dry biomass of *S. canadense* and *P. arundinacea*. However, AZY will exert an indirect effect on AMF.

b- Mesocosm experiment:

1- In AMF + pots treated with AZY, *C. album* will be at an advantage with respect to the other plants because it does not form mycorrhizal associations.

2- *G. canadense* will be at a disadvantage due to the direct toxicity of AZY in addition to direct toxicity to AMF.

3- All species will perform well in pots that were not exposed to AZY but have AMF present.

4- *C. album* will be at an advantage in pots not exposed to AZY where AMF is absent.

Chapter 2. Materials and Methods

Two main experiments were conducted. The first experiment comprised of each individual plant species (1 plant per pot) sprayed with 5 doses of AZY, with and without AMF (6 replicates per treatment) and act as a frame of reference for the second experiment. Various endpoints were measured including plant biomass and % AMF root colonization.

The second experiment consisted of the four plant species grown in the same pot with identical treatment in terms of doses and presence/absence of AMF, as well as number of replicates per treatment. Results were compared to those obtained in the first experiment in order to evaluate if there was a difference in the adverse effects between individual species exposed and the community as well as to examine whether the presence of competition between plants affected plant responses and susceptibility to fungicidal stress as studies have shown that competition between plant species can influence their response to stress (Moora and Zobel 1998) and plant community structure (Smith *et al.* 1999).

2.1 Experimental Protocol

Prior to the actual experiments, a direct toxicity test was carried out on the four plant species in order to determine the sensitivity range for each plant. Plants (n = 6) were transplanted without the AMF substrate in 10 cm plastic pots and were subjected to two sprays, one week apart, and at the same doses as in the main experiment. All above ground parts were harvested 30 days after the last spray date.

Shoots were then inserted into paper bags and placed in a forced air drier for 72 hrs at 70°C in order for dry mass measurements to take place. The experimental studies were carried out in the greenhouse at the National Wildlife Research Centre, Carleton University, Ottawa. Table 2 lists the greenhouse growth conditions.

Table 2: Greenhouse growth conditions

Min. Temperature	16°C
Max. Temperature	27°C
Relative Humidity	56%
Photoperiod	16:8 L:D
Light Intensity	1.8×10^{16} photons/ cm^2/ s

Pots were watered when the soil surface was dry. During the experiment, the greenhouse temperature fluctuated between 16°C to 27°C. *Coccinellidae septempunctata* was introduced into the greenhouse to control for possible aphid infestations.

The four plant species were inoculated either with an AMF inoculum, *Glomus intraradices* Schenck & Smith, or a control substrate without the inoculum (Add source of inoculum, Mycorhize, Premier Tech, Rivière-du-Loup, QC, Canada). All the plants were exposed to five concentrations of AZY: 0, 0.033, 0.069, 0.15, and 0.31 mmol (representing 0, 10, 21, 44, and 93% of the recommended label rate respectively). The recommended label rate ranges from 300 ml/ha to 1000 ml/ha depending on the crop to which the fungicide is to be applied and the disease it is

supposed to control. We chose an intermediate application rate of 800 ml/ha, which is used on potatoes to control early and late blight and on canola to control *Sclerotinia* stem rot and *Alternaria* black spot.

Germination requirements differed across plant species. *S. canadense* and *G. canadense*, both required 60 days of cold stratification in the dark. Seeds belonging to both species were sown on a moist soil mixture consisting of Premier Pro-Mix potting medium and sand, mixed until homogenous by a ratio of 2:1 (henceforth referred to as potting mixture). Seeds were then covered in black nylon and inserted into a refrigerator at 4°C for 60 days. *C. album* required a two-week stratification procedure following the same protocol as above. All seeds were placed in the greenhouse once stratification was complete. *P. arundinacea* seeds did not require any stratification and were sown in the potting mixture and placed in the greenhouse to germinate. All seeds were surface sown since they need light to complete germination (White and Boutin 2009).

All seedlings were transplanted within 14 days of germination in 2.84 L pots for the single species experiment and in 11.35 L pots for the mesocosm experiment. For the mesocosm experiment, seedlings from the different species were not ready to be transplanted at the same time. Therefore, *S. canadense* and *G. canadense* were transplanted in the pots first and *P. arundinacea* and *C. album* were transplanted 14 days later.

Pots for both experiments were filled with the potting mixture until it reached approximately 5 cm from the rim, then a 3 cm layer of substrate containing the AM fungal inoculum (15 propagules per gram) or the control substrate was added. Both substrates were provided by Premier Tech Inc. (Rivière-du-Loup, QC, Canada). A final 2 cm layer of the potting mixture was added on top to prevent the seedlings'

taproots from coming in to direct contact with the AM/non-AM substrates. All plants were allowed to grow and reach the 2-6 leaves stage before they were first exposed to AZY.

2.2 Fungicide Application

AZY is s systemic broad spectrum fungicide available under the commercial name Quadris®. All the plants were sprayed with 0, 10, 21, 44, and 93% of the recommended label rate that was defined as 200 g of the active ingredient per hectare. These correspond to 0, 0.33, 0.69, 1.45, and 3.07 mmol/L and to 0, 20, 42, 88, and 186 grams of active ingredient (a.i.) per hectare (ha). Plants were subjected to the first spray when they reached the 2-6 true leaves stage and a second spray treatment, one week later.

The fungicide was applied using a track spray booth (de Vries Manufacturing, Hallandale, MN, USA) supplied with a TeeJet 8002E flat-fan nozzle (Spraying Systems, Wheaton, IL, USA), that delivers 6.75 $ml.m^2$ at 206.84 kPa.

2.3 Harvest

Plants were harvested 30 days after the 2^{nd} spray date. Prior to the beginning of harvest, a visual assessment was performed and recorded. All above ground growth was cut and placed in paper bags. Soil was removed from roots by gentle shaking and then allowed to soak in still tap water until most of the soil and debris were detached; any remaining soil particles were removed manually. A fresh root sample of approximately 2 grams was taken from each sample and weighed on an analytical balance, stored in water in a growth chamber at 4°C until later staining for microscopic examination of mycorrhizal colonization %. The remaining roots were

then padded dry and inserted into brown paper bags. Shoot and root samples were placed in a forced air drier for 72 hours at 70°C for dry mass measurement.

2.4 Mycorrhizal Development

Root samples were bleached and stained for AMF colonization measurement according to the method of Dalpé (1993). Briefly, the root samples were bleached in 2.5% KOH (w/v) at 90°C for 40 minutes. The samples were then rinsed thoroughly in tap water and soaked in a 1% HCl solution for 10 minutes. After clearing with 1% HCl, roots were immersed in 0.02% aniline blue staining solution (6.78 mM Aniline blue; 450 ml deionized H_2O; 500 ml glycerol; and 50 ml 1% HCl solution) at 90°C for 15 minutes. Roots were then stored in plastic 50 ml centrifuge tubes filled with destaining solution (prepared identically to the staining solution with omission of the dye) until further microscopic examination at 10 and 40X.

2.5 Statistical Analysis

Data are presented as means ± SEs. For root and shoot mass, results were compared by two-way analysis of variance (ANOVA) for each plant species, with post-hoc multiple comparisons using the Bonferroni correction. Assumptions of normality and homogeneity of variance for an ANOVA were met for all species.

A *p*-value less or equal to 0.05 was considered significant. Statistical analyses were performed using S-Plus version 8.

Chapter 3. Results

3.1 Single species experiment

3.1.1 *Solidago canadensis* (S.C.)

Root mycorrhization levels for *S. canadense* plants sprayed with AZY were 40, 39, 41.7, 43 and 38.6 % for the respective doses. A positive main effect of AZY was observed on total biomass (figure 2) of S.C. ($p = 0.009$) without any significant main effect of AMF, and no interaction between the two factors (AZY and AMF). Therefore, multiple comparisons using the Bonferroni correction were performed over pooled values of AMF^+ and AMF^- samples, and showed that the positive effect of AZY was significant at doses of 0.0692 ($p = 0.035$), 1.45 ($p = 0.005$) and 3.066 ($p = 0.022$) mmol/L.

For the root biomass, there was no significant effect of AZY, however the presence of AMF exerted a positive effect on root biomass (figure 3) ($p = 0.013$), without any interaction. The shoot biomass was also significantly increased (figure 4) ($p = 0.031$) by AZY, although no effect of AMF or interaction was observed. Multiple comparison analysis of data pooled over AMF^+ and AMF^- samples showed the effects of doses of 1.45 ($p = 0.033$) and 3.066 ($p=0.032$) mmol/L to be significant.

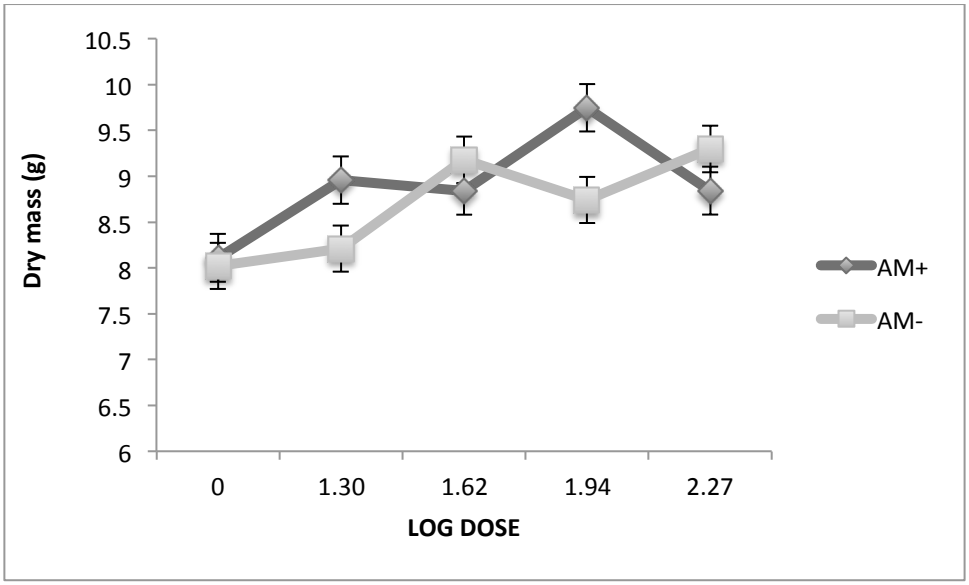

Figure 2: Total dry mass of *S. canadense* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

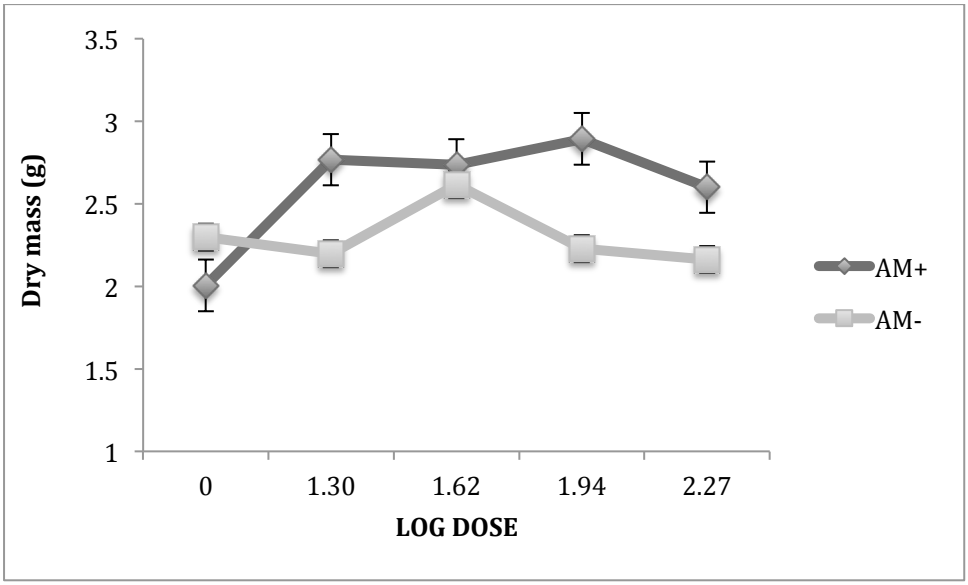

Figure 3: Root dry mass of *S. canadense* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

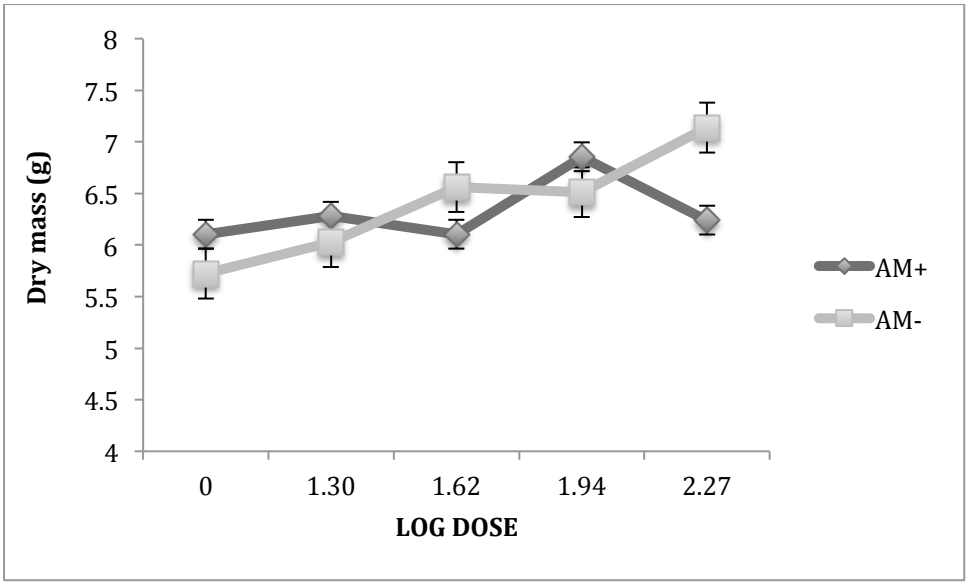

Figure 4: Shoot dry mass of *S. canadense* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.1.2 *Phalaris arundinacea* (P.A.)

Root mycorrhization levels for *P. arundinacea* plants sprayed with AZY were 13, 9, 8, 10 and 8 % for the respective doses. AZY (p=0.003) and AMF (p=0.001) had significant effects respectively on total biomass (figure 5), without any significant interaction between the two factors. Thus, multiple comparisons using the Bonferroni correction were carried out on pooled samples of AMF^+ and AMF^- and revealed that AZY had a positive effect at doses of 0.692 (p = 0.017), 1.45 (p = 0.003) and 3.066 (p = 0.04) mmol/L.

Root biomass was also positively affected by AZY (figure 6) (p = 0.014) and AMF (p = 0.016), although no significant interaction was found. Multiple comparisons performed over pooled values of AMF^+ and AMF^- showed that the effect of AZY

was significant (p = 0.004) only at 1.45 mmol/L. Similarly shoot biomass was positively affected by AZY (figure 7) (p = 0.008) and AMF (p = 0.001), without any significant interaction. When samples were pooled, it resulted that AZY was significant at 1.45 (p = 0.031) and 3.066 (p = 0.039) mmol/L.

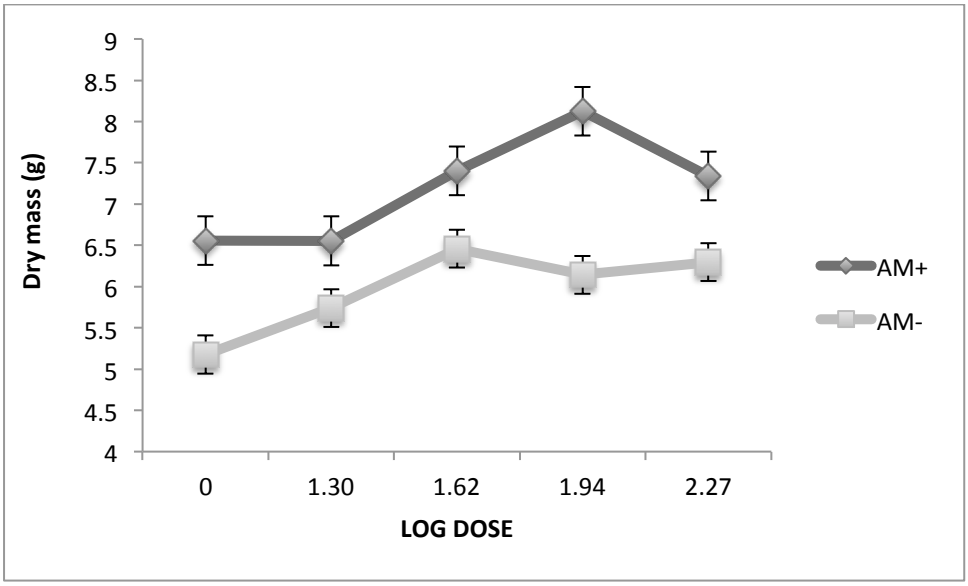

Figure 5: Total dry mass of *P. arundinacea* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

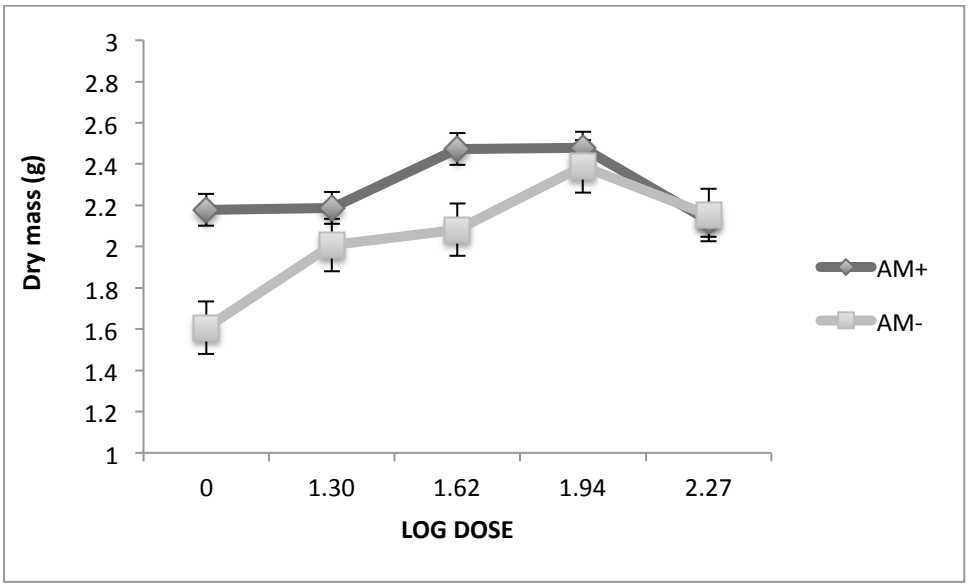

Figure 6: Root dry mass of *P. arundinacea* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

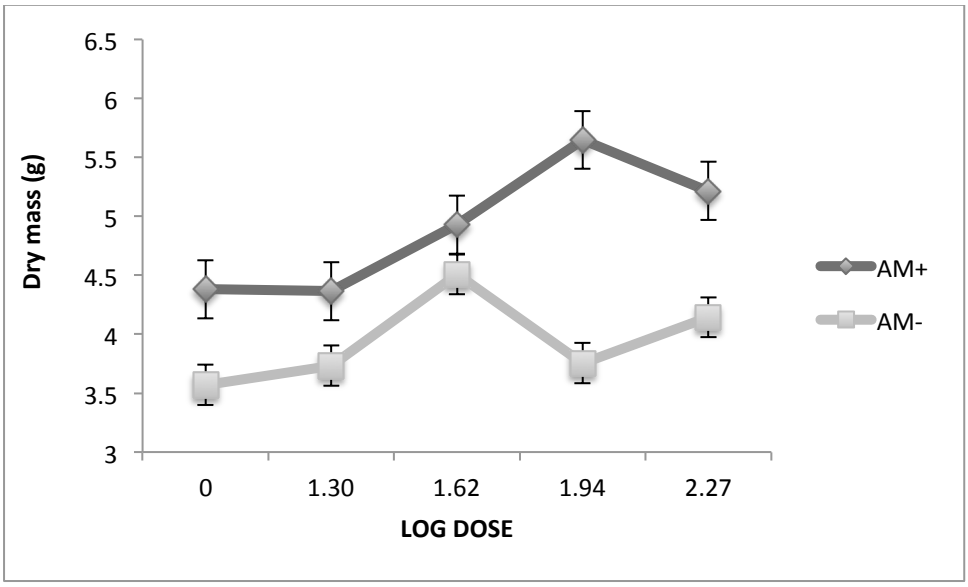

Figure 7: Shoot dry mass of *P. arundinacea* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.1.3 *Chenopodium album* (C.A.)

The roots of *C. album* did not undergo any mycorrhizal colonization. The effect of AZY on total biomass of C.A. was not significant (figure 8), but AMF had a significant positive effect ($p = 0.001$) without any significant interaction between the two factors.

Regarding root biomass of C.A., AZY did not have a significant effect (figure 9), however AMF had a significant positive effect ($p = 0.002$) and the interaction ($p=0.023$) between the two factors was also significant. Hence, multiple comparisons were performed on separate data sets (comprising AMF^+ and AMF^- samples) but no significant effect of AZY was found. As for shoot biomass of C.A., AZY exerted a significant (figure 10) ($p = 0.006$) main effect (graphs show an increase followed by a decrease), as well as a positive significant effect ($p=0.001$) of AMF. Moreover, the interaction was found to be significant ($p=0.036$). We therefore carried out multiple comparisons on separate AMF^+ and AMF^- data sets, but no significant effect of AZY was observed.

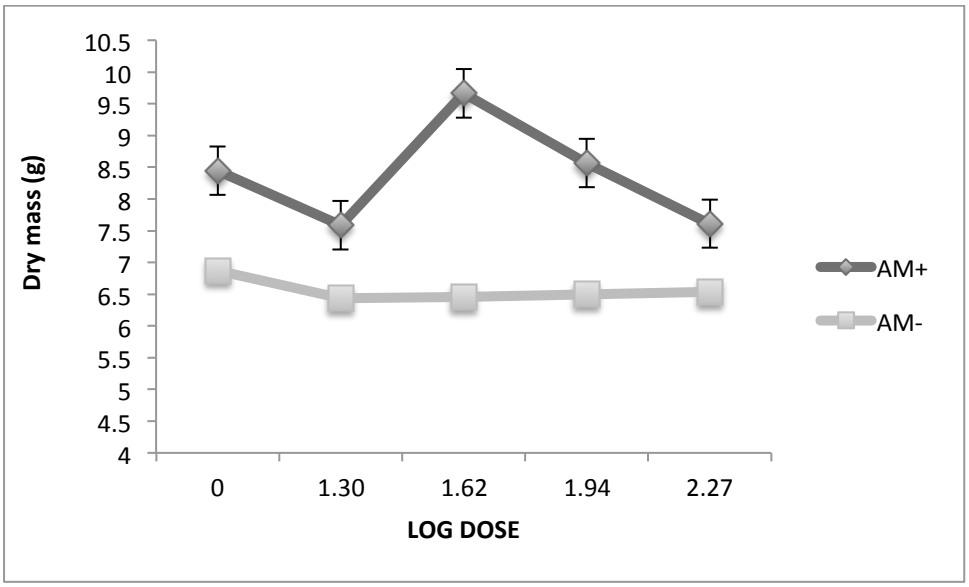

Figure 8: Total dry mass of *C. album* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

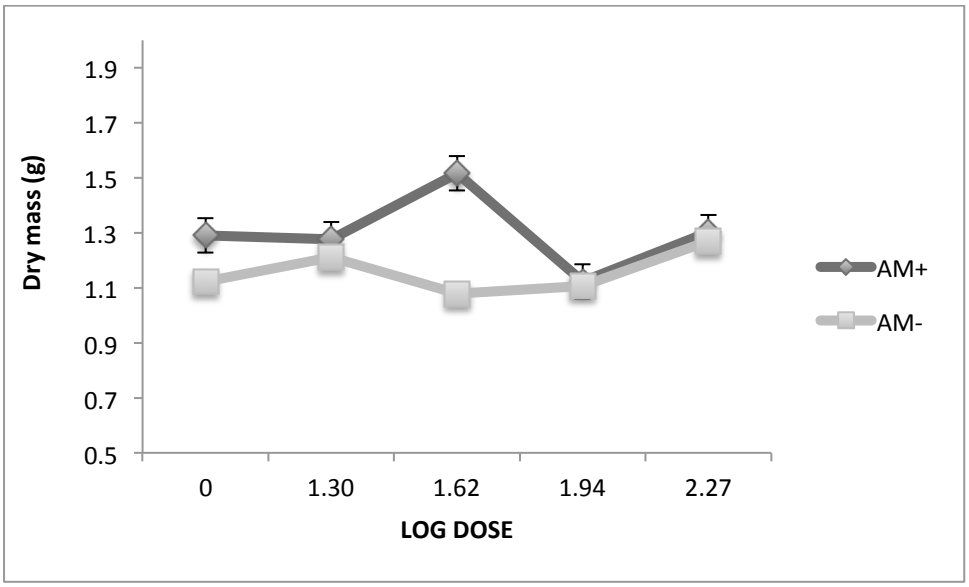

Figure 9: Root dry mass of *C. album* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

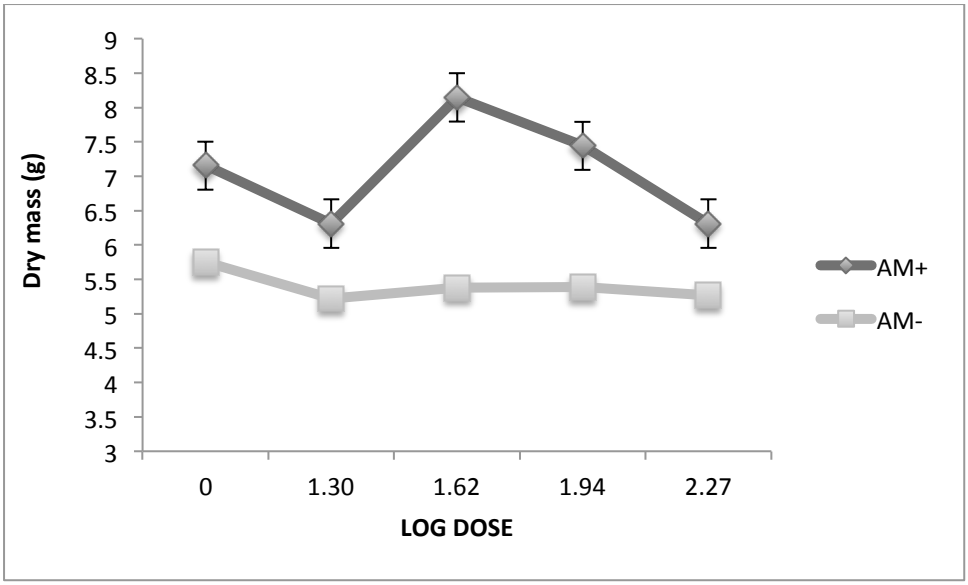

Figure 10: Shoot dry mass of *C. album* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.1.4 *Geum canadense* (G.C.)

Root mycorrhization levels for *G. canadense* plants sprayed with AZY were 6.8, 5.3, 5.6, 7.1 and 0.9 % for the respective doses. No significant effect of AZY or AMF was observed on the total biomass of G.C. (figure 11). Yet, the interaction between AZY and AMF was significant ($p = 0.033$). Multiple comparisons were performed on the separate AMF^+ and AMF^- data sets and they revealed that AZY exhibited a significant negative effect ($p = 0.007$) on AMF^+ plants at a dose of 3.066 mmol/L.

AZY and AMF did not have any significant effects on root biomass (figure 12) ($p=0.235$ and 0.051, respectively) without any significant interaction. Shoot biomass was not significantly affected by either AZY or AMF (figure 13). However, the interaction was found to be statistically significant ($p = 0.033$). Therefore,

multiple comparisons on the separate AMF^+ and AMF^- data sets were performed and it was shown that AZY exerted a statistically significant negative effect at 3.066 mol/L ($p = 0.008$) in AMF^+ plants.

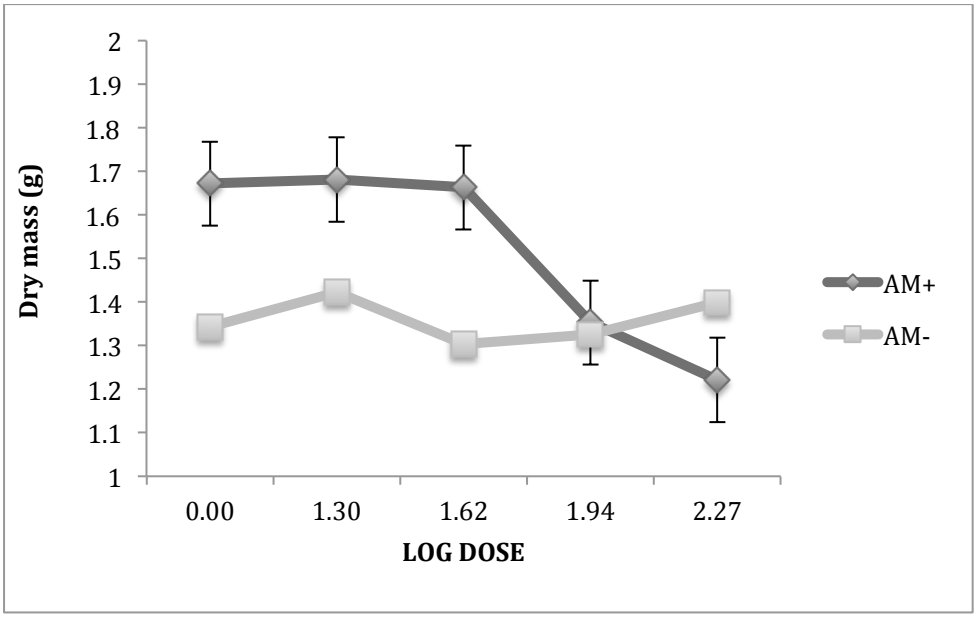

Figure 11: Total dry mass of *G. canadense* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

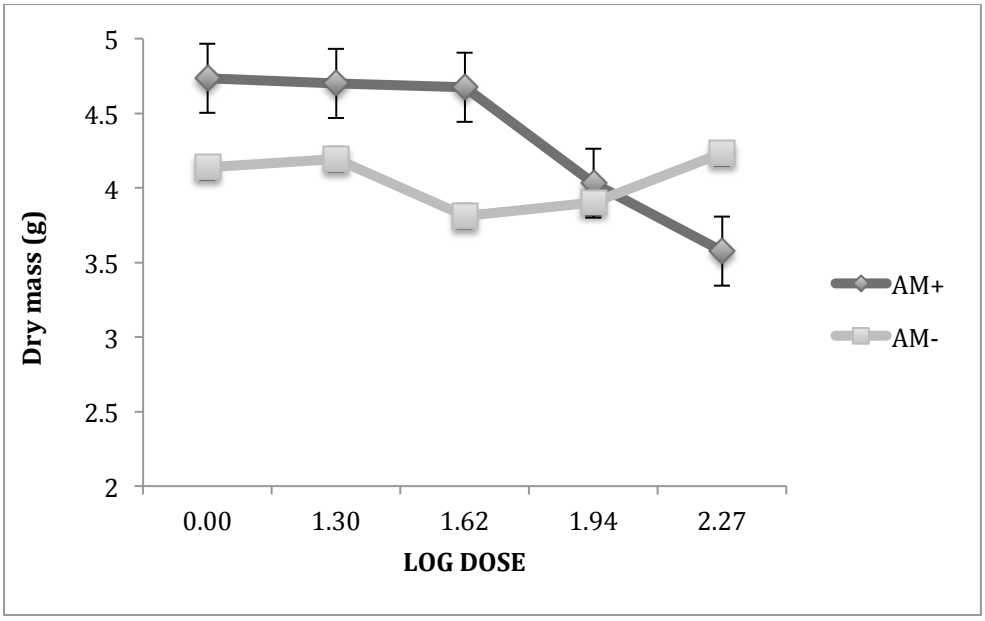

Figure 12: Root dry mass of *G. canadense* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

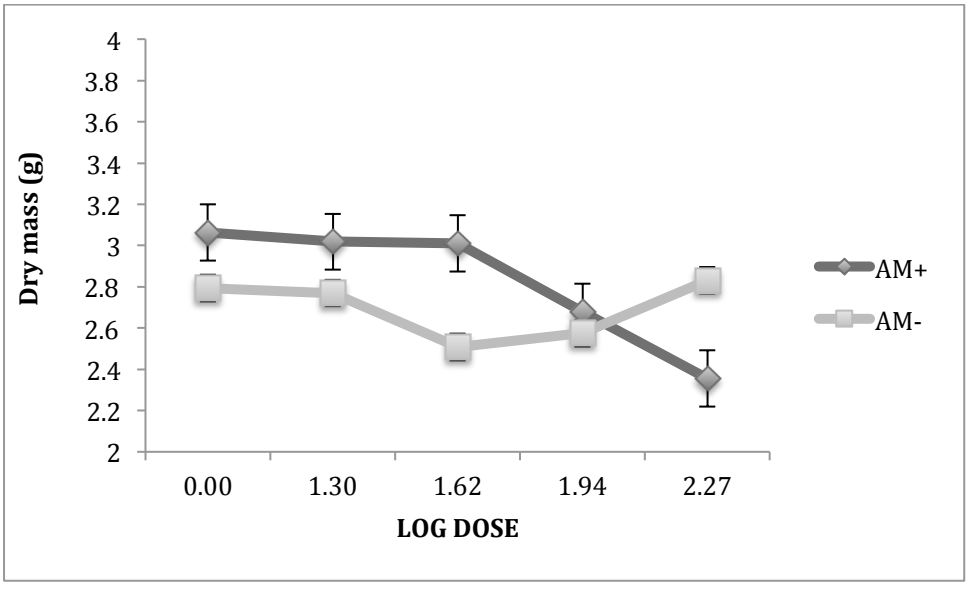

Figure 13: Shoot dry mass of *G. canadense* with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.2 Mesocosm Experiment

3.2.1 *Solidago canadensis* (S.C.)

Mycorrhization levels for *S. canadense* roots were 43, 46.3, 46, 42.6 and 45.4 % for the respective doses. No significant effect of AZY was observed on the total, root, and shoot biomass of S.C. (figures 14, 15, and 16 respectively). The presence of AMF exerted a statistically significant decrease in shoot biomass ($p = 0.017$) and in total biomass ($p = 0.022$). Furthermore, no significant interaction was observed between AZY and AMF.

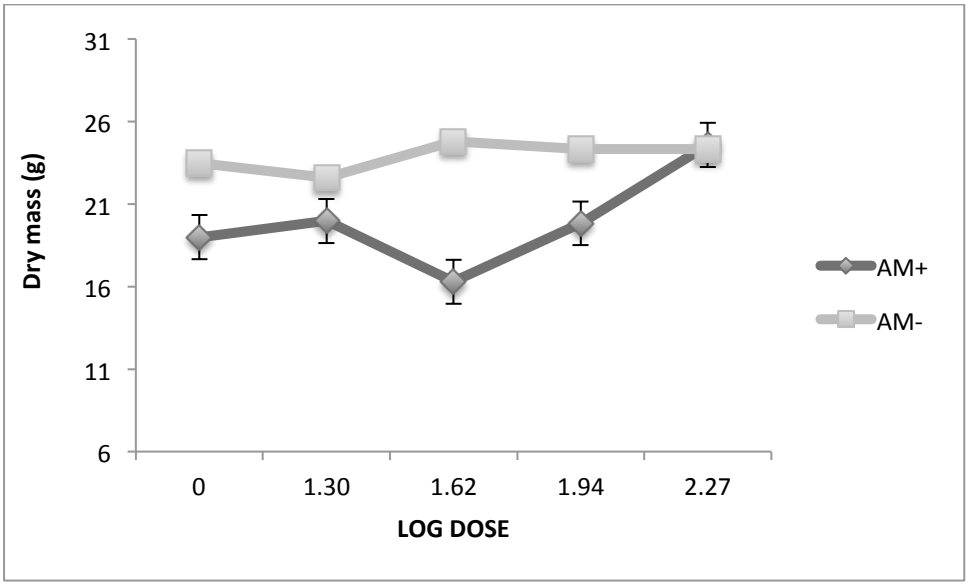

Figure 14: Total dry mass of *S. canadense* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

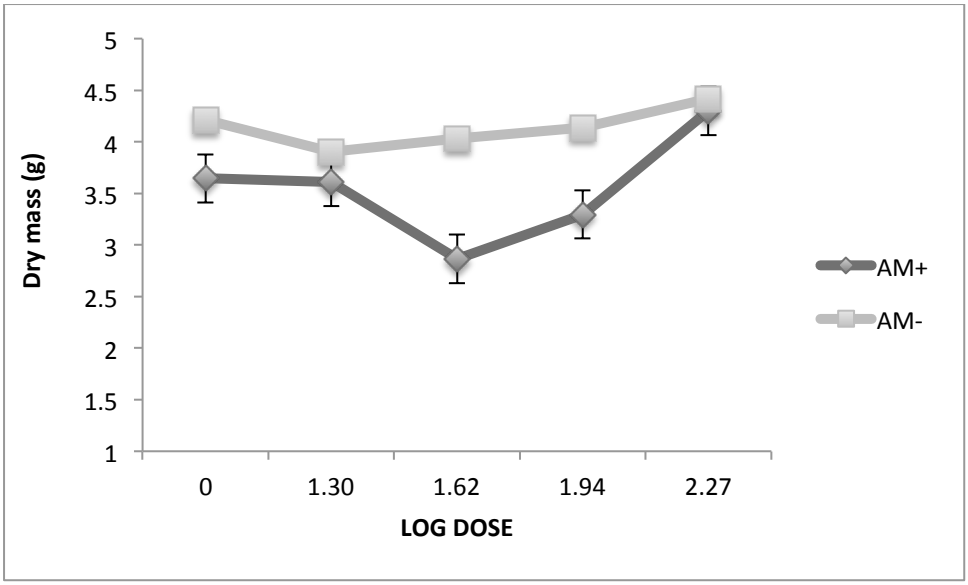

Figure 15: Root dry mass of *S. canadense* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

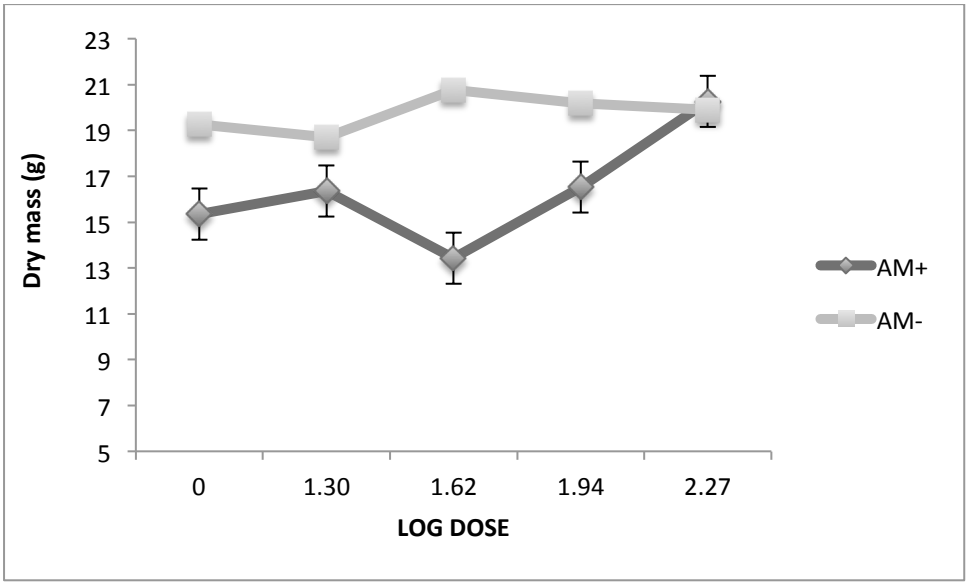

Figure 16: Shoot dry mass of *S. canadense* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.2.2 *Phalaris arundinacea* (P.A.)

Root mycorrhization levels for *P. arundinacea* plants sprayed with AZY were 45, 38.6, 41.3, 44.7 and 37 % for the respective doses. Furthermore, AZY did not exert a significant effect on total, root, and shoot biomass, of P.A.

However, the presence of AMF had a significant effect on total biomass (figure 17) (p=0.001), root biomass (figure 18) (p=0.001) and shoot biomass (figure 19) (p=0.001), with the interaction between AMF and AZY being insignificant across all three parameters.

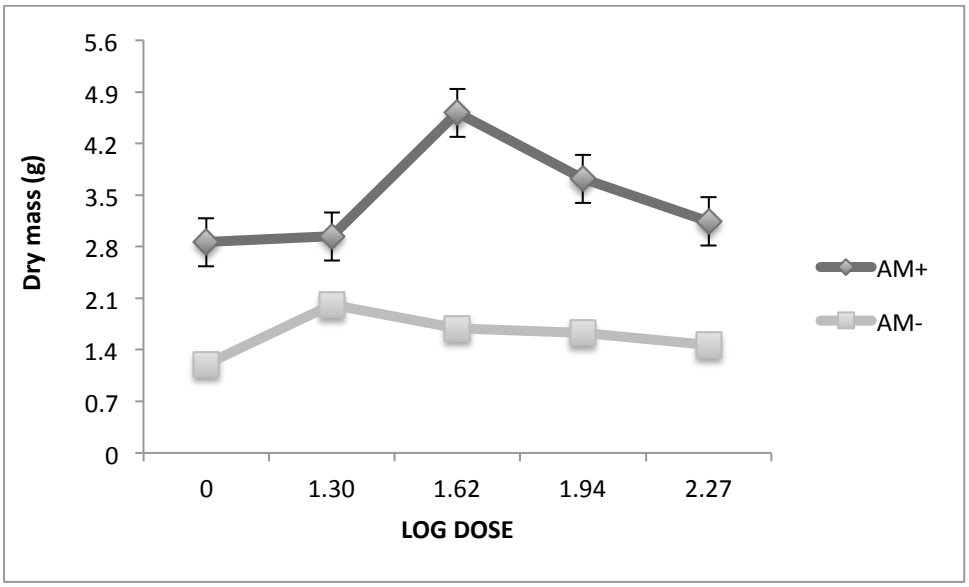

Figure 17: Total dry mass of *P. arundinacea* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

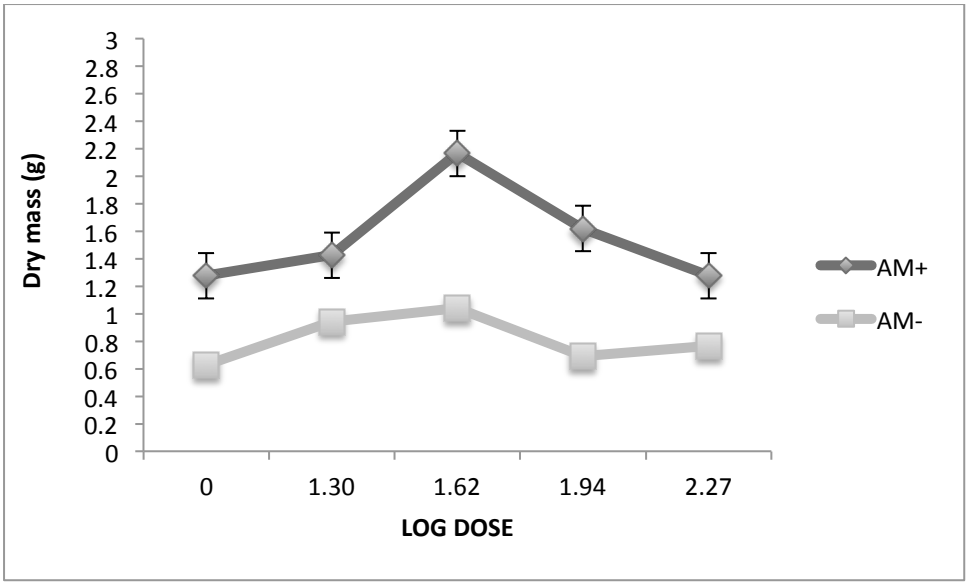

Figure 18: Root dry mass of *P. arundinacea* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

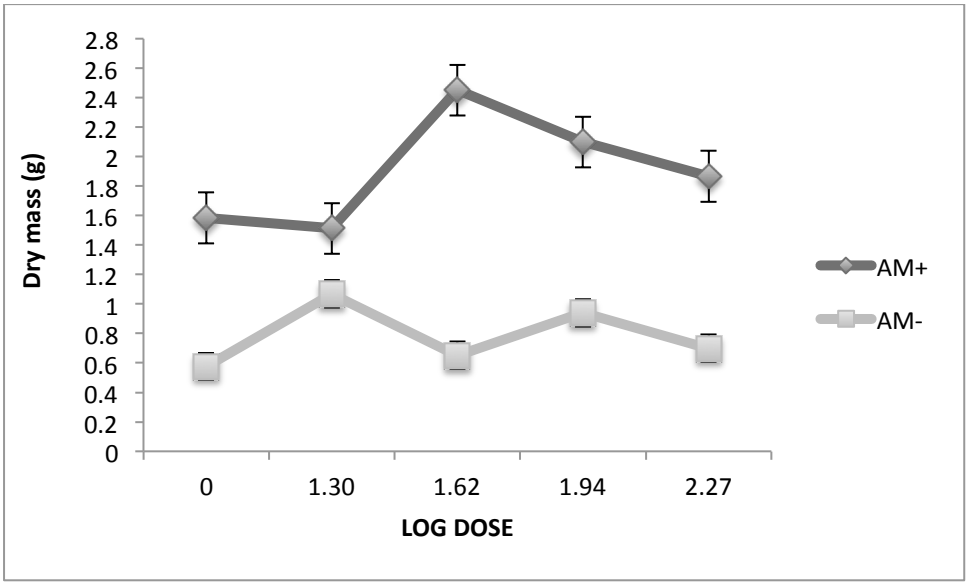

Figure 19: Shoot dry mass of *P. arundinacea* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.2.3 *Chenopodium album* (C.A.)

Root mycorrhization did not take place in *C. album* plants. Moreover, there was no significant effect of either AZY or AMF, nor their interaction on the total biomass (figure 20), root biomass (figure 21) and shoot biomass (figure 22).

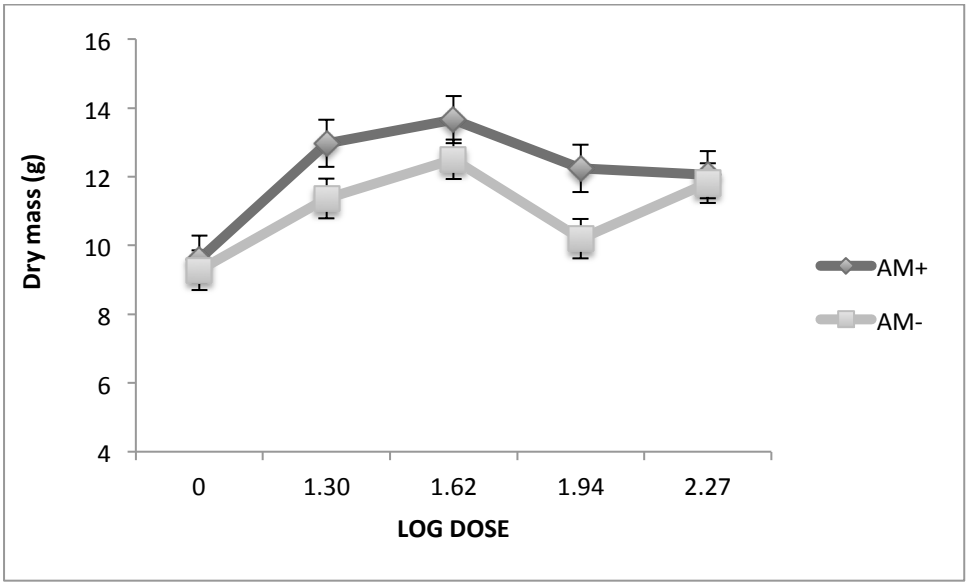

Figure 20: Total dry mass of *C. album* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

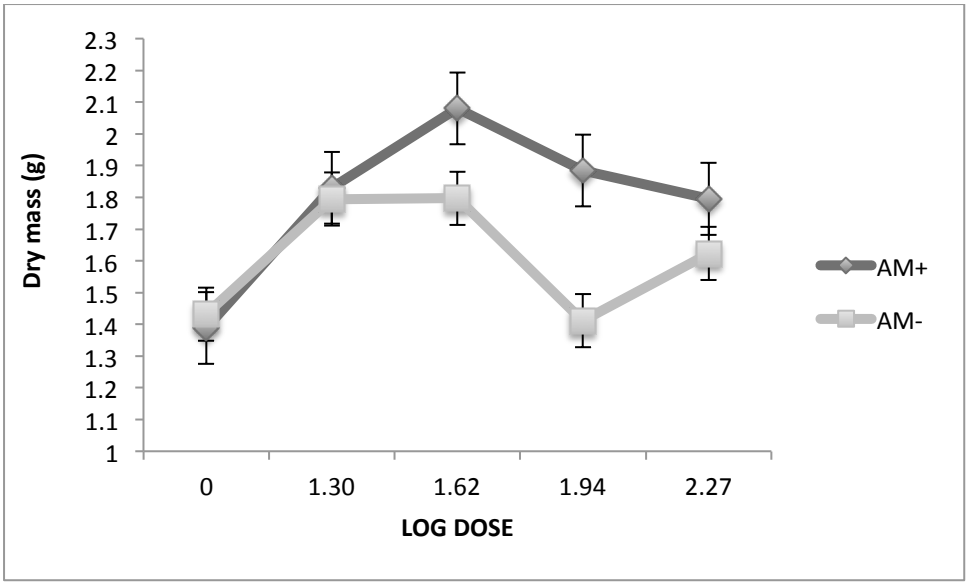

Figure 21: Root dry mass of *C. album* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

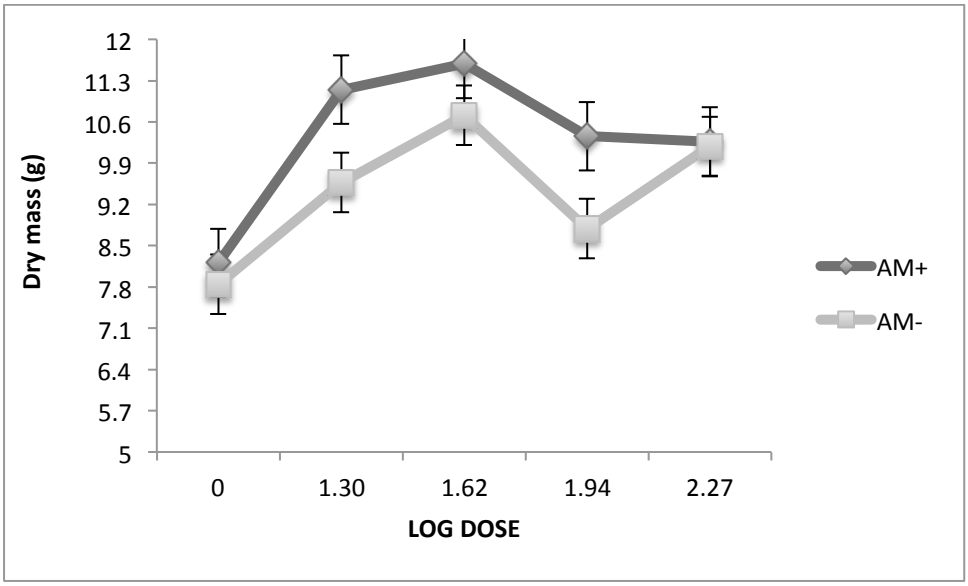

Figure 22: Shoot dry mass of *C. album* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

3.2.4 *Geum canadense* (G.C.)

Mycorrhization levels in *G. canadense* roots were 7, 3, 5, 3.7 and 6 % respectively for the doses. Furthermore, AZY did not have a significant effect on total, root, and shoot biomass of G.C. However, the presence of AMF resulted in a significant increase in total biomass (figure 23) ($p = 0.015$), root biomass (figure 24) ($p = 0.037$), and shoot biomass (figure 25) ($p = 0.012$).

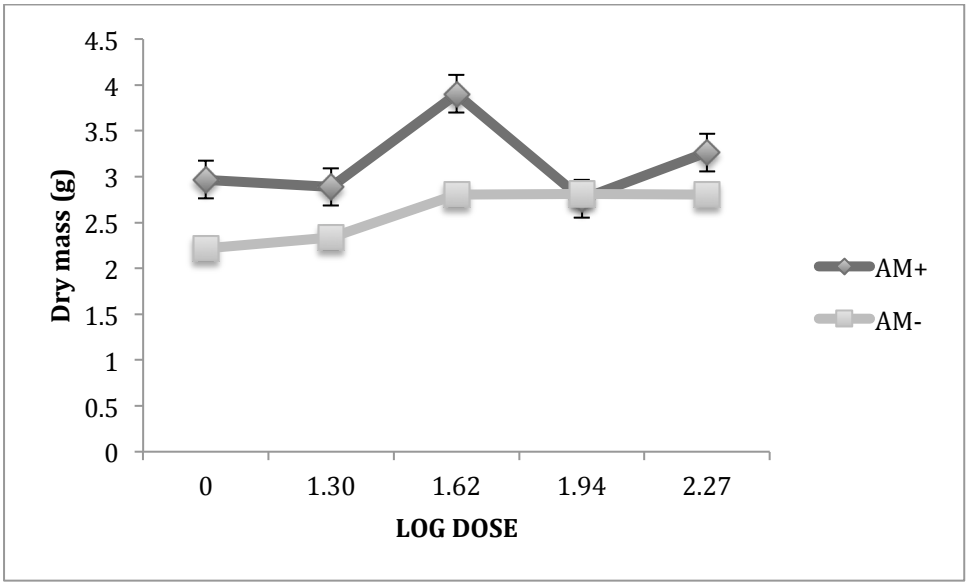

Figure 23: Total dry mass of *G. canadense* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

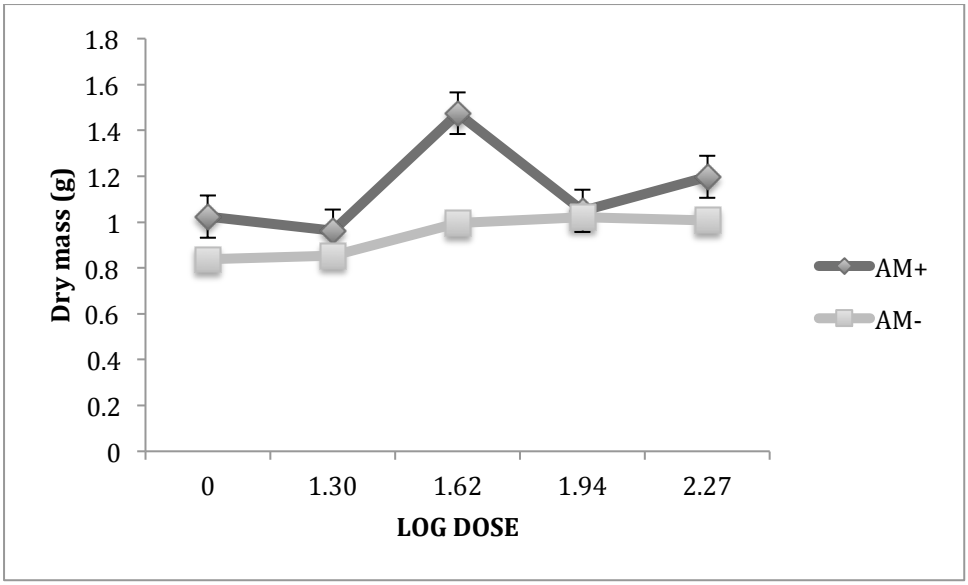

Figure 24: Root dry mass of *G. canadense* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

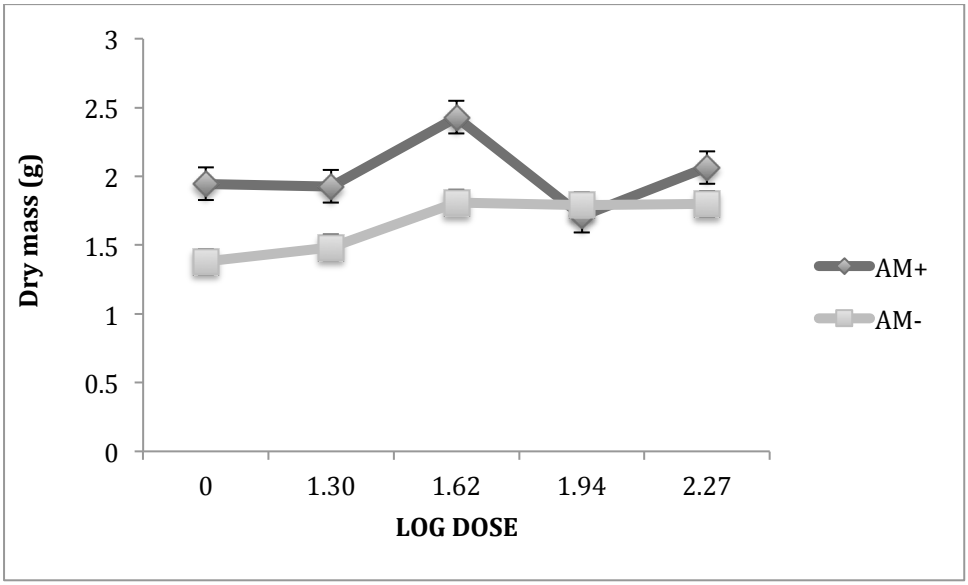

Figure 25: Shoot dry mass of *G. canadense* (Mesocosm) with or without AM colonization at different AZY doses (0, 0.33, 0.69, 1.45, and 3.07 mmol/L). Means (n=6) and SEs are shown.

Chapter 4. Discussion

Arbuscular mycohrrizal fungi (AMF) were shown to colonize a widespread variety of host-plant species with different life histories. However, the kind of interaction that ultimately takes place between host plants and AMF and the results of the symbiosis differ greatly depending on plant and fungus genotypes, both in isolation and in competitive environments (Smith and Smith 1995).

In the mesocosm experiment, AZY had no significant effect on any of the measured parameters for all four-plant species, in contrast with the single species experiment, where AZY's effects varied greatly between species. Most notably in *Geum canadense*, which underwent a decrease in shoot and total dry mass in AMF inoculated candidates under the highest AZY dose (3.07 mmol/L).

Because the plant candidates grew much more slowly in our screening test in autumn, we had not anticipated the surge in growth once the first two species (*Solidago canadense* and *G. canadense*) were transplanted in the mesocosm pot before *Phalaris arundinacea* and *Chenopodium album*. *S.canadense* and *G. canadense* were transplanted first because it was calculated that they needed more time to reach the growth stage at which they were to be sprayed. *S. canadense*, the plant with the highest percent colonization in this study seems to have taken advantage below ground to form AMF associations that altered AMF/root dynamics pertaining to the other plants in the pot. *S. canadense* had the highest biomass and quickly outgrew the rest of the plants, boosting light acquisition (Bray 2003). Following from that, *S. canadense's* foliage was exposed the most to AZY since it covered most of the pot area, followed by *C. album*, It is possible that *C. album* being a non-host for AMF, was able to redistribute carbon to shoot growth (Zhang

2010). AMF can demand up to 20% of total carbon under severe cases (Peng *et al*. 1993). This behaviour of *C. album* was enhanced by the fact that since it was the only annual plant in the experiment, it does not need to invest extensively in root formation.

There is subtle, but persuasive evidence that *P. arundinacea* and *G. canadense* were exposed to less AZY than *S. canadensis* and *C. album*.

It is important to note that plant responses to AMF are affected to a large extent by light exposure and soil available phosphorus (Gavito *et al.* 2000). This discrepancy in the plants' foliar exposure to AZY is more apparent when comparing AZY effects on the same species grown in isolation or in the mesocosm. A key finding in this study is that in *G. canadense*, the species most likely susceptible to AZY since it belongs to the same family as Macintosh apple trees (Rosaceae), AZY inflicted a significant decrease in shoot and total biomass in AMF inoculated plants under the highest dose (3.07 mmol/L), while in the mesocosm experiment, there was no effect of AZY on plant biomass but rather a significant effect of AMF presence which led to increased plant growth. We may infer that, among other factors, the near total masking of *G. canadense* by *S. canadense* and *C. album* foliage led *G. canadense* to be underexposed to AZY, hence the lack of AZY effect, in contrast with the single species experiment. This finding is concurrent with previous research conducted on the toxicity of AZY to Cortland and Macintosh apple trees, where it was found that exposure to doses as low as 10 ppb resulted in severe damage to well established trees (Lange 2004).

Similarly, AZY caused a significant increase in root, shoot, and total biomass of the single species experiment on *P. arundinacea*, but so did the presence of AMF. Under the mesocosm experiment, only AMF had a significant effect on *P.*

arundinacea. It is quite probable that the canopy cover provided by *S. canadense* and *C. album* reduced the amount of AZY to which *P. arundinacea* was exposed.

The increases in shoot and total biomass caused by AZY in *S. canadense* plants inoculated with AMF in the single species experiment can be perplexing, but could be attributed to the physiological effect of AZY on *S. canadense*. Research on the effect of a fungicide belonging to the same family as AZY, Pyraclostrobin, has shown that this fungicide can alter the phytohormonal balance of the exposed plant (in this case winter barley) and caused an increase in plant biomass (Köehle *et al.* 2002). The relatively high root colonization rate by AMF in *S. canadense* in both experiments is not uncommon for a known mycotrophic host (Werner *et al.* 1980) and the significant effect AMF had on root biomass of *S. canadense* (increase in growth for single species experiment) could be due to the fact that AMF colonization has been shown to increases root biomass (van der Heijden *et al.* 2003). Conversely, AMF exerted a decrease in shoot and total biomass of *S. canadense* when grown in competition in the mesocosm. One could argue that this effect is attributed to the competition for resources by the other plants and below ground interactions. Furthermore, it has been proposed that the relationship between host plants and the degree of root colonization by AMF is curvilinear, with the advantage to the host ultimately reaching a plateau at some colonization level (Gange and Ayres 1999).

Results from *C. album* seemed puzzling at first. While there were no observable AMF structures in our root samples, AMF had a significant effect on all measured parameters, with AZY affecting all parameters except root biomass. Since some experiments have shown that AMF can have antagonistic effects on *C. album* (Francis and Read 1995), a possible explanation could be that *C. album* reacted to the AMF as a stressor, and over allocated resources to counteract that stress, which

resulted in an increased growth. *C. album* grown solely underwent an increase in shoot biomass at the intermediate AZY dose (0.69 mmol/L) before dropping again at the highest applied dose (3.07). This phenomenon, known as hormesis, is not rare in nature and is essentially a dose-response relationship best described by stimulation at low doses of xenobiotics and inhibition at high doses (Calabrese and Baldwin 2003).

Another interesting finding is that, with the exception of AMF inoculated *G. canadense* exposed to the highest dose of AZY, the fungicide did not seem to have disrupted the plant-AMF association. Within the limits of our study there are two factors that may have affected this outcome. The first is the method of application of AZY and the limited basipetal translocation for most foliar fungicides. For the fungicide to reach the AMF, it would have to be absorbed by the leaf, and then translocated via the xylem to the roots, were it will be in contact with the AMF. Fungicides applied as soil drenches (AZY being one such fungicide) tend to have a more direct effect on the AMF. The second factor is the degree of the plant-AMF symbiosis at the time of spraying. Diedhio *et al.* (2004) demonstrated that, when applied to foliage of maize that had either well established mycorrhiza (defined at 5-weeks old) or newly established mycorrhiza (defined as 10 days old), AZY did not affect the mycorrhizal activity of the older better established plants but had an adverse effect on the AMF in the 10-day old transplants. Moreover, when AZY was applied as a soil drench to 5-week old plants, mycorrhizal activity decreased drastically and ceased completely after 21 days (Diedhiou *et al.* 2004).

Conclusions

This thesis aimed to investigate the effect of AZY, a systemic fungicide with known toxicity to certain varieties of apple trees and to aquatic organisms, on four non-target plant species and on the *Glomus intraradices*.

AZY exerted an increase in the biomass of *P. arundinacea* and *S. canadense* grown in single species experiments. This may be due in part to the strong ability of those species to withstand different types of stress. It can also result from the physiological effects that some fungicides can cause. AMF presence may have caused *C. album* to over allocate resources in order to counteract the antagonistic effect of AMF, which may explain the increase in dry mass.

A key finding in this research is the effect AZY had on *G. canadense*. In the single species experiment AZY was shown to cause a decrease in the biomass of plants exposed to the highest dose (93% label rate) in AMF inoculated plants. These results are concurrent with research on the effect that AZY may have on Rosaceae species. Conversely, in the mesocosm experiment, AMF were able to exert a positive effect on the biomass of *G. canadense.* This may be due to the other plant species shading *G. canadense* so that AZY did not reach *G. canadense*.

The exposure of non-target plant species in our experiment resulted in a myriad of effects and interactions in terms of biomass. Fungicides clearly posses the potential for disturbing ecosystem equilibrium. Therefore it follows that further understanding on the singular and synergistic effects of agrochemicals is required so long as the use of these products continues and is poised to increase when patented formulations become more readily available.

Future Directions

The impact of pesticides on species distribution and ecosystem composition is influenced by a myriad of factors. More studies should be conducted in order to further define the relationship between plants and AMF when exposed to pesticides, while taking the following into consideration:

- Special attention should be given towards ensuring that all plants are grown simultaneously.
- The measurement of soil pH should be conducted in order to better evaluate not only the presence of AMF, but also their metabolic activity.
- Future studies should be performed that target other plant species belonging to the Rosacea*e* family to ascertain if a pattern of toxicity from AZY does emerge.

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