# EFFECTS OF NATURAL/ANTHROPOGENIC STRESSORS AND A CHEMICAL CONTAMINANT ON PRE AND POST MYCORRHIZAL COLONIZATION IN WETLAND PLANTS

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Arbuscular mycorrhizal fungi, colonizing over 80% of all plants, were long thought absent in wetlands; however, recent studies have shown many wetland plants harbor arbuscular mycorrhizae (AM) and dark septate endophytes (DSE). Wetland services such as biodiversity, shoreline stabilization, water purification, flood control, etc. have been estimated to have a global value of $14.9 trillion. Recognition of these vital services is accompanied by growing concern for their vulnerability and continued loss, which has resulted in an increased need to understand wetland plant communities and mycorrhizal symbiosis. Factors regulating AM and DSE colonization need to be better understood to predict plant community response and ultimately wetland functioning when confronting natural and human induced stressors. This study focused on the effects of water quality, hydrology, sedimentation, and hurricanes on AM and DSE colonization in three wetland species (*Taxodium distichum*, *Panicum hemitomon*, and *Typhal domingensis*) and plant communities of coastal wetlands in Southeast Louisiana and effects of an antimicrobial biocide, triclosan (TCS), on AM (*Glomus intraradices*) spore germination, hyphal growth, hyphal branching, and colonization in fresh water wetland plants (*Eclipta prostrata, Hibiscus laevis, and Sesbania herbacea*) from bottom land hardwood forest in north central Texas. The former, mesocosm studies simulating coastal marsh vegetation ran for five years. In the latter studies, AM spores and wetland plants were exposed to 0 μg/L, 0.4 μg/L, and 4.0 μg/L TCS concentrations in static renewal and flow through exposures for 21 and 30

days, respectively. AM and DSE colonization was significantly affected by individual and interactions of four independent variables in mesocosm experiments. Similarly, spore germination, hyphal growth, hyphal branching, and AM colonization in selected wetland plants were significantly lowered by exposure to the TCS at environmentally relevant concentrations. However, levels of effects were plant species and fungal propagules specific. My results showed that natural and human induced alterations in environmental factors and chemical contaminants can significantly impact levels of mycorrhizal spore germination, colonization, and spore density in coastal and freshwater wetland plants. The resulting impacts on plant community structure and ecosystem function require further study.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Wetlands

Natural wetlands provide habitat for native and migratory birds, fish, and animals, a physical barrier for flood water, improvement of water quality by natural filtration, and are sources and sinks in biogeochemical cycles (Keddy, 2000; Mitsch and Gosselink, 2000). Likewise, manmade wetlands are used for wastewater treatment due to their efficiency of removing nutrients and environmental contaminants (Kadlec and Wallace, 2009), and provide habitat for water fowl, and recreation (EPA, 2000). As a result of these varied ecosystem services, wetlands are valued at $ 14.9 trillion USD globally (Costanza et al., 1997). The nature of the wetland ecosystems and services provided primarily depends on the wetland plant communities present (Zedler and Kercher, 2005). Any alteration in wetland plant communities such as plant death, or loss of species may have negative impacts on ecosystem services (Boesch et al., 1994; Zedler and Kercher, 2005) or lead to a loss of wetland ecosystems (Gough and Grace, 1998; Gibbs, 1999). Half of the world's wetlands have already been lost and remaining wetlands comprise less than 9% of the earth's land (Zedler and Kercher, 2005). There is a pressing need for sustainable use and conservation of existing wetlands and restoration of lost wetlands (Ramsar Convention; State and Federal Wetland Restoration Programs; Coastal Wetland Planning, Protection, and Restoration Act [CWPPRA]). The restoration and conservation of wetlands requires an understanding of vegetation response to biotic and abiotic changes (Dobson et al., 1997; Hobbs and Harris, 2001; Suding et al., 2004).

## 1.2 Mycorrhizas

Most terrestrial plants form symbiotic associations with soil fungi from diverse fungal taxa (Bever et al., 2001). Termed, mycorrhizas, these associations which develop around or within host plant roots are generally considered mutualistic (Kiers et al., 2006; Rodriguez et al., 2008). The fungus obtains carbohydrates from the host while the host is provided with inorganic nutrients and water (Peterson et al., 2004; Allen, 2007). Hyphae, thread-like extensions of the fungal body extend beyond the zone of depletion surrounding the host roots thereby providing access to resources otherwise unavailable to the host (Smith and Read, 2008). In addition, extracellular enzymes secreted by fungi enhance absorption of nutrients in the soil (Siddiqiu et al., 2008). Historically, mycorrhizae have been divided into two categories. In endomycorrhizal associations the fungal symbiont proliferates throughout the cortex of the host roots, while in ectomycorrhizal associations, the fungal symbiont does not enter the host plant roots beyond the epidermal layer (Peterson et al., 2004). This dissertation is focused on two types of endomycorrhizal association; arbuscular mycorrhiza (AM) and dark septate endophytes (DSE).

### 1.2.1 Arbuscular Mycorrhiza (AM)

Among the several types of mycorrhizal fungi, arbuscular mycorrhiza (AM), are the most widespread endomycorrhizal association (Bever et al., 2001). Arbuscular mycorrhizae are formed by aseptate, obligately symbiotic fungi from the order Glomales in the Zygometcetes (Smith and Read, 2008). It is estimated that more than 90% of terrestrial plants harbor AM (Strack et al., 2003). AM are characterized by the formation of arbuscules (Fig. 1.1A), highly conserved structures developed within the host cells but not penetrating host protoplasm

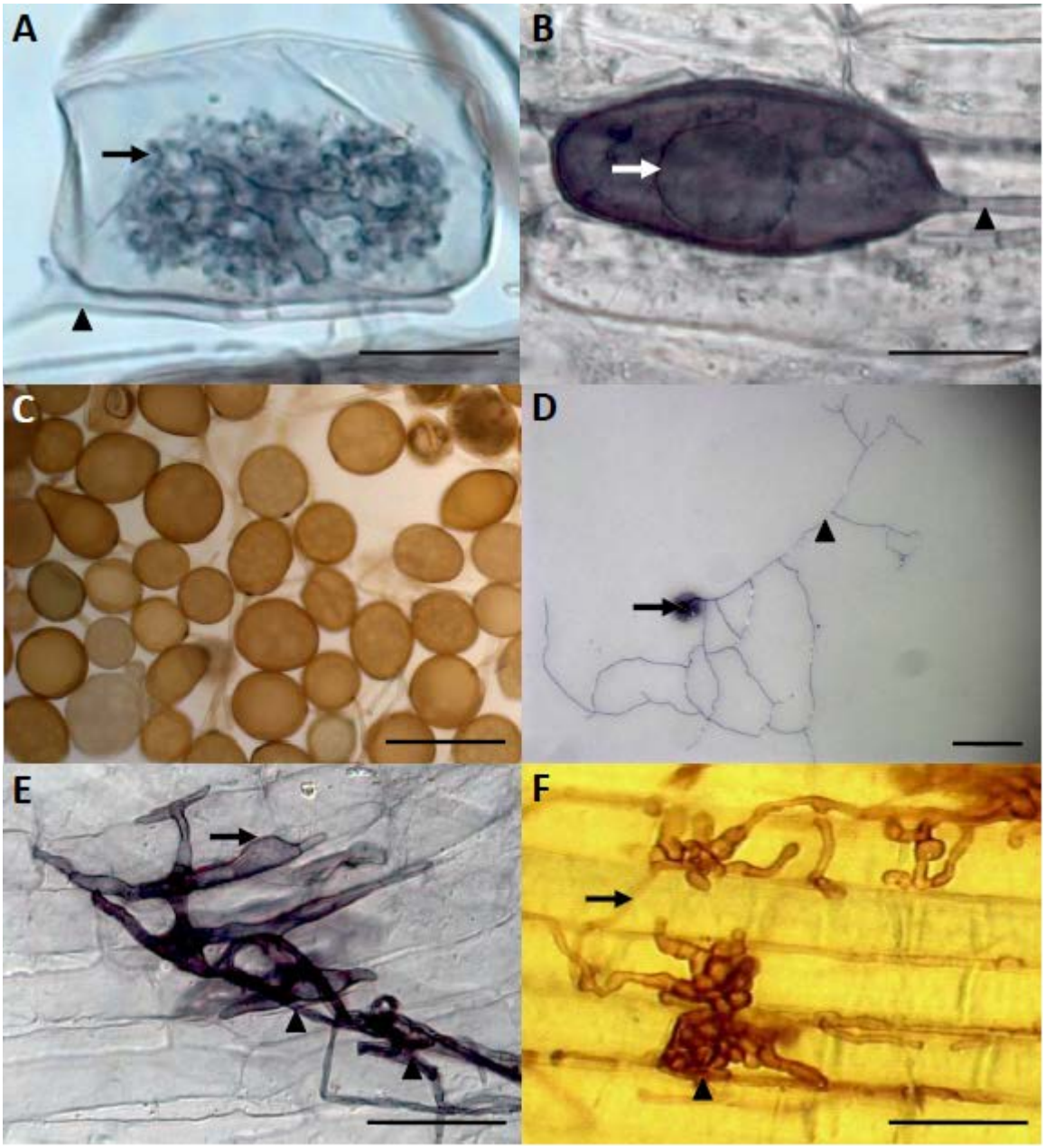


Figure 1.1 Arbuscular mycorrhizal fungi and dark septate endophytes. A-E arbuscular mycorrhizae. A: An arbuscule inside the host cortical cell (arrow) and intra-radical hypha (arrow head), scale bar 20 µm. B: A vesicle with lipid (arrow) and subtending hypha (arrow head), scale bar 20 µm. C: *Glomus intraradices* AM spores, scale bar 100 µm. D: A germinated spore (arrow) and a branched hypha (arrow head), scale bar 200 µm. E: An appresorium (arrow) and extraradical hyphae (arrow heads), scale bar 50 µm. F: A dark septate endophyte, hypha (arrow) and microsclerotia (arrow head), scale bar 50 µm.

(Smith and Read, 2008; Pumplin and Harrison, 2009). Arbuscule formation begins when a side branch of intraradical hyphae penetrates the cell wall and divides dichotomously to develop an

arbuscular “tree”. This structure increases the surface area of the interface between two symbionts enhancing nutrient and organic carbon exchange (Toth and Miller, 1984; Smith and Gianinazzi-Pearson, 1988; Harrison, 2005). In addition to arbuscules, AM develop spores and depending on the taxa, vesicles. Vesicles (Fig. 1.1B) are oval or round, thick-walled, multinucleated, and lipid containing structures developed at the tip of hyphae or hyphal branches in the host cortex. They are usually formed at the end of the growing seasons and act as propagules for the next season (Peterson et al., 2004). Asexual spores (Fig. 1.1C), a second type of propagule, are produced usually on extra-radical hyphae. Spores contain numerous nuclei, lipid droplets, and other organelles and are protected by pigmented and impermeable wall layers (Eskandari and Danesh, 2010). Spores may be dispersed by air, water and animals, and following germination can colonize fresh and newly formed roots of the host plants (Janos et al., 1995; Warner et al., 1987).

### 1.2.2 Colonization of Roots

A newly developed root of a host plant may be colonized by germinating spores, previously colonized root fragments, or hyphae in the soil (Klironomos and Hart, 2002; Smith and Read, 2008). Colonization involves a series of stages starting from germination of spores (Fig. 1.1D). Spores can germinate readily after undergoing a period of dormancy and hyphae will grow in absence of host roots; however, host root exudates will stimulate spore germination, hyphal growth and hyphal branching (Tsai and Phillips, 1991; Akiyama et al., 2005; Harrison, 2005). Host root exudates indicate root presence inducing, hyphae from the germinating spore or pre-existing hyphal branches in soil to grow towards the host root. Fungal hyphae increase respiration in response to the root exudates within 2-3 h (Harrison, 2005).

Once contact is made with the host root at the epidermal layer, appressoria (Fig 1.1E), specialized elliptical, elongated, and multinucleated structures, are formed through which the hypha enters host epidermal cells (Harrison, 2005; Requena et al., 2006). Epidermal cell penetration is followed by development of intraradical mycelia. Arbuscules in the colonized roots are developed within 2-3 d of infection. In species forming vesicles, vesicles can develop 4-5 d following colonization; however, extensive vesicular formation usually takes place at the end of the growing season (Brundrett et al., 1985; Alexander et al., 1988; Peterson et al., 2004). At maturation, intra- or extra-radical hyphae develop asexual spores after 3-4 weeks but reach maximal production in 3-4 months (Chabaud et al., 2006); however, sporulation was observed within 2 weeks in a root organ culture in *Daucus carota* (Hillis, 2009). During each stage in the colonization process, signaling occurs between the host plant and fungus and colonization may be aborted at any stage in the process (Harrison, 2005). Any external factors that impact colonization may have consequences in terms of plant performance and the ecosystem services they provide (Gianinazzi et al., 2010; Stevens et al., 2007).

### 1.2.3 Dark Septate Endophytes (DSE)

Dark septate endophytes (Fig. 1.1F) are a heterogeneous group of sterile fungi and thought to be ascomycetous fungi (Jumpponen, 1998). DSEs are characterized by darkly pigmented and melanized septate hyphae; however, non-melanized hyaline hyphae are developed in host tissue (Newsham, 1999). DSE colonize roots by extending their septate hyphae through inter and intracellular spaces in the root tissues without causing any harm to the host (Jumpponen and Trappe, 1998). They produce intracellular spherical clusters of structures called microsclerotia (Fig. 1.1F). Microsclerotia accumulate and store enough

reserves like glycogen, protein, and polyphosphate and function as propagules to facilitate further colonization (Yu et al., 2001). Like AM, they are found in the roots of angiosperms, gymnosperms, and ferns from tropical to alpine ecosystems and are common in cold, nutritionally poor, alpine or subalpine ecosystems with stressful environments (Read and Haselwandter, 1981; Jumpponen and Trappe, 1998; Rains et al., 2003). In addition, DSE have been found in vegetation from degraded wetlands of Louisiana (Kandalepas et al., 2010) and bottomland hardwood forests in north central Texas (Stevens et al., 2010).

## 1.3 Functions of Mycorrhizal Fungi

In the terrestrial ecosystems, the primary role of the AM fungi is in the acquisition and transportation of inorganic phosphate (P) and other nutrients from substrate to the plants roots, enhancing plant physiology and biomass production (Smith and Read, 2008). The benefits of harboring AM fungi, however, extend beyond nutrient uptake. Through their uptake and transport of water to their host AM fungi increase drought resistance (Auge, 2001), delay wilting, and elevate stomatal conductance (Zhu et al., 2010). Increased stomatal conductance improves the gas exchange via stomata, contributing to enhanced photosynthesis in mycorrhizal plants (Allen et al., 1981; Dunham et al., 2003; Sheng et al., 2008). AM also help to reduce the effects of plant pathogens and nematodes possibly by the release of mycorrhizal metabolites that reduce nematode attraction or by increase in the cell wall thickness in the tissues at the site of infection increasing physical barrier for pathogen invasion (Ingham, 1988; Rodriguez et al., 2003; de la Pena et al., 2006), and inducing systemic resistance as in the tomato plant (Vos et al., 2012). AM have also been shown to provide protection from salt stress

(Evelin et al., 2009) by reducing intake of $Na^+$ ions, inducing the expression of aquaporin (a specific protein on the plasma membrane regulating the flow of water) genes to maintain a favorable osmotic gradient, detoxifying reactive oxygen species developing from salt stress, and increasing hydraulic conductivity (Giri and Mukerji, 2004; Bothe, 2012).

Similarly, increased root, shoot, and total biomass of plants colonized by DSE is also believed to result from increased nutrient acquisition (Haselwandter and Read, 1982; Newsham, 1999; Newsham, 2010). A meta-analysis of plant responses to DSE by Newsham (2010) has found 19 plant species from 8 families to increase average shoot P and N content by 26 and 103% respectively thereby increasing total, root, and shoot biomass by 138, 79, and 109% respectively without additional inorganic nitrogen supply. DSE have been shown to reduce pathogen infection by consuming organic carbon sources that would otherwise be available as a pathogen substrate (Mandyam and Jumpponen, 2005), increasing the physical barrier to pathogens by wall thickening of exodermal cells adjacent to hyphae in asparagus (Yu et al., 2001), and production of toxic compounds, periconisins (antibacterial) (Kim et al., 2004). Given these varied roles and contributions, it can be surmised that if DSE colonization is affected by adverse environmental factors, impacts on the plant performance, plant communities and ecosystem services may ensue.

### 1.3.1 Mycorrhizas in Wetlands

Mycorrhizal fungi were long thought absent in wetland plants (Khan and Belik, 1995) in part stemming from the belief that AM fungi are unable to survive the anaerobic conditions typical of wetland soils (Cooke et al., 1993; Peat and Fitter, 1993). Extensive studies of AM in aquatic systems in the past few decades, however, have revealed that many wetland plants are

colonized by endophytes (Radhika and Rodrigues, 2006). Kandalepas et al. (2010) found all 18 plants in the degraded wetlands in Louisiana marsh colonized by AM fungi, DSE or both. Similarly, in an investigation of 290 species of flowering plants studied in variety of Connecticut fresh water habitats, all plants were found colonized by endomycorrhiza (Cooke and Lefor, 1998). Plants in wetland ecosystems ranging from bottomland hardwood forest (Stevens et al., 2010), marshlands, saltmarshes (Daleo et al., 2008), oligotropic wetlands, prairie potholes, everglades, recently rehabilitated wetlands (Radhika and Rodrigues, 2006), degraded cypress swamps (Kandalepas et al., 2010), and submerged macrophytes (Clayton and Bagyaraj, 1984) harbor symbiotic fungi. The presence of AM fungi and DSE in wetland plants is now expected; however, the factors that affect levels of colonization in wetland habitats and the role that mycorrhizae play in structuring wetland plant communities are poorly understood (Stevens et al., 2002; Muthukumar et al., 2004; Stevens and Peterson, 2007).

Colonization levels are known to differ among wetlands and are influenced by hydrology, nutrients, oxygen, and other factors (Miller and Bever, 1999; Miller, 2000; Bohrer et al., 2004; Escudero and Mendoza, 2004). As in terrestrial ecosystems, mycorrhizal fungi in wetlands help plants in nutrient acquisition (Wigand and Stevenson, 1994), plant growth, plant performance, and seedling establishment (Stevens et al., 2011). They are also found to increase drought resistant in the wetland plants during seasonal water fluctuation (Khan, 2004). Although seldom measured, mycorrhizal responsiveness is a valuable metric to gauge the contribution of AM fungi to overall plant performance (Janos, 2007). In one of the few studies to assess mycorrhizal responsiveness in wetland species, Stevens et al. (2011) found that mycorrhizal responsiveness differed among two closely related species (*B. frondosa* and *E.*

*prostrata*) and furthermore that mycorrhizal dependency was affected by water availability. Given that mycorrizal responsiveness is species and environment specific and that the majority of wetland species assessed harbor AM fungi, it can be expected that environmental factors that impact mycorrhizal colonization will impact the host plant species to different degrees. This in turn could significantly alter plant community structure and valuable ecosystem functions. Understanding the effects of natural and anthropogenic stressors on wetland mycorrhizae may therefore, provide greater insight into the factors shaping wetland plant community structure and the ecosystem services they provide.

## 1.4 Factors Affecting Mycorrhizal Colonization

Colonization levels and functionality of mycorrhizal fungi in upland plants are directly dependent on edaphic factors such as soil temperature, moisture, pH, salinity, ionic condition, soil depth, and rhizosphere organisms (Al-Agely and Reeves, 1995; Entry et al., 2001; Sharma and Johri, 2002). While most of the mycorrhizal species studied to date appear to colonize at temperatures ranging from 18 °C to 40 °C (Entry et al., 2002), optimal temperatures for spore germination range from 18 to 25 °C for *Glomus epigaeus* (Daniel and Trappe, 1980). Sporulation has been found to be positively correlated with redox potential, soil pH, and warmer seasons (Sharma and Johri, 2002; Sivakumar, 2012), while factors such as high nutrients (P and N), flooding and organic pollutants have shown to reduce mycorrhizal spore density (Ortega-Larrocea et al., 2001; Sharma and Johri, 2002; Cheeke et al., 2011). Extreme flooding and drought have been shown to reduce mycorrhizal colonization (Miller, 2000; Auge, 2001) as has elevated levels of soil nutrients including nitrogen and phosphorus (White and Charvat, 1999;

Tang et al., 2001). Colonization levels are further affected through interactions with other soil orgamisms. Many rhizosphere bacteria are found to promote mycorrhizal colonization, while soil animals grazing fungal hyphae negatively impact colonization (Ingham, 1988). Colonization is also negatively impacted by anthropogenic pollutants including polyaromatic hydrocarbons (e.g. anthracene), diesel fuel, pesticides (e.g. benomyl, chlorothalonil, dimethoate), and metal contaminants (e.g. Al, Ni) (Cairney and Meharg, 1999; Titus and Leps, 2000; Grigera and Oesterheld, 2004; de Oliveira and de Oliveira, 2005; Harner et al., 2009).

In contrast to the rather well understood effects of biotic and abiotic factors affecting AM colonization in terrestrial environments, studies on the factors affecting mycorrhizal fungi in wetlands are scanty. Mycorrhizal colonization in wetland plants has been shown to be affected by flooding, reduced oxygen, phosphorus availability, salinity, and change in seasons (Auge, 2001; Stevens et al., 2002; Bohrer, et al., 2004; Khan, 2004; McHugh and Dighton, 2004; Ray and Inouye, 2006; Stevens et al., 2011). More importantly, in one of the recent studies, Hillis et al. (2008) found significant reduction in fungal growth and spore production in *G. intraradices* grown with *Daucus carota* in agar media exposed to pharmaceuticals and personal care products (doxicycline, carbamazepine, and 17 $\alpha$-ethynylestradiol). The lack of understanding regarding the factors affecting mycorrhizal associations in wetlands, the potential for anthropogenic and natural factors to impact mycorrhizal associations, and the recognition of the value and threats of our remaining wetlands demand that a deeper understanding of mycorrhizal dynamics in wetlands be obtained.

## 1.5 Problem Statement and Objectives

Mycorrhizas are important in upland plant nutrition, water relations, ecosystem establishment, plant diversity, productivity of plants, plants resistance to pathogens, and anthropogenic and environmental stressors (Siddiqiu et al., 2008). Their prevalence in wetland plants suggests that they may play important roles in wetland ecosystems and they have been shown to influence plant performance, seedling growth, and seedling establishment in wetland plants (Stevens et al., 2011). Given their ecological importance, understanding the effects of natural and anthropogenic stressors on mycorrhizal fungi in wetland plants may have important ecological implications. This thesis focuses on two distinct aspects of mycorrhizal functioning in wetland ecosystems. The first area quantifies the effects of natural and anthropogenic stressors on mycorrhizal associations found in coastal wetlands of the southern United States, the second focus is on quantifying the effects of an urban contaminant, triclosan, on mycorrhizal development and colonization of bottomland hardwood forest vegetation.

In the last 200 years, more than 50% of Louisiana's coastal wetlands have been converted into open waters (Day et al., 2007). These losses lead to the loss of ecosystem services such as primary and secondary productivity, habitat for costal fauna, flood protection, storm protection, and functioning as barriers between salt water and inland waters (Coreil, 1993). Anthropogenic factors contributing to this loss included changes in hydrology, water quality, and sedimentation (Barras et al., 2004; Gedan et al., 2009). Hurricanes play a great role in destruction of vegetation and in salt-water intrusion in coastal wetlands both of which lead to wetland loss (Morton and Barras, 2008; Palaseanu-Lovejoy, et al., 2013). To minimize coastal wetland loss, new science-based approaches to coastal wetland conservation and restoration

are in demand (Steyer and Llewellyn, 2000). Given the potential role of mycorrhizas in wetlands, understanding the mycorrhizal ecology of the wetland plants may have implications to plant survival and ultimately a role in restoration and conservation.

An increasing number of compounds originating from the pharmaceutical and personal care product (PPCP) industry are being discharged to fresh water wetlands. Major sources of these contaminants include manufacturer release, runoff from animal and agriculture farms, and household and hospital discharges to municipal waste-water (Ellis, 2006). Recent attention and studies on toxicological issues concerning these chemicals have revealed that PPCPs are toxic to humans, aquatic animals, plants, and ecosystems as a whole (Orvos et al., 2002; CADTSC, 2007; Stevens et al., 2009). Triclosan (TCS) is one of the most ubiquitous PPCP contaminants displaying toxicological effects on aquatic and terrestrial organisms such as algae, crustaceans, early developmental stages of fish, duckweed, and wetland macrophytes (Fulton et al., 2009; Ishibashi, 2004; Orvos et al., 2002; Tatarazako et al., 2004; Stevens et al., 2009; Wilson et al., 2003). TCS disrupts fatty acid synthesis (FAS) by inhibiting the enoyl-acyl carrier protein reductase activity encoded by the *fab I* during Type II FAS (Heath et al., 1999; Newton et al., 2005); a pathway shared between bacteria and plants. Hillis et al. (2008), however, found no significant effects of TCS exposure on AM hyphal growth and spore production at nominal concentrations of up to 1000 µg/L TCS. This study used a static non-renewal exposure system with TCS dissolved in the agar media, and transformed carrot roots as the host organism. The growth conditions used in this study may not reflect exposure dynamics in water bodies receiving wastewater treatment plant effluents or responses of more typical wetland vegetation. Due to the increasing use of TCS and its potential impact on fungal taxa, this

dissertation has examined the effects of TCS on AM spore germination, hyphal growth, hyphal branching, and colonization in three wetland species common in bottomland hardwood forest in north central Texas.

To understand the importance of AM in structuring and maintaining wetland ecosystem services and given the lack of information regarding the effects of natural and anthropogenic stresses on AM associations, this dissertation has three broad objectives.

1) To study the individual and interaction effects of natural and anthropogenic stresses (water quality, hydrology, sedimentation, and a hurricane simulation) on rhizosphere spore density and mycorrhizal colonization in three coastal wetland plant species (*Typha domingensis* [Pers]*, Taxodium distichum* [L]*,* and *Panicum hemitomon* [Schult]) and mixed roots of plant communities in mesocosm experiments.
2) To assess the effects of TCS on development of AM associations in three freshwater emergent wetland plant species (*Eclipta prostrata* [L.]L.*, Hibiscus laevis* All.*,* and *Sesbania herbacea* Mill. [McVaugh]) utilising a continuous flow-through exposure system.
3) To examine the effects of TCS exposure on spore germination, hyphal growth, and hyphal branching of AM fungi prior and during colonization investigating TCS mycotoxic and/or impediment of fungal-plant signaling by using treatments with and without a root wash containing water soluble root exudates in a static renewal experiments.

# CHAPTER 2

# EFFECTS OF WATER QUALITY, HYDROLOGY, SEDIMENTATION, AND A SIMULATED HURRICANE EXPOSURE ON ARBUSCULAR MYCORRHIZA (AM) AND DARK SEPTATE ENDOPHYTE (DSE) COLONIZATION IN COASTAL MARSH VEGETATION (*Typha domingensis* [Pers], CHAPTER 3*Panicum hemitomon* [L], *Taxodium distichum* [Schult])

## 3.1 Abstract

Arbuscular mycorrhizal associations are among the most widespread symbioses estimated to occur in over 80% of all plants and have been found in fossils of the earliest land plants. Although well studied in terrestrial habitats, they were long thought absent in wetland plants. Recent studies, however, have shown many wetland plants harbor arbuscular mycorrhizal (AM) fungi and a less well understood group of root endophytes referred to, in general, as dark septate endophytes (DSE). The factors that regulate AM and DSE colonization are poorly understood but this understanding is necessary to predict plant community response and ultimately ecosystem functioning to human induced stressors. My study focused on the effects of water quality, hydrology, hurricanes, and sedimentation on AM and DSE colonization in coastal marsh vegetation. Identical plant communities were established in 200L mesocosms then treatments imposed. Treatments consisted of four levels of water quality (fresh water control, fresh water with fertilizers, 3 parts per thousand (ppt) salinity, and 6 ppt salinity), three levels of water availability (permanently flooded, continuous flow of water [throughput], and mesic soil [moist, but not flooded]), sediment application (+,-) and exposure to hurricane (+,-), yielding a total of 24 different treatment combinations. After five years, roots of three plant species (*Taxodium distichum*, *Typha domingensis*, and *Panicum hemitomon*) and two soil cores

were obtained from each mesocosm. Roots of three plant species and from soils were separated, cleared, stained, and levels of AM and DSE colonization quantified. Subsamples of soils were assessed for AM spore density. AM colonization was significantly affected by treatments; however, this differed among types of AM propagules and plant species. Hyphal colonization was affected by hydrology and interaction of water quality × hurricane exposure and hydrology × sedimentation. Arbuscular colonization decreased with increasing salinity and water availability. Vesicular colonization was affected by the interaction of water quality × hydrology × hurricane exposure. Similarly, DSE hyphal colonization was significantly lower in flooded treatments compared to mesic and also was affected by interaction of water quality × hurricane. Spore density was significantly lower in mesic treatments compared to constantly flooded and throughput treatments. My results show that natural and human induced alterations in environmental variables have significant impacts on levels of AM colonization and spore density in marsh vegetation. The resulting impacts on plant community structure and ecosystem function require further study.

## 3.2 Introduction

Arbuscular mycorrhizal (AM) fungi were long thought absent in wetlands (Khan and Belik 1995); however, recent evidence has challenged this prevailing thought. In the past few decades, AM have been found colonizing plants in wetland ecosystems ranging from bottomland hardwood forests (Jurgensen et al., 1997; Stevens et al., 2009), marshlands (Radhika and Rodrigues, 2006; Kandalepas et al., 2010), salt marshes (Cooke et al., 1993; Carvalho et al., 2001), oligotropic lakes (Beck-Nielsen and Madsen, 2001), prairie potholes

(Wetzel and van der Valk, 1996), everglades (Aziz and Sylvia, 1995), and peat swamp forests (Tawaraya et al., 2003). Although less studied, dark septate endophytes (DSE) have also been documented in wetland species from fen meadow and peat bogs (Fuchs and Haselwandter, 2004), calcareous fens (Weishampel and Bedford, 2006), bottomland hardwood forests (Stevens et al., 2009), degraded wetlands (Kandalepas et al., 2010), and from polarregions (Newsham et al., 2009). Dark septate endophytes, conidial or sterile (Jumpponen and Treppe, 1998), are found highly colonized in monocots roots compared to dicots (Weishampel and Bedford, 2006; Kandalepas et al., 2010) and found more frequently than AM in polarregions (Newsham et al., 2008). In an assessment of 18 wetland plant species from a degraded marsh in Southeastern Louisiana, Kandalepas et al., (2010) found all 18 species were colonized by AM, DSE or both. This included native plant species targeted for restoration efforts such as *Taxodium distichum* and *Typha domingensis* (Pers)*,* and introduced species *Triadica sebifera* (L.) Small and *Alternanthera philoxeroides* (Mart.) Griseb. While the importance of AM and DSE to marsh vegetation community structure and ecosystem function has yet to be ascertained, if their impact is comparable to that suggested for terrestrial ecosystems (Brundrett et al., 1996; van der Heijden et al., 1998; Escudero and Mendoza, 2005), understanding the potential effects of anthropogenic stressors on AM and DSE may be crucial to understanding wetland vegetation dynamics.

Currently it is estimated that over 50% of Louisiana's wetlands that existed prior to European settlement have been lost, and without intervention, it is projected that the majority of Louisiana's remaining wetlands will be lost in next 200 years (USGS, 2013). Several factors have been identified as contributing to the conversion of Louisiana's wetlands to open waters.

Geological subsidence (settlement or sinking land into sea) causes relative sea level rise (RSLR; sea level rise due to subsidence and polar ice melts) at the rate of 1.09 cm/yr in Louisiana, which has to be recovered by sediment deposition, plant growth forming organic soils, and mineral sediments (Penland and Ramsey, 1989; Cahoon et al., 1995). Construction of levees on the Mississippi river and isolation of rivers has prevented overbank flooding, reducing freshwater input, sediment deposition, and nutrient loading on wetlands. In addition construction of dams for floodwater reservoirs in the Mississippi river has remarkably reduced the supply of suspended and bed-load sediment to the wetlands (Day et al., 2000). Dredging of canals for navigation, drainage, and logging has changed the hydrology of the marsh allowing salt water to intrude further inland via deep and straight navigation canals, causing the death of fresh water vegetation (Day et al., 2000).

Intensities of hurricane destruction are greatly reduced by wetland forest canopies and shallow water by reducing frictional forces (van Heerden et al., 2006; Day et al., 2007). Massive vegetation die-offs have left inland vegetation vulnerable to hurricane damage (McDonald, 1955). Regular hurricanes damage marsh vegetation by converting marshes into open water in its path; however, under certain conditions, runoff generated by hurricane precipitation provides freshwater with nutrients which reduce salinity and enhance productivity of the wetlands (Conner et al., 1989). Furthermore, hurricanes deposit sediments on wetlands helping to recover marshes (Cahoon et al., 1995).

Wetland loss leads to the loss of ecosystem services such as habitat for costal fauna, flood protection, storm protection, and barriers between salt water and inland waters (Coreil, 1994). The restoration and conservation of Louisiana wetlands require an understanding of

vegetation response to biotic and environmental changes. In terrestrial ecosystems, AM fungi influence plant community structure and consequently ecosystem services (Brundrett et al., 1996; van der Heijden, 1998; Escudero and Mendoza, 2005). If they also influence plant community structure in wetlands, understanding their responses to stress could be crucial to marsh management and conservation efforts. While the role of DSE in aquatic ecosystems is poorly understood, it is currently thought that they contribute to enhanced plant performance (Newsham, 2010); therefore, understanding their responses to biotic and abiotic factors may also have management implications.

Kandalepas et al. (2010) showed that the AM and DSE are widespread in degraded coastal wetlands in southeast Louisiana. This suggests that these fungi have important functions in wetlands, thereby stressing the urgency to understand the effects of environmental pressures on plant fungal interactions in these vulnerable wetlands.

Despite the increasing evidence that AM and DSE are abundant in wetlands, little is known about the impacts of climate change and altered hydrology on these fungi, or their relationship with their hosts. Effects of abiotic factors associated with climate change and human disturbance, such as nutrient availability (Stevens and Peterson, 1996; White and Charvat, 1999), increased salinity (Saint-Etienne et al., 2006; Evelin et al., 2009), and altered hydrology (Miller, 2000; Khan, 2004; Ray and Inouye, 2005) on AM communities have been assessed independently; however, I was unaware of any studies that have examined the combined effects of these factors. Furthermore, no studies have examined the effects of hurricanes on this relationship in degraded wetlands, though hurricanes are an intricate component of many coastal systems and are predicted to increase in intensity, if not frequency,

with the progression of climate change (Hoyos et al., 2006; Mann and Emanual, 2006; Emanuel and Sundararajan, 2008; Saunders and Lea, 2008). Finally, I was unaware of any studies assessing any of these factors on DSE colonization.

In this study, I assessed the effects of coastal processes, including hurricanes, on colonization by AM and DSE in native Louisiana wetland plants, using a mesocosm approach. I examined mixed roots of mesocosm communities, each consisting of the same twelve plant species, collected from soil cores. Among twelve plant species in mesocosm community, I also determined level of colonization in roots of three important marsh plants *(Typha domingensis, Panicum hemitomon,* and *Taxodium distichum*). In addition, I assessed AM propagules such as hyphae, arbuscules, vesicules, spores, and DSE hyphae as well. To date, this is the first study to examine the combined effects of salinity, nutrients, hydrology, and hurricanes on root colonizing fungi in wetland plants.

## 3.3 Materials and Methods

### 3.3.1 Experimental Design

The experiment was a completely randomized design, in which I manipulated salinity, hydrology, water quality, sediment deposition, and hurricane-force winds. Each experimental unit consisted of one 200L polyethylene mesocosm (552 mm diameter $\times$ 851 mm height) filled with one hundred and fifty liters mixed (peat moss and top soil) soil to simulate the wetland soils in the upper Lake Pontchartrain Basin and a plant community established with 12 of the most dominant plant species in the Manchac Swamp, Manchac, Louisiana (Table 2.1). To minimize damage from full sunlight, mesocosms were maintained under a shade cloth with

approximately 70% shade, mimicking a relatively dense swamp canopy. This study was conducted at the Horticultural Center, Southeastern Louisiana University, located in Hammond, LA. Mesocosm plant communities were grown in one of four water quality levels [fresh water (0), fresh water with fertilizer (0F), fresh water with 3 parts per thousand (ppt) salinity (3), and fresh water with 6 ppt salinity (6)], and one of three hydrologic conditions [permanently flooded (P), constantly flowing water- throughput (T), and moist, but not flooded soil -mesic (M)]. In addition, I applied 2 mm of sediment slurry, originating from the Bonnet Carre Spillway in southeast Louisiana, to half the vessels every month. In total, the experiment consisted of 24 treatment combinations with six true replicates, for a total of 144 mesocosm vessels. After four years of exposure to the above treatments, I simulated a hurricane to affect half the vessels in the middle of hurricane season in Aug, 2007. Half the vessels were "protected" from the hurricane as controls. A wall was installed immediately prior to the hurricane simulation to shield the controls from the manipulated wind, salinity, hydrology, and sedimentation. This halved the number of true reps to three, and doubled the number of treatments to 48, with the number of mesocosm vessels maintained at 144. The simulation was implemented by flooding vessels with salt water, increasing salinity of each vessel by 9 ppt (i.e., vessels with fresh water were increased to 9 ppt, vessels with 3 ppt were increased to 12 ppt, and those with 6 ppt were increased to 15 ppt salinity). Also, 5 centimeter of slurred river silt from Bonnet Carre Spillway was added to the vessels exposed to hurricane. Finally, hurricane-force winds were imposed on half the vessels using an airboat. Maximum wind speed was 251 km/hr, with sustained winds at 201 km/hr. The duration of the simulation was six hours, after which pre-hurricane hydrology was restored, salinities were gradually (over two weeks) returned to pre-hurricane

levels, and plants were left to regenerate for one year before soil and roots were sampled. These conditions were modeled after Hurricane Katrina, as this was one of the most devastating storms in Louisiana's recent history (Congleton, 2006).

Table 2.1 Wetland plant species established in mesocosm experiments (species in bold were collected for mycorrhizal study).

| **Family** | **Species** | **Common name** |
|---|---|---|
| **Liliopsida (Monocotyledons)** | | |
| Alismataceae | *Sagittaria lancifolia* L. | Bulltongue arrowhead |
| Araceae | *Peltandra virginica* (L.) Schott | Green arrow arum |
| Juncaceae | *Juncus roemarianus* | Needlerush |
| Cyperaceae | *Cladium jamaicense* (Crantz) Kük. | Jamaica swamp sawgrass |
| Poaceae | ***Panicum hemitomon* Schult** | Maidencane |
| | *Spartina patens* (Aiton) Muhl. | Saltmeadow cordgrass |
| | *Spartina alterniflora* Loisel. | Smooth cordgrass |
| Potenderaceae | *Pontederia cordata* L. | Pickerelweed |
| Typhaceae | ***Typha domingensis* Pers.** | Southern cattail |
| **Magnoliopsida (Dicotyledons)** | | |
| Cornaceae | *Nyssa aquatica* L. | Water tupelo |
| Rubiaceae | *Cephalanthus occidentalis* L. | Common buttonbush |
| **PINOPHYTA (CONIFERS)** | | |
| Cupressaceae | ***Taxodium distichum* (L.) Rich.** | Baldcypress |

Figure 2.1 Mesocosm experiments

### 3.3.2 Soil Sampling and Processing

Two soil cores (6 cm diameter × 24 cm height) were obtained from the top 24 cm of each mesocosm. Half of each core was placed in ziplock bags and transported on ice to the University of North Texas (UNT), located in Denton, TX then refrigerated until processing. The second half of the samples was utilized for soil chemistry (Data not shown). Approximately 20 gm of soil was obtained from each sample and dried to allow an estimation of soil's dry weight. The remaining soil was sifted through a 500 µm sieve and any roots present were collected and stored in 50% ethanol for assessment of AM and DSE colonization level in the mixed roots of

mesocosm plant community. Spore extraction followed Brundrett et al. (1996). Soil was passed through a series of stacked sieves (250 µm, 106 µm, and 45 µm diameter). All material collected on the sieves was transferred to 50 ml centrifuge vials and de-ionized water added to achieve a volume of 50mL. Each sample was centrifuged at 2000 rpm for 1 minute then the supernatant discarded. The remaining pellet was resuspended in 50% sucrose and centrifuged again at 2000 rpm for 1 minute. The supernatant containing spores and debris was poured out of the centrifuge tubes onto the surface of a 50 µm mesh screen and rinsed with DI water. After washing, spores and debris collected on the filters were transferred into 20 mL clear vials containing DI water and refrigerated at 4°C. For assessing spore density, a 1 mL subsample was added to 4 mL of DI water and 1 mL of this solution was transferred to 15 cm petridish. Spores were then counted using a Zeiss Stemi 2000-C dissection scope (Carl Zeiss Inc., USA) at 4.0× magnifications. Four 1 mL subsamples were processed for each soil core and the average spore density per soil core was calculated. Spore density was expressed as the number of spores per gm of dry soil.

### 3.3.3 Root Sampling and Processing

Roots of 3 most predominant plant species in the mesocosms (*P. hemitomon, T. domingensis,* and *T. distichum*) were collected, bagged, and transported on ice to UNT. At UNT, roots were rinsed, then fixed and stored in 50% ethanol. Roots were cleared in 5% potassium hydroxide at 80°C for 1-2 hours, rinsed, then stained with 0.1% Chlorazol Black E at 80°C for 1 hour (Brundrett et al., 1996). Roots were destained and stored in 50% glycerol prior to mounting on slides in 50% glycerol (Phillips and Hayman, 1970). Due to heavy pigmentation, *T. distichum* roots were treated with 0.5% commercial bleach prior to clearing with KOH. Slides

were viewed with at 200× magnification using a Zeiss Axio image microscope with images obtained with a Zeiss Axiocam MRC-5 camera. Colonization levels were assessed using a modified grid line intersect procedure (McGonigle et al., 1990). A total of 100 fields of view were assessed for each sample.

### 3.3.4 Plant Species Selection

Among three wetland plant species studied, *T. distichum* (baldcypress) is one of the most important woody deciduous conifers abundant in Southeastern and Gulf Coastal Plains of US. This large woody tree is resistant to hurricane wind (Wilhite and Toliver, 1990), salinity and flooding (Allen et al., 1996). Beside resistance to the environmental stresses, it has important functions such as storing surface water to reduce downstream flood, maintenance of hydrophytic plant community, retention of sediments and nutrients, and maintaining habitat for other plants and animals (Parresol, 2002) and used in restoration of swamps.

*Panicum hemitomon* (maidencane), a fresh marsh dominant grass species, is distributed along the coastal plains of the Southeastern and Eastern US. It is a dominant emergent macrophyte in fresh marshes of Southeastern deltaic plain (Chabreck, 1972). This species was found to be dominant species in freshwater mesocosms with nutrients augmentation, while it became virtually extinct in saltwater treated experiments (Carrell, 2009).

*Typha domingensis* (cattail), a prolific wetland emergent macrophyte, is a wetland graminoid native to the Florida Everglades, which can tolerate wide range of hydrology and other wetland stresses including anthropogenic disturbances to make it invasive and out-competing other vegetation in Everglades (Lagerwall et al., 2012). This species is also widely used in constructed wetlands due to its high uptake of nutrients from wastewater (Chen et al.,

2013); however, in Louisiana its distribution is restricted to areas where an invasive, highly destructive rodent, *Myocastor coypus* (Shaffer et al., 1992), is not present.

### 3.3.5 Data Analysis

Data analysis was conducted using Proc Mixed in SAS 9.1 (SAS Institute Cary, NC). For mesocosm mixed root colonization, the main effects and interaction of water quality (4 levels), hydrology (3 levels), hurricane (2 levels), and sedimentation (2 levels) were assessed. Spore densities were analyzed in the same way as mesocosm colonization; however, since two samples were obtained from each mesocosm, subsampling was included in the analysis. Due to large missing values of three species in some treatment combinations, only the main effects of a hurricane, water quality, hydrology, and sedimentation were included in the assessment of colonization of individual species. If significant main effects and/or interaction effects were detected, multiple comparisons were conducted using contrasts (Steel and Torrie, 1980).

## 3.4 Results

All plants were colonized by AMF, DSE or both (Table 2.2). The highest colonization of AM hyphae exceeded 50% in *T. distichum*, while *P. hemitomon* and *T. domingensis* had maximum colonization levels of 36.7±5.92% and 24.3±5% respectively. *Taxodium distichum* had the highest colonization levels of arbuscules and coils with average percent colonization being 16±4.73% and 33.3±8.39% respectively, whereas arbuscular colonization in *P. hemitomon* and *T. domingensis* did not exceed 3% (Table 2.3). Similarly, maximum vesicular colonization in *T. distichum, P. hemitomon*, and *T. domingensis* were 18±6.97%, 6.35±2.3%, and 1±1% respectively. *Panicum hemitomon* and *T. domingensis* had maximum 43.5±5.17% and

37.9±4.83% DSE hyphal colonization and 63.4±5.84% and 45.8±5.71% total colonization respectively, while *T. distichum* had relatively low DSE hyphal of 6.8±2.71% with total colonization of 60.5±11.8% (Table 2.3).

Table 2.2 Summary of ANOVA showing the effects of water quality, hydrology, hurricane, and sedimentation on AM and DSE colonization in three wetland plant species (*Panicum hamitomon, Typha domingensis,* and *Taxodium distichum*). Significant effects (*p* =<0.05) in bold.

| | | **Water quality** | | **Hydrology** | | **Hurricane** | | **Sedimentation** | |
|---|---|---|---|---|---|---|---|---|---|
| | **Freq.** | **F** | **F>pr** | **F** | **F>pr** | **F** | **F>pr** | **F** | **F>pr** |
| ***P. hemitomon*** | | | | | | | | | |
| Hyphae | 58/63 | **4.44** | **0.0072** | 1.37 | 0.2627 | **10.77** | **0.0018** | 0.34 | 0.5645 |
| Arbuscles | 19/63 | 1.34 | 0.2707 | 0.89 | 0.4181 | **18.62** | **<0.000** | **5.96** | **0.0179** |
| Arb. Coils | 36/63 | 0.72 | 0.5468 | 1.75 | 0.1828 | **17.58** | **0.0001** | 1.01 | 0.3197 |
| Vesicles | 30/63 | 1.88 | 0.1440 | 1.69 | 0.1934 | **7.31** | **0.0091** | 1.33 | 0.2532 |
| DSE | 62/63 | **8.69** | **<0.0001** | 2.60 | 0.0831 | 0.05 | 0.8268 | 0.55 | 0.4634 |
| Total Col. | 63/63 | **8.45** | **0.0001** | 2.82 | 0.0684 | 2.80 | 0.1000 | 0.10 | 0.7577 |
| ***T. domingensis*** | | | | | | | | | |
| Hyphae | 64/84 | **3.57** | **0.0179** | 0.70 | 0.4997 | 3.25 | 0.0754 | 0.52 | 0.4741 |
| Arbuscles | 1/84 | **--** | **--** | -- | -- | -- | -- | -- | -- |
| Arb. Coils | 2/84 | -- | -- | -- | -- | -- | -- | -- | -- |
| Vesicles | 7/84 | -- | -- | -- | -- | -- | -- | -- | -- |
| DSE | 82/84 | **7.65** | **0.0002** | **4.64** | **0.0126** | 0.34 | 0.5613 | 1.50 | 0.2251 |
| Total Col. | 84/84 | **5.53** | **0.0017** | **3.46** | **0.0364** | 0.04 | 0.3118 | 0.66 | 0.4183 |
| ***T. distichum*** | | | | | | | | | |
| Hyphae | 31/35 | 1.13 | 0.3556 | 0.18 | 0.8376 | 0.80 | 0.3794 | 0.27 | 0.6100 |
| Arbuscles | 22/35 | 0.95 | 0.4327 | 0.50 | 0.6125 | 0.39 | 0.5385 | 0.02 | 0.8992 |
| Arb. Coils | 27/35 | 1.98 | 0.1407 | 0.80 | 0.4608 | 0.05 | 0.8190 | 0.07 | 0.8000 |
| Vesicles | 23/35 | 1.13 | 0.3534 | 0.94 | 0.4048 | 0.73 | 0.3998 | 0.05 | 0.8287 |
| DSE | 27/35 | 0.69 | 0.5667 | 0.14 | 0.8693 | 0.27 | 0.6085 | 0.18 | 0.6726 |
| Total Col. | 35/35 | 1.05 | 0.3877 | 0.37 | 0.6930 | 0.89 | 0.3540 | 0.02 | 0.9017 |

Table 2.3 Effects of water quality, hydrology, hurricane, and sedimentation on AM and DSE colonization in three wetland plant species (*Panicum hamitomon, Typha domingensis,* and *Taxodium distichum)* grown in four water quality [Control (0), control with nutrients (0F), 3 parts per thousand (ppt) salinity (3), and 6 ppt salinity (6)], three levels of hydrology [permanently flooded (P), throughput (T), and mesic (M)], two levels of hurricanes (control and hurricane +), and two levels of sedimentation (control and sediment +). Significant effects ($p$<0.05) in bold. Different superscript lowercase letters on mean=significant). Data shown are % mean ± one standard error and sample size in parantheses.

| | **Water Quality** | | | | **Hydrology** | | | **Hurricane** | | **Sedimentation** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0** | **0F** | **3** | **6** | **P** | **T** | **M** | **Control** | **Hurricane +** | **Control** | **Sediment +** |
| ***P. hemitomon*** | | | | | | | | | | | |
| Hyphae | 17.5$^{a}$ ±3.61(23) | 36.7$^{b}$ ±5.92(20) | 24.1$^{a}$ ±5.66(9) | 8.45$^{a}$ ±2.82(11) | 15.6±4.71(17) | 23.5±4.92(24) | 28±4.4(22) | 14.7$^{a}$ ±3.17(32) | 31.5$^{b}$ ±4.07(31) | 21.1±3.84(28) | 24.4±3.95(35) |
| Arbuscles | 1.61±0.63(23) | 2.3±1.14(20) | 0.88±0.51(9) | 0±0(11) | 0.41±0.24(17) | 1.25±0.61(24) | 2.45±1.03(22) | 0.06$^{a}$ ±0.04(32) | 2.87$^{b}$ ±0.82(31) | 1.17$^{a}$±0.76(28) | 1.66$^{b}$±0.51(35) |
| Arb. Coils | 6.69±2.24(23) | 11.4±3.61(20) | 6.11±2.07(9) | 1.63±1.03(11) | 2.94±1.44(17) | 6.91±2.55(24) | 10.9±2.89(22) | 2.25$^{a}$ ±0.96(32) | 12.4$^{b}$ ±2.56(31) | 5.36±1.84(28) | 8.71±2.23(35) |
| Vesicles | 2.48±0.96(23) | 6.35±2.3(20) | 3.11±1.31(9) | 0.09±0.09(11) | 2.59±1.95(17) | 2.41±0.91(24) | 5.04±1.69(22) | 2.03$^{a}$ ±1.14(32) | 4.77$^{b}$ ±1.27(31) | 3.75±1.66(28) | 3.09±0.83(35) |
| DSE | 20.9$^{ac}$ ±3.78(23) | 43.5$^{b}$ ±5.17(20) | 33.9$^{ab}$ ±9.44(9) | 12.7$^{c}$ ±2.2(11) | 23.2±6.5(17) | 26±3.73(24) | 35.3±5.17(22) | 25.4±3.89(31) | 31.6±4.34(32) | 28±4.77(28) | 28.9±3.66(35) |
| Total Col. | 34.5$^{ac}$ ±5.17(23) | 63.4$^{b}$ ±5.84(20) | 50.2$^{ab}$ ±8.93(9) | 18.5$^{c}$ ±3.36(11) | 30.7±6.77(17) | 42.1±5.93(24) | 53.8±5.38(22) | 34.9±4.68(31) | 51.6±5.11(32) | 41.6±5.28(28) | 44.3±4.96(35) |
| ***T. domingensis*** | | | | | | | | | | | |
| Hyphae | 8.43$^{a}$ ±3.46(14) | 24.3$^{b}$ ±5(20) | 8.96$^{a}$ ±2.96(24) | 11.2$^{a}$ ±2.4(26) | 11.1±2.45(33) | 11.8±2.62(28) | 18±4.72(23) | 10±2.54(32) | 15.2±2.51(52) | 12.4±2.52(45) | 14.1±2.71(39) |
| Arbuscles | 0±0(14) | 0.4±0.4(20) | 0±0(24) | 0±0(26) | 0±0(33) | 0±0(28) | 0.35±0.34(23) | 0±0(32) | 0.15±0.15(52) | 0±0(45) | 0.2±0.2(39) |
| Vesicles | 1±1(14) | 0.55±0.42(20) | 0.42±0.37(24) | 0.11±0.08(26) | 0.24 ±0.24(33) | 0.54 ±0.5(28) | 0.65±0.41(23) | 0.06±0.06(32) | 0.69±0.35(52) | 0.29±0.2(45) | 0.64±0.41(39) |
| DSE | 25.9$^{ac}$ ±5.82(14) | 24.2$^{a}$ ±4.12(20) | 13$^{b}$ ±3.42(24) | 37.9$^{c}$ ±4.83(26) | 22.9$^{ab}$ ±3.27(33) | 19.4$^{a}$ ±3.95(28) | 36.8$^{b}$ ±5.39(23) | 24.4±4.44(32) | 26.2±2.91(52) | 24.8±3.85(45) | 26.4±2.92(39) |
| Total Col. | 30.9$^{ab}$ ±5.79(14) | 37.5$^{a}$ ±5.19(20) | 19$^{b}$ ±4.39(24) | 44$^{a}$ ±4.78(26) | 29.8$^{ab}$ ±3.65(33) | 26.6$^{a}$ ±4.35(28) | 45.8$^{b}$ ±5.71(23) | 30.2±4.62(32) | 34.9±3.27(52) | 31.9±4.11(45) | 34.6±3.34(39) |
| ***T. distichum*** | | | | | | | | | | | |
| Hyphae | 41.1±7.92(17) | 59.7±11.9(10) | 47.3±16.2(3) | 23.6±15.4(5) | 36.9±9.43(11) | 46.7±9.31(13) | 49.4±12.3(11) | 38.6±8.97(15) | 48.9±7.79(20) | 48.8±9.05(18) | 39.9±7.45(17) |
| Arbuscles | 16±4.73(17) | 15.1±7.59(10) | 8±5.03(3) | 3.8±3.8(5) | 9.72±5.38(11) | 14.7±4.88(13) | 15.3±6.93(11) | 9±3.38(15) | 16.6±5(20) | 13.7±4.56(18) | 12.9±4.7(17) |
| Arb. Coils | 19.6±4.69(17) | 33.3±8.39(10) | 24±10.1(3) | 7.4±5.27(5) | 16.6±5.34(11) | 23.3±5.8(13) | 26.3±8.21(11) | 22.6±6.26(15) | 21.8±4.5(20) | 23.7±5.91(18) | 20.5±4.36(17) |
| Vesicles | 3.82±1.07(17) | 15.1±5.76(10) | 4±3.05(3) | 12.8±12.6(5) | 1.82±0.68(11) | 5.69±1.75(13) | 18±6.97(11) | 5±2.71(15) | 10.9±3.85(20) | 13.2±4.52(18) | 3.18±1.09(17) |
| DSE | 6.29±2.03(17) | 4±1.26(10) | 1.33±1.33(3) | 6.8±2.71(5) | 5±2.79(11) | 5.23±1.64(13) | 5.64±1.49(11) | 3.93±1.24(15) | 6.3±1.74(20) | 6.11±1.81(18) | 4.41±1.35(17) |
| Total Col. | 42.9±7.85(17) | 60.5±11.8(10) | 47.3±16.2(3) | 28±14.3(5) | 38.8±9.32(11) | 48.5±9.06(13) | 50.8±11.9(11) | 40.3±8.58(15) | 50.7±7.68(20) | 49.8±8.9(18) | 42.4±7.18(17) |

Mycorrhizal colonization in *T. distichum* was not affected significantly by any factors tested in this study. In contrast, colonization in *P. hemitomon* was significantly affected by water quality, hurricane exposure, and sedimentation, while colonization in *T. domingensis*s was significantly affected by water quality and hydrology (Table 2.2).

Arbuscular mycorrhizae and DSE hyphal, as well as total colonization in *P. hemitomon* were significantly affected by water quality (Table 2.2). Hyphal colonization was highest in the 0F treatments compared to 0, 3, and 6 ppt salinity treatments (Table 2.3). DSE hyphal and total colonization levels followed similar trend being 0F treatments highest compared to the 0 and 6 ppt salinity and higher in 3 ppt salinity compared to 6 ppt salinity treatments. Furthermore, AM hyphal, arbuscular, coil, and vesicular colonization were significantly higher in the hurricane exposure compared to controls (Table 2.3). Arbuscular colonization also was significantly higher in the treatments receiving sediment; however, colonization levels did not exceed 2%.

In *T. domingensis*, AM and DSE hyphal, as well as total colonization were affected by water quality. In addition, DSE hyphal and total colonization were also affected by hydrology (Table 2.2). Hyphal colonization was significantly greater in the 0F treatments compared to 0, 3, and 6 ppt treatments (Table 2.3). In contrast, 6 ppt salinity had higher DSE hyphal colonization than 3 ppt and 0F treatments, where 3 ppt had the lowest DSE hyphal colonization (Table 2.3). Total colonization was lowered to half in 3 ppt treatments compared to 0F and 6 ppt treatments (Table 2.3). Significant effects of hydrology on DSE hyphal and total colonization had resulted higher colonization in mesic soils compared to throughput (Table 2.3). Hurricane and sedimentation had no significant effects on AM and DSE colonization in *T. domingensis*.

Table 2.4 Summary table of four-way ANOVA assessing the effects of water quality (WQ), hydrology (HD), hurricane (HR), and sedimentation (SD) on AM and DSE colonization in the roots of mesocosm plant communities. Significant effects ($p$ <0.05) in bold.

| | | Hyphae | Vesicles | Arbuscles | Coils | DSE | Total | Spores |
|---|---|---|---|---|---|---|---|---|
| **WQ** | F | 1.800 | **2.900** | **2.740** | 1.410 | **3.850** | **3.600** | **7.250** |
| | Pr>F | 0.150 | **0.040** | **0.050** | 0.250 | **0.010** | **0.020** | **0.000** |
| **HD** | F | **13.150** | **6.560** | **14.180** | **7.660** | **4.810** | **11.990** | **4.620** |
| | Pr>F | **<0.0001** | **<0.01** | **<0.0001** | **<0.01** | **0.010** | **<0.0001** | **0.010** |
| **HR** | F | 0.810 | 0.290 | 0.990 | 0.700 | **7.470** | **4.250** | 0.940 |
| | Pr>F | 0.370 | 0.590 | 0.320 | 0.400 | **0.010** | **0.040** | 0.330 |
| **SD** | F | 1.180 | 0.180 | 2.430 | 1.810 | 0.070 | 0.310 | **6.870** |
| | Pr>F | 0.280 | 0.680 | 0.120 | 0.180 | 0.790 | 0.580 | **0.010** |
| **WQ*HD** | F | 1.110 | 1.020 | 1.860 | 1.970 | 0.330 | 0.380 | 1.120 |
| | Pr>F | 0.360 | 0.420 | 0.100 | 0.080 | 0.920 | 0.890 | 0.360 |
| **WQ*HR** | F | **5.320** | 1.460 | 2.290 | **5.690** | **3.010** | **4.400** | **0.800** |
| | Pr>F | **<0.01** | 0.230 | 0.080 | **<0.01** | **0.040** | **0.010** | 0.500 |
| **WQ*SD** | F | 0.780 | 0.450 | 1.670 | 0.640 | 0.710 | 0.210 | 0.830 |
| | Pr>F | 0.510 | 0.720 | 0.180 | 0.590 | 0.550 | 0.890 | 0.480 |
| **HD*HR** | F | 0.040 | 1.840 | 0.240 | 0.450 | 0.190 | 0.340 | **3.350** |
| | Pr>F | 0.960 | 0.170 | 0.790 | 0.640 | 0.820 | 0.710 | **0.040** |
| **HD*SD** | F | **3.960** | **3.710** | 0.160 | 0.390 | 1.040 | 2.550 | 1.660 |
| | Pr>F | **0.020** | **0.030** | 0.850 | 0.680 | 0.360 | 0.080 | 0.200 |
| **HR*SD** | F | 0.820 | 0.200 | 0.280 | 1.040 | 0.000 | 0.190 | 0.230 |
| | Pr>F | 0.370 | 0.660 | 0.600 | 0.310 | 0.980 | 0.660 | 0.630 |
| **WQ*HD*HR** | F | 1.750 | **2.730** | 0.160 | 1.510 | 0.860 | 0.790 | **2.580** |
| | Pr>F | 0.120 | **0.020** | 0.990 | 0.190 | 0.530 | 0.580 | **0.020** |
| **WQ*HD*SD** | F | 1.940 | **2.390** | 0.650 | 1.440 | 1.370 | 2.050 | 0.710 |
| | Pr>F | 0.080 | **0.040** | 0.590 | 0.210 | 0.240 | 0.070 | 0.640 |
| **HD*HR*SD** | F | 1.220 | 0.800 | 0.390 | 0.340 | 0.490 | 0.150 | 0.080 |
| | Pr>F | 0.300 | 0.450 | 0.680 | 0.710 | 0.620 | 0.860 | 0.920 |
| **WQ*HD*HR*SD** | F | 0.990 | 0.820 | 8.930 | 1.370 | 0.880 | 1.300 | 1.240 |
| | Pr>F | 0.460 | 0.600 | 0.500 | 0.220 | 0.550 | 0.250 | 0.280 |

In the mixed roots of mesocosm plant communities, AM hyphal colonization was significantly affected by the interaction of water quality × hurricane and hydrology × sedimentation (Table 4). In absence of sedimentation, throughput and mesic treatments had higher AM hyphal colonization compared to 0, while mesic had higher compared to

permanently flooded and throughput following sedimentation. Within a given level of hydrology, permanently flooded treatments with sedimentation had higher colonization compared to those without sedimentation (Fig. 2.2). In absence of hurricane exposure, AM hyphal colonization was higher in 0F compared to 0, 3, and 6 ppt treatments, while 0F following hurricane had lower colonization compared to 0, and 6 ppt (Fig. 2.3a).

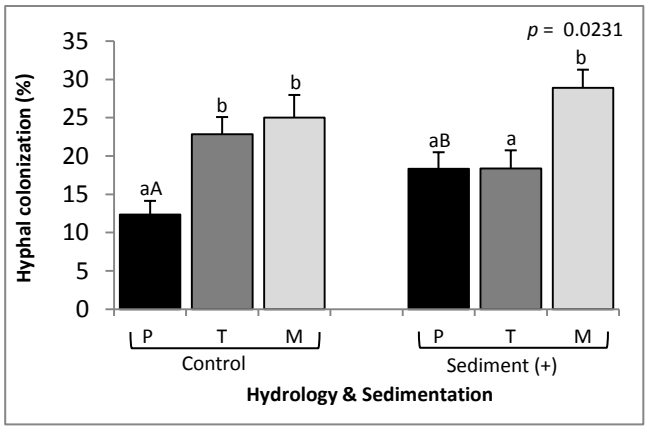


Figure 2.2 Effects of the interaction of hydrology × sedimentation on AM hyphal colonization in the roots of mesocosm plant communities grown under three levels of hydrology [permanently flooded (P), throughput (T), and mesic (M)], and two levels of sedimentation [control and sediment (+)]. Different lowercase letters indicate significant difference ($p$<0.05) among hydrology treatments within same sediment conditions, uppercase letters indicate significant difference across sedimentation with same hydrology. Raw means are presented with bars indicating ± one standard error.

Vesicular colonization was affected by the interaction of water quality × hydrology × sedimentation and the interaction of water quality × hydrology × hurricane (Table 2.4). Within the levels of hydrology and water quality, the 0F in throughput without sedimentation was significantly higher compared to 0F with sedimentation (Fig. 2.4). Similarly, 0 in throughput and 0F in mesic had significantly higher vesicular colonization compared to those without with hurricane exposure. In contrast, 0 in permanently flooded increased with hurricane exposure (Fig. 2.5a).

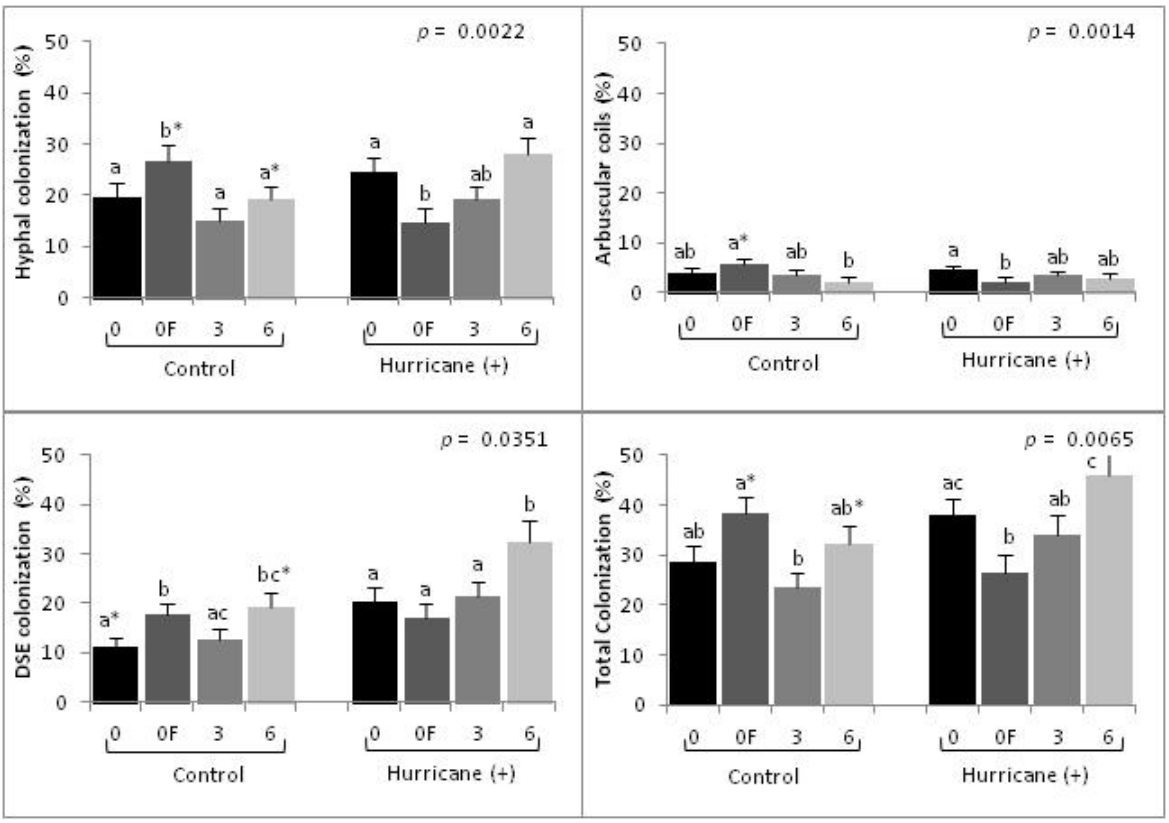


Figure 2.3 Effects of the interaction of water quality × hurricane on AM hyphal (a), coils (b), DSE hyphal (c), and Total colonization (d) in the roots of mesocosm plant communities grown under four levels of water quality [control (0), control with fertilizers (0F), 3 parts per thousand (ppt) salinity (3), and 6 ppt salinity (6)], and hurricane condition [control and hurricane (+)]. Different letters indicate significant differences ($p$<0.05) among water quality treatments within same hurricane condition and *Asterisks* indicate significant differences between hurricane treatments with same water quality. Raw means are presented with bars indicating ± one standard error.

Within a given level of water availability, the effect of water quality differed among treatments exposed to sedimentation or hurricane exposure. In the permanently flooded mesocosms following sedimentation, the 0 had a significantly higher level of vesicular colonization compared to the 3 ppt (Fig. 2.4), while in the hurricane exposed treatments, vesicular colonization in the 0 was significantly higher than all other treatments (Fig. 2.5a). In the treatments not exposed to hurricane or sedimentation, there were no significant

differences in colonization among levels of water quality with permanently flooded hydrology. In the throughput treatments, vesicular colonization was significantly higher in the 0F treatments compared to 0 and 6 ppt without sedimentation; however, there were no significant differences in vesicular colonization among water quality treatments receiving sediments (Fig. 2.4). In contrast, there were no significant differences among water quality treatments in non-hurricane exposed throughput treatments, but vesicular colonization was significantly higher in the 0F compared to the 0 following hurricanes. In mesic soils, there were no significant difference among water quality treatments not receiving sediments; however, vesicular colonization was significantly lower in 6 ppt compared to 0 and 0F receiving sediments (Fig. 2.4). In contrast, vesicular colonization was significantly greater in the 0 in mesic soils compared to 0F following hurricanes, while in the treatments not receiving hurricanes, vesicular colonization was significantly higher in the 0F compared to 3 and 6 ppt (Fig. 2.5a).

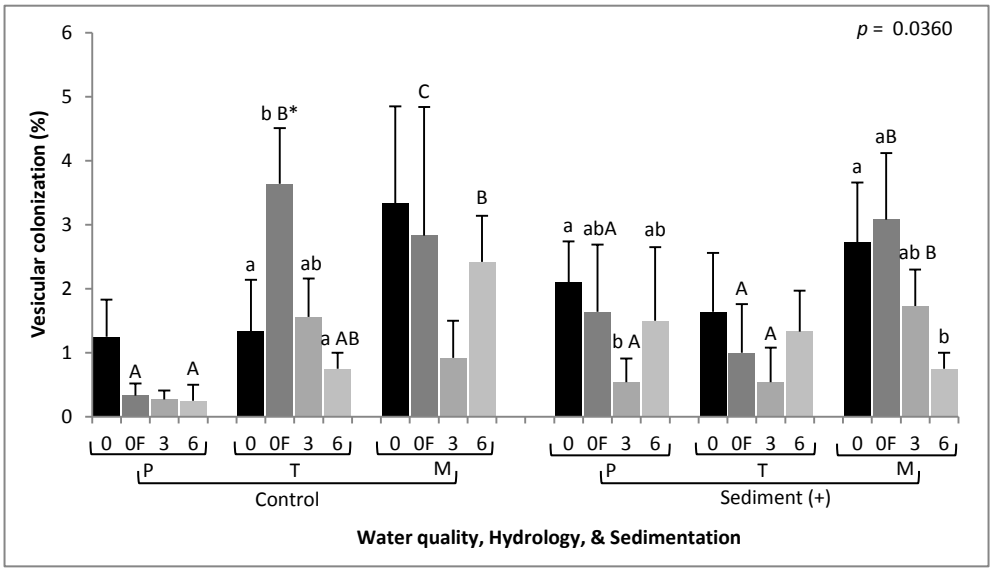


Figure 2.4 Effects of the interaction of water quality × hydrology × sedimentation on vesicular colonization in the roots of mesocosm plant communities grown under four levels of water quality [control (0), control with fertilizers (0F), 3 parts per thousand (ppt) salinity (3), and 6 ppt salinity (6)], three levels of hydrology [permanently flooded (P), throughput (T), and mesic (M)],

and two levels of sedimentation (sediment (+) and control). Different lowercase letters indicate significant difference ($p<0.05$) among water quality treatments within same sediment and hydrology conditions, uppercase letters indicate significant difference among hydrology treatments with same water quality and sediment condition, and *Asterisks* indicate significant difference across sedimentation with same water quality and hydrology. Raw means are presented with bars indicating $\pm$ one standard error.

Within a given level of water quality and sedimentation or hurricane exposure, vesicular colonization differed across hydrology. The vesicular colonization was significantly lower in the 0F and 6 ppt with permanently flooded compared to mesic soils not receiving sediments; however, 0F in throughput had higher compared to those in permanently flooded and mesic soils (Fig. 2.4). Similarly, 0F in permanently flooded without hurricane exposure had lower vesicular colonization compared to 0F in mesic soils (Fig. 2.5a). In treatments receiving sediments, vesicular colonization was significantly higher in 0F and 3 ppt with mesic soils compared to those in permanently flooded and throughput (Fig. 2.4). In hurricane exposed treatments, vesicular colonization was significantly lower in the 0 with throughput compared to permanently flooded and mesic soils (Fig. 2.5a).

Table 2.5 Effects Hydrology on AM and DSE colonization in the mixed roots of mesocosm plant communities grown under three hydrology conditions [permanently flooded (P), throughput (T), and mesic (M)]. Significant effects ($p<0.05$), different superscript lowercase letters on mean=significant. Data shown are raw mean ± one standard error with sample size in parantheses.

| | **Hydrology** | | |
|---|---|---|---|
| **Propagules** | **P** | **T** | **M** |
| Arbuscles | 0.65±0.22(91)[a] | 1.83±0.62(84)[b] | 3.24±0.65(94)[c] |
| Coils | 2.15±0.44(91)[a] | 3.88±0.63(83)[b] | 4.68±0.61(94)[b] |
| DSE | 15.2±1.62(91)[a] | 18.74±1.71(84)[ab] | 23.18±1.97(94)[b] |
| Total Col. | 26.02±2.06(91)[a] | 33.4±2.1(84)[b] | 41.11±2.26(94)[c] |

Arbsucular colonization was significantly affected by water quality and hydrology (Table 2.4); however, colonization levels did not exceed 4% for any treatment combinations. Arbuscular colonization was significantly lower in 6 ppt (0.88±0.31%) compared to 0 (2.57±0.71%). Similarly, arbuscular colonization was lowest in permanently flooded, followed by throughput, and greatest in mesic soils (Table 2.5).

Coils were significantly affected by hydrology and interaction of water quality × hurricane (Table 2.4). Coil colonization was significantly lower in permanently flooded compared to throughput and mesic (Table 2.5). In absence of hurricane exposure, coil colonization was significantly greater in 0F compared to 6 ppt, while coil colonization was significantly reduced in 0F following hurricane exposure and remains lower compared to 0 and 6 ppt (Fig. 2.3b).

Dark septate endophyte hyphae were found in all mesocom treatments and were significantly affected by hydrology and the interaction of hurricane exposure × water quality (Table 2.4). DSE hyphal colonization was significantly lower in permanently flooded (15.2±1.62%) compared to the mesic hydrology (23.18±1.97%); neither differed significantly from the throughput treatments (18.74±1.71%). In absence of hurricane exposure, DSE colonization was significantly lower in 0 compared to 0F and 6 ppt, while in the hurricane exposed treatments DSE colonization was significantly higher in 6 ppt compared to 0, 0F, and 3 ppt (Fig. 2.3c).

Total colonization was significantly affected by hydrology and interaction of water quality × hurricane (Table 2.4). Total colonization of AM and DSE was lowest (26.02±2.06) in permanently flooded, which is significantly increased from throughput (33.4±2.10) to mesic

(41.11±2.26) soils. Within given water quality conditions, 0F treatments not exposed to hurricane had significantly higher total colonization compared to 0F following hurricane exposure. In contrast, 6 ppt without hurricane had lower colonization compare to those

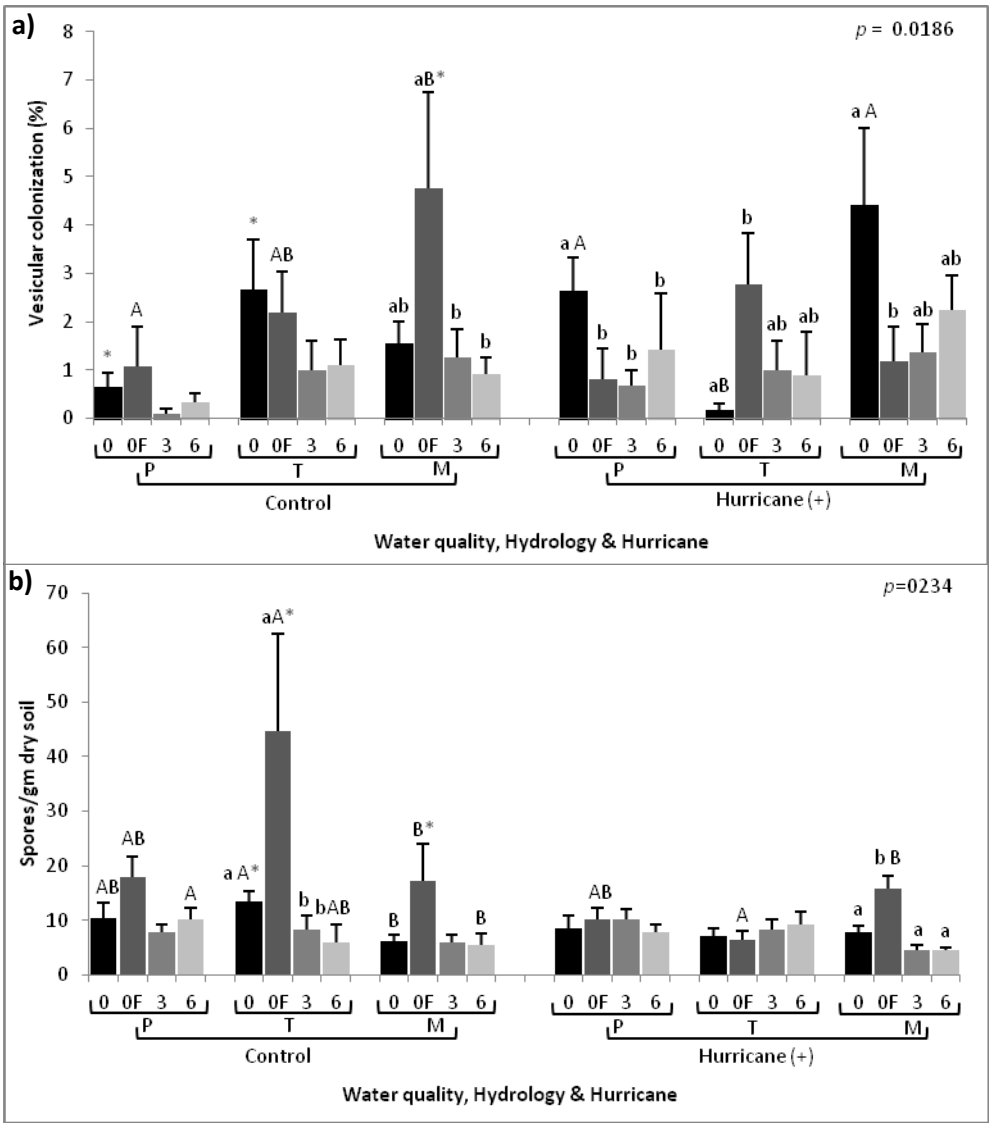


Figure 2.5 Effects of the interaction of water quality 1hygrology 1 hurricane on vesicular (a) and spore density (b) on roots and soils of mesocosm plant community grown under four levels of water quality [control (0), control with nutrients (0F), 3 parts per thousand (ppt) salinity (3), and 6 ppt salinity (6)], three levels of hydrology [permanently flooded (P), throughput (T), and mesic (M)], and two hurricane conditions [control and hurricane (+)]. Different lowercase letters indicate significant difference ($p<0.05$) among water quality treatments within same hurricane and hydrology, different uppercase letters indicate significant difference among hydrology with same water quality and hurricanes, and asterisks indicate significant difference between two hurricane conditions with same water quality and hydrology. Raw means are presented with bars indicating 1 one standard error.

following hurricane exposure. At a given hurricane condition, 0F had higher total colonization compare to 3 ppt without hurricane exposure, while 0F exposed to hurricane had lower colonization compared to 6 ppt (Fig. 2.3d).

Spores were found in all treatments and mesocoms. Spore density was significantly affected by sedimentation, and the interaction of water quality × hydrology × hurricane exposure (Table 2.4). The mesocosms receiving sediment had lower spore density (8.59±0.75 spores/gm dry soil) compared to control treatments (12.33±1.68 spores/gm dry soil).The highest spore density was found in the 0F treatments receiving throughput without hurricane exposure (44.6±18.02 spores/gm dry soil) and the lowest in the 6 ppt mesic soils with hurricane exposure (4.48±0.48 spores/gm dry soil). A hurricane exposure resulted in significantly lower spore density in the 0 and 0F with throughput and the 0F mesic compared to those without hurricanes (Fig. 2.5b).

In absence of hurricane exposure, the interaction of water quality and hydrology significantly affected spore density. The spore density was greater in 0 and 0F treatments compared to the 3 and 6 ppt salinity with throughput. Furthermore, in absence of hurricane exposure, the hydrology affected spore density. In 0 and 0F treatments spore density was significantly greater in throughput treatments compared to the mesic soils, while in 6 ppt salinity spore density was significantly greater in the flooded compared to mesic treatments (Fig. 2.5b). In hurricane exposed mesocosm soils within mesic condition, spore density was greater in 0F compared to 0, 3, and 6 ppt. Similarly, 0F with mesic soils had greater spore density compared to 0F with throughput (Fig. 2.5b).

## 3.5 Discussion

It is becoming accepted that mycorrhizal colonization is widespread in wetland plants, despite past prevailing thoughts that mycorrhizal fungi were rare in wetland soils. While there is a growing body of literature on the effects of individual and in some cases the two way interaction of environmental factors on AM fungi in wetland plants (Stevens and Peterson, 1996; Miller, 2000; Miller and Sharitz, 2000; Carvalho et al., 2003; Stevens et al., 2003; Ray and Inouye, 2005; Stevens et al., 2011), studies including multiple main effects and their interactions that more closely to simulate natural conditions, are absent. Furthermore, studies assessing sedimentation and hurricane effects, common occurrences in southern wetlands, are lacking all together. The interaction of the various treatment conditions in this study simulated hydrology and salinity gradients that correspond to various stages of tides and saltwater intrusion, storms, sea level rise, as well as proximity to the Mississippi River in the wetlands of Southeastern Louisiana, common and dominant factors known to impact wetlands in Louisiana (Kandalepas, 2012). This study has found that the coastal wetland plants grown in mesocosms of all treatment combinations harbor AM, DSE or both; however, levels of colonization differed among treatments and plant species.

Water availability has been identified as one of the single most important factors in structuring wetland plant communities (Casanova and Brock, 2000; Todd et al., 2010). Several studies have identified hydrology, as the predominant factor impacting AM fungi in wetlands (Rickerl et al., 1994; Stevens and Peterson, 1996; Auge, 2001; Ray and Inouye, 2006). Generally, colonization levels are inversely proportional to water availability and are reduced or absent in flooded soils (Rickerl et al., 1994; Stevens and Peterson, 1996; Miller, 2000; Escurado and

Mendoza, 2005). In agreement with previous findings, this study also found reduced overall colonization levels of AM and DSE in permanently flooded treatments compared to drier soils. The mechanisms underlying reduced colonization in flooded soils are unclear but may be related to increased hypoxia (Khan and Belik, 1995), toxic ion accumulation, and high phosphorus availability (Stevens and Peterson, 1996). Water availability may not act independently affecting colonization; the impact of increased water availability may be influenced by other co-occurring factors. For example, Stevens et al. (2002) found that phosphorus availability rather than water availability had a greater impact on AM colonization in the wetland plant *Lythrum salicaria*, while salinity was identified as the major determinant affecting AM colonization in *Aster tripolium* (Carvalho et al., 2003). In many wetlands, factors affecting hydrology and water qualities are linked and the individual effects difficult to disentangle. This study has shown that, while hurricane exposure affects overall colonization, the magnitude of the effect is dependent on water quality. This is the only study to date that has manipulatively examined the effects of hurricane exposure on mycorrhizal symbioses. The only other studies I am aware of that observed effects of hurricanes on mycorrhizal colonization found lower AM colonization and spore densities after a hurricane (Hasselquist et al., 2010; Vargas et al., 2010), but both of these studies have focused on temperate forests in Yucatan Peninsula, Mexico.

Fungal hyphae are important components of this association and function in the transport nutrients and water from soil to the host plants. Previous studies have found main effects of water availability, nutrients, and salinity on hyphal colonization (Rickerl et al., 1994; Auge, 2001; Ray and Inouye, 2006; Saint-Etienne et al., 2006; Stevens et al., 2011). In this study,

however, the effects of hydrology and water quality were dependent on sedimentation and hurricane exposure respectively. Therefore, AM hyphal colonization was affected by the interaction of many environmental factors rather than simple main effects. Overall, levels of hyphal colonization in this study were within the range of colonization levels found in a field study in Louisiana wetlands (Kandalepas et al., 2010).

Arbuscules are highly conserved AM structures with a very short life span and are the major sites of resource exchange between the two symbionts (Harrison, 2005). Arbuscular coils are developed in some types of AM fungi and also facilitate resource exchange (Peterson et al., 2004). Brown and Bledsoe (1996) suggest that arbuscular coils might respond to environmental variables in a similar manner as arbuscules. Partial support for this hypothesis is provided by this study. While both were significantly lower in the flooded compared to the mesic treatments, and both were affected by water quality, the effects of water quality on arbuscular coil colonization was dependent upon hurricane exposure while this was not the case for arbuscular colonization. It must be noted however that, neither arbuscular nor arbuscular coil colonization exceeded 6% and consequently level of functioning of the AM symbiosis and the biological significance of this response is unclear. That is, while there was a statistically significant difference in colonization, it is unclear if a difference at such relatively low levels of colonization results in a biological effect. This low level of arbuscular colonization is in contrast to levels exceeding 25% noted in the study of Kandalepas et al. (2010).

AM fungi can overwinter as dormant vesicles or spores. They are developed in the later stages of colonization and remain viable during adverse environmental conditions (Smith and Read, 2008). They store the organic reserves and act as propagules for next season (Biermann

and Linderman, 1983). Consequently, an adverse impact on either vesicular colonization or spore density may have longer term impacts that span seasons. The significant three way interaction effects of water quality × hydrology × sedimentation for affecting vesicle production and water quality × hydrology × hurricane affecting vesicular colonization and spore density suggest that, both are under complex control and are susceptible to several anthropogenic disturbances. Overall, vesicular colonization was similar to most of the species studied in the field (Louisiana wetlands) by Kandalepas et al. (2010). In addition, reduced spore density by sedimentation may be due to low spores in the sediments added with lack of vegetation (Anderson et al., 1983) or AM supplying less energy in sporulation while it colonizes new roots developed in treatments with added sediments (Harner et al., 2009).

Despite the scanty literature on DSE in the wetlands, they have been found in various wetland habitats including bottomland hardwood forest, and degraded wetlands of Louisiana (Kandalepas et al., 2010; Stevens et al., 2010). They are believed to function similar to AM fungi (Jumpponen, 2001); however, the potential roles of DSE in wetland plants are unclear (Kandalepas et al., 2010). In permanently flooded mesocosms, DSE responded similarly to the AM fungi, where colonization levels were significantly reduced compared to the mesic treatments. The reasons for this reduction may be similar to those proposed for the reduced AM fungi; inability to survive hypoxia and susceptibility to toxic ions in inundated soils (Khan and Belik, 1995; Stevens and Peterson, 1996). Similarly, combined effects of water quality and hurricane also followed AM hyphal colonization favoring salinity treatment in hurricane exposed mesocosms. Unlike AM fungi, however, DSE colonization was higher in high salinity treatments regardless of hurricane. This may suggest that DSE in degraded wetlands prefer or

are better adapted to saline environments that present conditions unfavorable to AM survivorship.

The effects of anthropogenic stressors on AM and DSE colonization in the roots of three wetland plant species observed in this study differed among plant species. There was no significant effect of any treatment on AM or DSE colonization in *T. distichum*; however, colonization in *T. domingensis* was affected by hydrology and water quality while colonization in *P. hemitomon* was affected by hydrology, water quality and hurricane exposure. Furthermore, while *T. distichum* had the highest levels of AM colonization, most notably arbuscules and coils, colonization of these structures did not exceed 2% in *P. hemitomon* or *T. domingensis*. It should be noted that levels of AM hyphal colonization (59.7%) in *T. distichum* grown in this study correspond to those found in the field (55.67%), while DSE colonization was little higher compared to the field (6.29% compared to 0.33%) (Kandalepas et al., 2010). It has been suggested that monocots support higher levels of DSE colonization, while dicots support higher levels of AM colonization (Weishampel and Bedford, 2006; Kandalepas et al., 2010) this relationship has not yet been explored for gymnosperms. This relationship may have significant implications, factors that limit AM colonization may have a greater impact on *T. distichum* compared to either monocot species and this may have significant ecosystem implications. *T. distichum* (Bald Cypress), an iconic species, is the defining species in cypress swamps in the U.S. This species is a woody conifer tree that has been shown to resist intense storms during hurricanes (Shaffer and Day, 2007; Shaffer et al., 2009) and is important as barrier of hurricanes (van Heerden et al., 2006; Day et al., 2007). The higher prevalence of AM in *T. distichum* and

near absence in either monocot suggest that a reduction in AM colonization or AM functioning would have the greatest effect on *T. distichum*.

## 3.6 Conclusions

Although in an early stage of understanding, studies thus far examining the role AM fungi in the wetland plants have shown that they are beneficial to their hosts in areas of nutrient acquisition, reduction of salt stress, providing resistance to periodic drought, improving plant performance, and influencing wetland plant community structure (Stevens et al., 2002; Khan 2004; Wolfe et al., 2006; Evelin et al., 2009). The roles of DSE in relation to plant performance in wetlands have not yet been explored (Kandalepas et al., 2010; Stevens et al., 2010). This study has shown that natural and anthropogenic factors such as hydrology, water quality, hurricanes, and sedimentation have significant impacts on root fungal colonization in several of Louisiana's coastal wetland plants, albeit in a species specific manner, and affect overall levels of community colonization. If the functions of AM fungi and DSE are also impaired, this may have substantial community level effects and alter the capacity of wetland plant communities to perform valued ecosystem services. Since human activities have and will continue to affect hydrology, water quality, sediment deposition and hurricane frequency and severity in the southern United States, understanding how these changes may in turn affect wetland plant community dynamics is necessary for effective wetland management and conservation efforts.

CHAPTER 4

# TRICLOSAN INHIBITS ARBUSCULAR MYCORRHIZAL COLONIZATION IN THREE WETLAND PLANTS[1]

## (*Eclipta prostrata* (L.) L., *Sesbania herbacea* (Mill.) Mcvaugh, AND *Hibiscus laevis* All)

## 4.1 Abstract

The ubiquitous and pseudo-persistent antimicrobial, triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol; TCS), is one of the most common urban contaminants found in municipal wastewater treatment plant discharges. Potential routes of environmental exposure include not only biota of receiving streams, but also agricultural areas using municipal effluent for irrigation purposes or biosolids for fertilizer and constructed wetlands designed for polishing effluent before delivery to drinking water reservoirs. TCS has been reported to have toxic effects on wide variety of biota and has a mode of action that interrupts lipid biosynthesis in prokaryotes and plants. However, TCS effects on colonization of arbuscular mycorrhizal (AM) fungi in plant roots have not previously been examined in wetland plants. Mycorrhizal fungi are common symbionts found in over 90% of terrestrial plants and are now recognized to play an important role influencing plant community composition in aquatic ecosystems as well. AM colonization benefits wetland vegetation increasing productivity and helping to ameliorate the effects of environmental and anthropogenic stresses. Given that TCS is a recognized antifungal agent, I examined whether TCS limits AM fungal growth resulting in reduced AM colonization in three wetland plants: Eclipta prostrata, Hibiscus laevis, and Sesbania herbacea. Seeds of three plant species collected from the wetlands of North Texas were germinated in growth room

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conditions on filter papers in petri dishes. Seedlings were inoculated with ~200 Glomus intraradices AM spores during transplantation to exposure trays. Plants were exposed to 0 ppb TCS (control), 0.4 ppb TCS (a concentration typical in a wastewater receiving stream), and 4 ppb TCS (an elevated concentration typical of an agricultural area receiving biosolids and/or effluent) in a flow-through system with supplement of 1/64th Long Ashton nutrient solution at the rate of 2.5 ml/minute under green house conditions. Plants were harvested at days 10, 20, and 30 for evaluation of roots for AM colonization. AM propagules (arbuscles, vesicles, and hyphae) were quantified under 200X magnification. Three-way ANOVA showed significant ($p<0.05$) reduction of hyphal and arbuscular colonization in all three plant species treated with 0.4 ppb and 4 ppb TCS compared to controls. Vesicles were not affected by exposure however levels were consistently low in all TCS treatments. Further studies will be required to understand the mechanism of this TCS inhibition of mycorrhizal colonization in wetland plant species as well as the potential ecological consequences that a decline in the benefits of this symbiotic relationship may represent.

## 4.2 Introduction

Triclosan (5-chloro-2-[2, 4-dichlorophenoxy] phenol; TCS) is a widely used antibacterial found in pharmaceuticals and personal care products (PPCPs) ranging from soaps and detergents to clothing and kitchen aids (Dann and Hontela, 2011). As a result of the many consumer products containing TCS and their usage, TCS is considered a “down the drain” contaminant. Consequently, the primary source of TCS input to the environment is via wastewater treatment plant (WWTP) effluent (Oulton et al., 2010). Although TCS

concentrations can be reduced by up to 98% of influent water depending on WWTP processing (Lishman et al., 2006; Thompson et al., 2005), effluent concentrations of up to 0.36 μg/L (Lee et al., 2005) and 2.7 μg/L (McAvoy et al., 2002) have been found in Canadian and US studies, respectively. Additionally, runoff from agricultural soils receiving sewage sludge as a soil amendment provides a second route of TCS entry into the environment (Macherius et al., 2012). TCS in sewage sludge from two North American studies were found at concentrations of 28.2 mg/kg in Canada (Lee and Peart, 2002) and 15.6 mg/kg (Chu and Metcalfe, 2007) in US, while TCS in agricultural soil amended with biosolids has been measured at the range of 0.160 to 0.960 mg/kg (Kinney et al., 2008).

TCS is among the most widely detected PPCPs in surface waters (Halden and Paull, 2005; Kolpin et al., 2002) and reported toxic to benthic invertebrates (Orvos et al., 2002), crustaceans (Tatarazako et al., 2004), fish (Ishibashi, 2004), algae (Wilson et al., 2003), duckweed (Fulton et al., 2009), and wetland macrophytes (Stevens et al., 2009). Algae have been identified as particularly sensitive to TCS exposure with an NOEC of 0.69 μg/L (Orvos et al., 2002); a value less than current TCS concentrations in US wastewater effluent (Dann and Hontela, 2011). Wetland vascular plants may share a similar sensitivity to TCS exposure. Stevens et al. (2009) found that root development of three emergent vascular plants was inhibited by measured TCS concentrations of approximately 0.6 μg/L, the lowest concentration tested. The effects of TCS on soil fungi have been largely neglected despite the significant role they play in nutrient cycling, soil stability and maintaining plant community structure.

The term “mycorrhiza” describes an association that develops between plant root systems and specific soil fungi. The association is widespread throughout the plant kingdom;

more than 90% of terrestrial plants are estimated to form mycorrhizal associations (Strack, 2003). Arbuscular mycorrhizae (AM) are the most abundant mycorrhizal fungi, colonizing root cortical cells and forming specialized structures within the root systems including hyphae, arbuscules, and vesicles (Brundrett et al., 1996; Smith and Read, 2008). Through their accession of, and translocation of nutrient sources normally unavailable to the plant, primarily phosphorus and nitrogen, AM improve plant nutrient uptake. In exchange the heterotrophic fungus, an obligate symbiont, is provided with host-produced photosynthates.

It is well recognized that AM play significant roles in terrestrial ecosystems due to their impacts on nutrient cycling, improvement in soil quality, carbon transport (Brundrett et al., 1996), providing a food for soil invertebrates (Fogel, 1988), and limiting erosion due to the mechanical aggregation of soil particles (Andrade et al., 1998). More recently, AM have been shown to influence plant community structure by mediating competitive interactions (Hartnett and Wilson, 1999) by plant competition (John and Coleman, 1983), influencing soil microbial community structure, and altering host plant physiology (Rillig, 2004). The importance of AM to wetland plant communities and their role in wetland ecosystem services is largely unknown. AM were long thought absent in wetland plants (Khan and Belik, 1995), however, they have been found in many major wetland ecosystems including Cypress Swamps (Kandalepas et al., 2010), bottomland hardwood forests (Stevens et al., 2010), nutrient poor fens (Cornwell et al., 2001), tropical river flood plains (de Marins et al., 2009), and tropical marshes (Radhika and Rodrigues, 2006). AM have been shown to impact *E. prostrata* seedlings (Stevens et al., 2011), *Lythrum salicaria* (Stevens et al., 1996), and *Cladium jamaicense* (Lin et al., 2011) wetland plants and several wetland species. Consequently, any impacts on AM associations in wetlands

may have substantial repercussions in terms of wetland plant community dynamics and wetland ecosystem functions.

TCS disrupts fatty acid synthesis (FAS) by inhibiting the enoyl-acyl carrier protein reductase activity encoded by the *fab I* during Type II FAS (Heath et al., 1999; Newton et al., 2005); a pathway shared between bacteria and plants. In contrast, animals and fungi undergo Type I FAS (Lee et al., 2006) and should be unaffected by TCS. The single study to date that examined TCS exposure on AM hyphal growth and spore production found no significant effects at concentrations of up to 1000 µg/L TCS (Hillis et al., 2008), however, TCS is listed by the EPA as a fungicide and fungistat (Jones et al., 2000). Given the importance of AM in structuring and maintaining ecosystem services and lack of information regarding TCS impacts on AM associations, my goal was to assess the effects of TCS on early development of AM associations in three emergent wetland plant species (*E. prostrata, H. laevis,* and *S. herbacea*) utilizing a continuous flow-through exposure system.

## 4.3 Methods and Materials

### 4.3.1 Plants

Based upon a preliminary assessment of the AM status of wetland plants in North Central Texas and their abundance in local wetlands, three rooted emergent wetland plant species were selected for this study: *E. prostata* (L.) L., false daisy, in the family Asteracea; *S. herbacea* (Mill.) McVaugh, big pod sesbania, in the family Fabaceae; and *H. laevis* All, halberd leaf rosemallow in the family Malvaceae (Taxonomy follows Diggs et al., 1999).

### 4.3.2 Chemicals

Neat native TCS (Irgasan) was purchased from Fluka Laboratories (Buchs, Switzerland). The internal standard, $^{13}C_{12}$ TCS, was obtained from Wellington Laboratories (Guelph, ON, Canada). Analytical grade hexane (HEX), ethyl acetate (ETAC), chloroform (CHLF), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Fisher Scientific (Houston, TX, USA).

### 4.3.3 Flow-Through Exposure System

A flow-through exposure system (Appendix E) was established in the Institute for Applied Sciences, Environmental Greenhouse at the University of North Texas, Denton, TX. Exposure solutions were obtained by dissolving neat TCS in deionized (DI) water without the use of carrier solvents. Exposure solutions were mixed in 22 L HDPE reservoirs and replenished after 36 hours. Nutrients were added to obtain 1/64$^{th}$ strength Long Ashton nutrient levels (Hewitt, 1966) in the exposure solution. This concentration of nutrients resulted in phosphorus level comparable to level present in the Trinity River, Denton, TX, and is level previously found to promote mycorrhizal associations in native Texas wetland plants (Stevens et al., 2011). Controls received 1/64$^{th}$ strength Long Ashton nutrients. Exposure solutions were delivered to non-draining plastic potting trays (54 $\times$ 28 $\times$ 6 cm, Summit Plastic Company) via a 12 channel peristaltic cassette pump (12/6 Thermo scientific, Barrington, IL) at a constant flow rate of 2.5 mL/min resulting two turnovers per day. Four channels on the pump were utilized for each treatment. Seedling growth inserts (4 $\times$ 6 $\times$ 6 cm; Dillen Products, Rochester, NY) were placed in the trays. Each insert was filled with approximately 115 g of commercial sand (Sakrete Natural Sand, Bonsal American, Charlotte, NC, USA) and the sand surface was covered with light

impenetrable fabric to inhibit algal growth. A small opening in the fabric permitted the shoots to pass through. To prevent algal growth in the 0.55 mm ID PTFE microbore (Cole-Palmer, Vernon Hills, IL) delivery tubes from each peristaltic pump cassette were inserted into 1 cm diameter black tubing. All reservoirs and the peristaltic pump were shielded from the light by a shade tent made from light impenetrable fabric.

Seeds of experimental plants were germinated in petri dishes on the surface of filter paper moistened with DI water. Immediately after radical emergence, seedlings were transplanted to the seedling growth inserts and inoculated with approximately 1 ml of *Glomus intraradices* spores in liquid suspension (BioSyneterra Solutions Inc. Quebec, Canada). One mL spore suspension contained approximately 200 AM spores. Nine seedlings of each species were randomly assigned a location in the seedling growth inserts. Plants were maintained under greenhouse conditions (16/8 light dark cycle and temperature 24-30 °C) for 30 days.

### 4.3.4 Root Harvesting, Processing and AM Quantification

Three randomly selected plants of each species from inoculated trays were harvested at days 10, 20 and 30. Harvested roots were rinsed in tap water then fixed and stored in 50% ethanol. Staining for visualization of AM structures followed Brundrett et al. (1996). In brief, roots were cleared in 5% potassium hydroxide at 80 °C for 1-2 h, rinsed in DI water, then stained with 0.1% Chlorazol Black E at 80 °C for 1 h. Roots were then de-stained and stored in 50% glycerol prior to mounting on slides (Phillips and Hayman, 1970). Ten to twenty, first-order fine roots were selected with fine forceps and mounted on microscope slides (25 $\times$ 75 mm) with 50% glycerol and covered with a cover glass (25 $\times$ 60 mm). Prepared slides were viewed at 200$\times$ magnification using a Zeiss Axio Imager A1 microscope (Carl Zeiss Inc., Germany) and images

obtained with a Zeiss Axiocam MRC-5 camera (Carl Zeiss Inc., Germany). Colonization levels were assessed using a modified intersects procedure (Brundrett et al., 1996). The percentage of hyphal, arbuscular, and vesicular AM colonization was calculated after assessing a total of 100 fields of view for each sample.

### 4.3.5 Exposure Water Preparation for TCS Concentration Analysis

All TCS exposure concentrations were verified by instrumental analysis (see section 3.7) of water samples collected from corresponding trays prior to seedling transplant after equilibration of the exposure system. Additional water analyses were performed at day 15 and day 30.

Two water samples from the middle of trays of each channel were collected after running the whole flow-through system for several turnovers. Ten mL of water samples for 4 µg/L exposures and 100 mL of water for 0.4 µg/L exposures and control treatments were collected in 50 mL Teflon cap glass centrifuge vials and 150 mL conical flasks, respectively. Water samples collected from exposure trays were extracted immediately after collection. Five µL of TCS internal standard ($^{13}C_{12}$ TCS) at 10 ppm was added to each sample before extraction. Each sample was extracted three times by liquid-liquid extraction with 1:1 HEX:ETAC (10 mL for each extraction) and the solvent was evaporated under nitrogen. Evaporated extracts were transferred in 1mL CHLF to 2 mL auto-sampler vials where they were re-evaporated under a gently nitrogen stream and derivatized with 50 µL of MSTFA for 30 min at 60 °C. After derivatization, each sample was re-evaporated to dryness, re-solubilized in 100 µL CHLF and transferred to a 200 µL auto-sampler vial insert for final analysis.

### 4.3.6 Quality Assessment/Quality Control

Quality control samples were included with each sampling episode. The analysis included two replicate method blanks (laboratory DI water spiked with internal standards only), and two replicates of blank analyte spikes (DI water spiked with internal standards and TCS). Two additional samples from control exposure water were also spiked with internal standards and TCS, serving as matrix spikes. All quality control samples received the same extraction preparation as experimental samples.

### 4.3.7 Instrumental Analysis

Instrumental analysis of TCS was conducted by isotope dilution gas chromatography (GC)-mass spectrometry (MS) on an Agilent 6890 GC couple with a 5973 mass selective detector (70 eV). Instrumental analysis of TCS was conducted by isotope dilution gas chromatography (GC)–mass spectrometry (MS) on an Agilent 6890 GC couple with a 5973 mass selective detector (70 eV). An eight point standard calibration curve was established with TCS analyte concentrations ranged from 5 pg/µL to 1,000 pg/µL with internal standard concentrations at 500 pg/µL. The MS was operated in the single ion monitoring mode (SIM) with 3 confirmatory masses monitored (50 msec dwell time) for quantification. A helium gas at 480 hPa was used as carrier gas in GC with inlet temperature at 260 °C (2µl, pulsed pressure at 1,700 hPa for 0.5 min, splitless injection). The GC column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d., 0.25 µm film) temperature was programmed initially at 40 °C with a 1-min hold followed by a 50 °C per min ramp to 140 °C with a 5- min hold followed by a 10 °C per min ramp to 300 °C with a final 17-min bakeout. Transfer line temperature was maintained at 265 °C (Coogan, 2007).

### 4.3.8 Statistical Analysis

A generalized linear mixed model (GLMM) using Proc MIXED (SAS 9.2) was used to fit the data. The design was a split plot with the fixed-effect whole plot concentration treatment applied to trays fed from a single channel from the peristaltic pump. The split factor was the fixed-effect of harvest time. The model included sub sampling within harvests and an interaction between concentration and harvest. Random effects consisted of channels nested within concentrations, trays nested within channels, and harvest within trays. If random effects were not significant at p-values >0.25 they were removed from the model. Although data were collected over time (harvest), this is not repeated measures as samples were taken destructively; hence the harvest times are independent.

To assess the ANOVA assumptions, comprehensive residual analyses were conducted. This included formally testing the residuals for normality using the four tests offered by SAS (Kolmogorov-Smirnov, Shapiro-Wilk, Cramer-von Mises, Anderson-Darling). The residuals were plotted against the predicted values and explanatory variables used in the model (including most random effects variables). Such analyses may reveal outliers or other problems with the data set. To meet ANOVA assumptions hyphal colonization was square-root transformed, arbuscular colonization and vesicular colonization were cube-root transformed. Although the analysis was conducted on transformed data, graphs and means presented in the results are raw means and ± one standard error. Since I was interested only in specific comparisons among treatment means and not in all possible pair-wise comparisons, multiple comparisons were conducted using the lsmeans statement in SAS without specifying adjustment.

## 4.4 Results

Measured exposure concentrations exceeded targeted levels at day 0 for 0.4 and 4 µg/L exposure concentrations but fell below expected levels at day 15 and 30 (Table 3.1). Averaged across all harvests, measured concentrations were slightly below target levels. TCS detected in control exposures ranged from <0.05 to 0.075 µg/L, while blank samples measured from <0.05 to 0.08 µg/L (Table 3.1) with PQL of 0.05 µg/L (Stevens et al., 2009). The average recovery of TCS in blank and matrix spikes was 107.6%. Background detection of TCS in control exposures remained consistent throughout the study and did not display a correlation with sampling period.

Table 3.1 Nominal and measured triclosan (TCS) concentrations (µg/L) in the exposure trays. Data shown are means ± one standard error.

| TCS | Day 0 | Day 15 | Day 30 | Average |
|---|---|---|---|---|
| Blank | < 0.05 | < 0.05 | < 0.05–0.13 | < 0.05 |
| 0 µg/L | < 0.05 | 0.07 ± 0.012 | < 0.05 | < 0.05 |
| 0.4 µg/L | 0.59 ± 0.008 | 0.32 ± 0.09 | 0.08 ± 0.02 | 0.334 |
| 4.0 µg/L | 6.35 ± 0.53 | 2.44 ± 0.81 | 1.12 ± 1.02 | 3.305 |
| Blk + MS Recovery (%) | 100.4 ± 25.45 | 90.9 ± 23.36 | 131.49 ± 3.79 | 107.6% |

Blk + MS = blank + matrix spike.
Practical quantification limit (PQL) = 0.05 (Stevens et al., 2009).

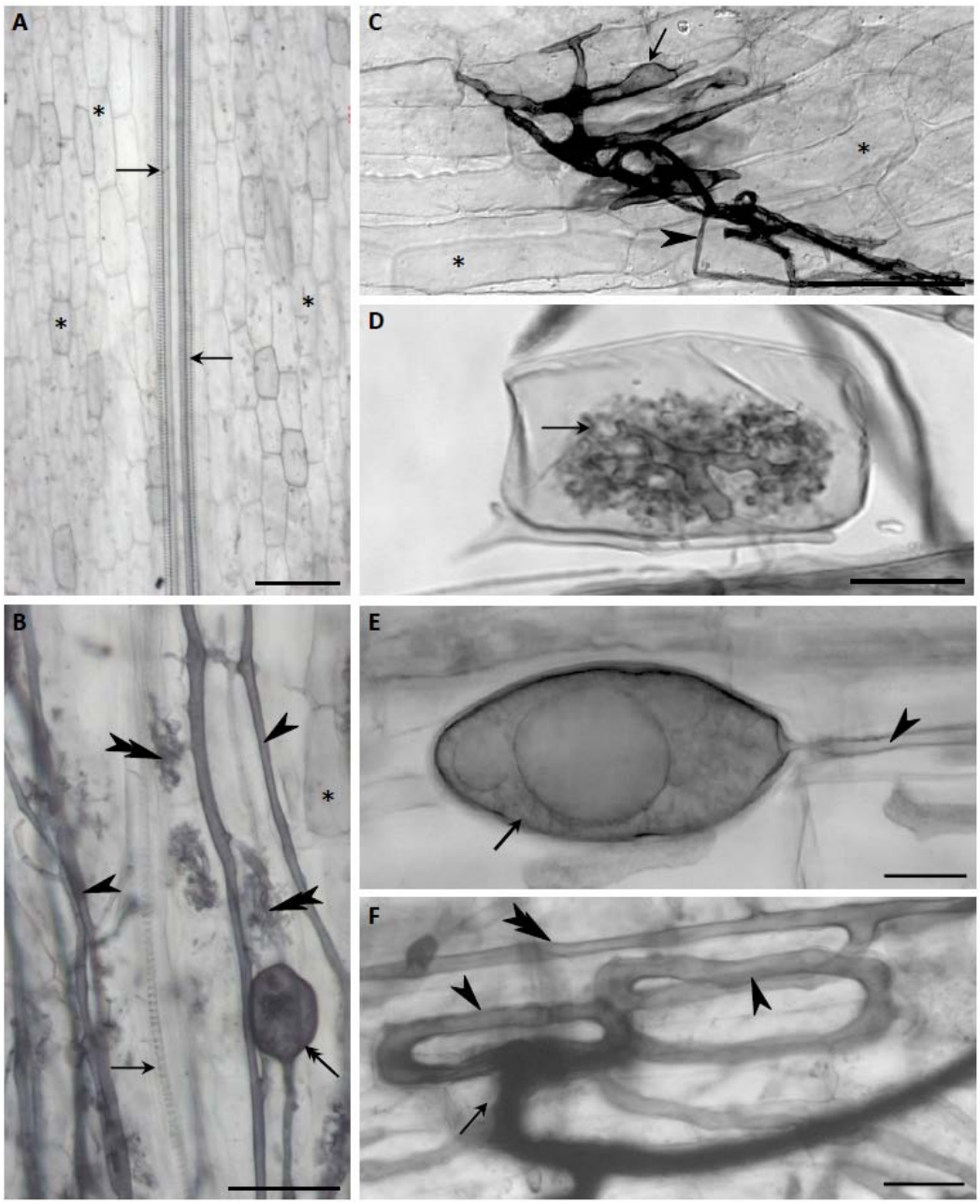


Figure 3.1 Cleared and stained roots of three wetland plant species abundant in North Texas wetlands. Roots were cleared in 10% KOH and stained with 0.1% Chlorazol Black E. A is a non-colonized root; B–F are colonized by arbuscular mycorrhizal (AM) fungi. A: Non-colonized area of a root from *Eclipta prostrata*. Non-colonized cortical cells appear translucent (*). The vascular cylinder is visible as a dark central structure with helical secondary cell wall thickenings in xylem tracheary elements (arrows). Scale bar=100 µm. B: Colonized section of *Sesbania herbacea* with visible xylem tracheary elements (arrow), cortical cells (*), intercellular hyphae (arrow heads), Arum-type arbuscules (double arrow heads), and a vesicle (double arrow). Scale bar=50 µm. C: Epidermal cells (*), an appressorium (arrow), and extra-radical hyphae (arrow head) on the surface of an *E. prostrata* root. Scale bar=50 µm. D: Arum-type arbuscule (arrow) within a cortical cell of Hibiscus laevis. Scale bar=20 µm. E: A vesicle (arrow), and subtending

hypha the cortex of an *S. herbacea* root. F: Hyphal coils (arrow heads) and hyphae (double arrow head) in cortical cells of an *S. herbacea* root. Scale bar=20 µm.

Hyphae, arbuscules and vesicles were found in all treatments for all three test species. Images of AM structures are shown in Figure 3.1; for comparison, a non-colonized area of an *E. prostrata* root is shown in Figure 3.1A. Hyphal colonization was evident in all species 10 days following inoculation. Arbuscules were found in *H. laevis* and *S. herbacea* 10 days after inoculation but were not detected in *E. prostrata* until 20 days post inoculation. Vesicles were noted in *H. laevis* 10 days after inoculation but were not found in *S. herbacea* and *E. prostrata* until 20 days after inoculation.

Table 3.2 Summary table of three-way ANOVA assessing the effects of triclosan concentration (TCS), plant species (Sp), and harvest time (Harv) on AM colonization in three wetland plant species (Eclipta prostrata, Hibiscus laevis, and Sesbania herbacea) (significant effects ($p \leq 0.05$) are in bold).

| | Hyphae | | | Arbuscules | | | Vesicles | | |
|---|---|---|---|---|---|---|---|---|---|
| variables | ndf/ddf | F | Pr > F | ndf/ddf | F | Pr > F | ndf/ddf | F | Pr > F |
| Sp | 2/282 | **6.31** | **0.0021** | 2/69.7 | **10.17** | **0.0001** | 2/282 | **4.95** | **0.0077** |
| TCS | 2/8.97 | **5.09** | **0.0333** | 2/8.81 | **7.18** | **0.0141** | 2/9.07 | 2.52 | 0.1350 |
| Harv | 2/282 | **118.40** | **< 0.0001** | 2/69.7 | **50.81** | **< 0.0001** | 2/282 | **57.49** | **< 0.0001** |
| Sp × TCS | 4/282 | 0.56 | 0.6886 | 4/69.7 | 1.42 | 0.2366 | 4/282 | 0.36 | 0.8345 |
| Sp × Harv | 4/282 | **5.50** | **0.0003** | 4/69.6 | **15.24** | **< 0.0001** | 4/282 | 1.51 | 0.1991 |
| TCS × Harv | 4/282 | 0.79 | 0.5354 | 4/69.7 | 1.18 | 0.3292 | 4/282 | 1.42 | 0.2289 |
| Sp × TCS × Harv | 8/282 | 0.50 | 0.8581 | 8/69.7 | 0.34 | 0.9494 | 8/282 | 0.41 | 0.9165 |

### 4.4.1 Hyphal Colonization

Percent hyphal colonization differed among species, TCS exposure, harvest and the interaction of species × harvest (Table 3.2; Fig. 3.2). Overall, hyphal colonization was

significantly higher in controls (18.58 ± 1.84%) compared to 0.4 and 4 μg/L (10.20 ± 1.34% and 9.86 ± 1.32% respectively). Hyphal colonization increased over time for all species; however, relative levels of colonization among species differed over time. After 10 days, hyphal colonization was significantly greater in *E. prostrata* (3.77 ± 0.91%) compared to *H. laevis* (0.67 ± 0.23%) and after 20 days colonization of *H. laevis* (18.22 ± 3.23%) was significantly greater than both *S. herbacea* (11.28 ± 2.33%) and *E. prostrata* (8.92 ± 1.55%). However, after 30 days there was not a significant difference between hyphal colonization of *E. prostrata* (34.94 ± 5.03%) and *H. laevis* (33.44 ± 4.31%), while both were significantly greater than *S. herbacea* (17.17 ± 2.83%).

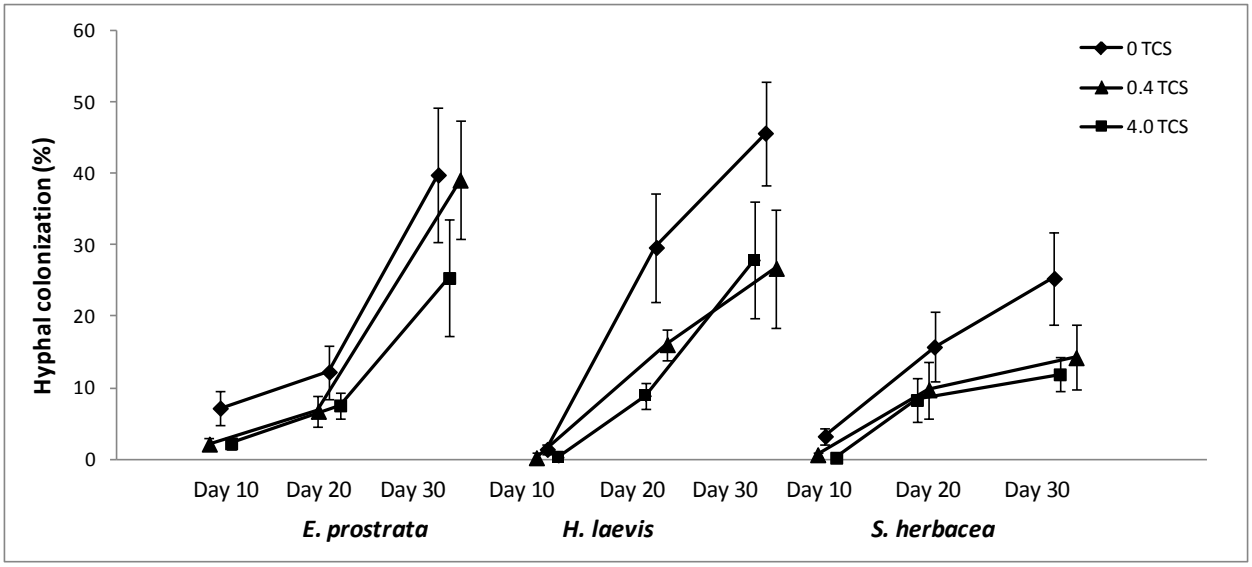


Figure 3.2 Hyphal colonization in three wetland plant species (*E. prostrata*, *H. laevis*, and *S. herbaceae*) grown for 30 days under exposure to control water and two environmental relevant concentration of TCS (0.4 and 4.0 μg/L). Data shown are means ± one standard error.

### 4.4.2 Arbuscular Colonization

Arbuscular colonization differed among species, TCS exposure, harvest and the interaction of species × harvest (Table 3.2; Fig. 3.3). Overall, arbuscular colonization was

significantly higher in the controls (4.58 ± 0.75%) compared to 0.4 µg/L (2.20 ± 0.38%) and 4 µg/L (1.22 ± 0.24%) TCS exposure. Arbuscular colonization increased over time for *E. prostrata* with means of 0%, 1.47 ± 0.39% and 9.03 ± 2.03% at days 10, 20 and 30 respectively. Colonization in *S. herbacea* was significantly greater at day 20 (2.11 ± 0.55%) compared to day 10 (0.31 ± 0.17%) and day 30 (0.47 ± 0.19%) with no significant differences in colonization detected between day 10 and 30. Arbuscular colonization in *H. laevis* differed at each sampling period and was lowest at day 10 (0.19 ± 0.90%) peaked at day 20 (8.22 ± 1.87%), then declined by day 30 (2.14 ± 0.66%). There were no significant differences in arbuscular colonization among species ten days after inoculation (0% for *E. prostrata*, 0.19 ± 0.09% for *H. laevis* and 0.31 ± 0.17% for *S. herbacea*). After 20 days arbuscular colonization was significantly greater in *H. laevis* (8.22 ±1.87%) compared to *S. herbacea* (2.11 ± 0.55%) and *E. prostrata* (1.47 ± 0.39%). At day 30 colonization levels were significantly different among all species and were highest in *E. prostrata* (9.03 ± 2.03%), intermediate in *H. laevis* (2.14 ± 0.66%) and lowest in *S. herbacea* (0.47 ± 0.19%).

### 4.4.3 Vesicular Colonization

Vesicular colonization differed among species and harvest (Table 3.2; Fig. 3.4). Overall, vesicular colonization was significantly lower in *S. herbacea*e (0.80 ± 0.20%) compared to *H. laevis* (1.95 ± 0.39%) while *E. prostrata* did not differ significantly from either (0.95 ± 0.18%). Vesicular colonization increased at each successive harvest from 0.20 ± 0.02% at 10 days to 1.04 ± 0.19% at 20 days and 1.94 ± 0.31% after 30 days of TCS exposure. There was not a detectable effect of TCS exposure on vesicular colonization.

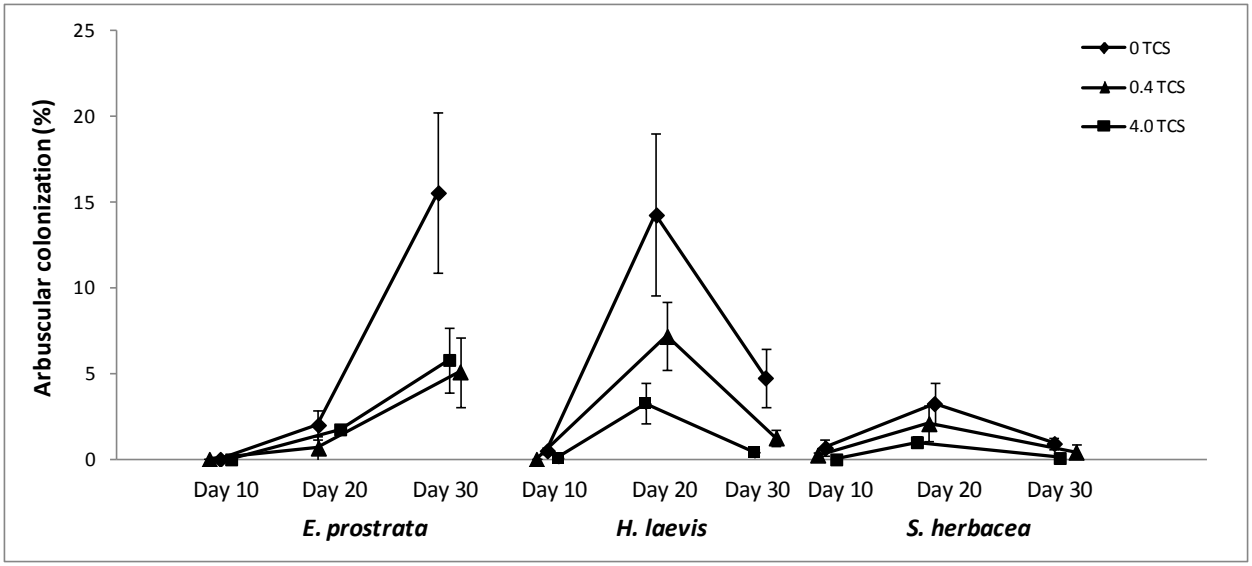


Figure 3.3 Arbuscular colonization in three wetland plant species (*E. prostrata*, *H. laevis*, and *S. herbaceae*) grown for 30 days under exposure to control water and two environmental relevant concentration of TCS (0.4 and 4.0 μg/L). Data shown are means ± one standard error.

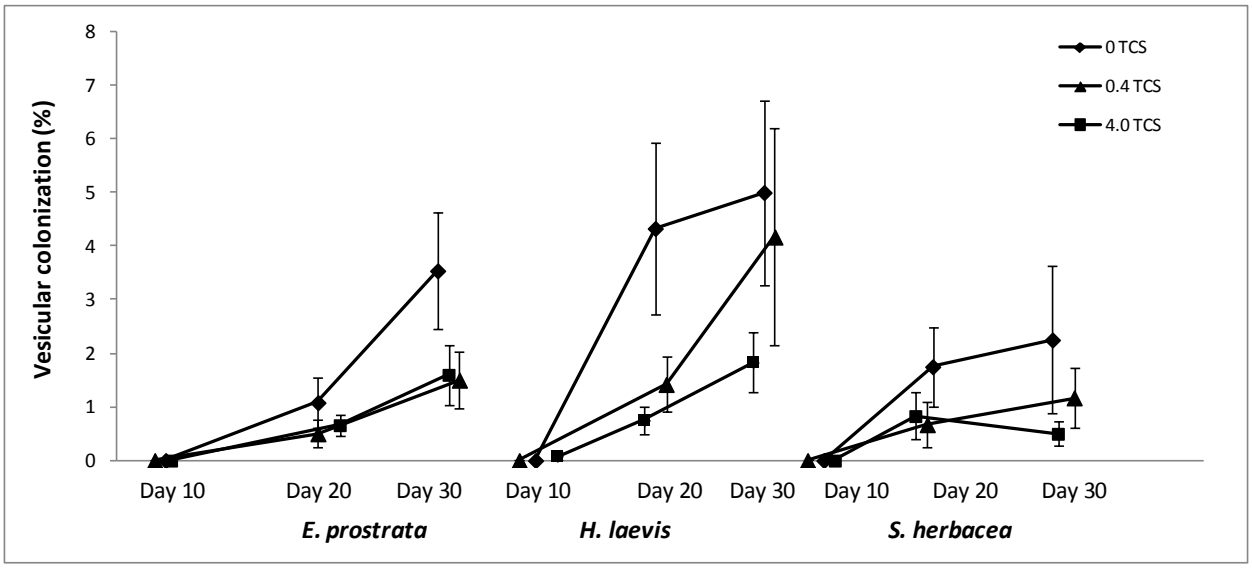


Figure 3.4 Vesicular colonization in three wetland plant species (*E. prostrata*, *H. laevis*, and *S. herbaceae*) grown for 30 days under exposure to control water and two environmental relevant concentration of TCS (0.4 and 4.0 μg/L). Data shown are means ± one standard error.

## 4.5 Discussion

The exposure system was successful in delivering concentrations that, overall, were reasonably close to the target concentration levels of 0.4 and 4 µg/L. Achieving consistent levels prior to the introduction of the test plants required a substantial equilibrium period following each adjustment of the stock concentrations and the entire system was sensitive to slight changes in stock concentrations. Consequently, once exposure concentrations close to the target concentrations were obtained, there was no further adjusting of the stock concentrations even though exposure concentrations dropped below target values 15 days following seedling transplant. Reductions in exposure concentrations coincided with plant root development and it is likely that these reductions were due to increased sorption sites on the developing root systems and/or increased uptake by the plant. These changes over the course of the experiment reinforce the need for periodic monitoring. Because of its ubiquitous environmental distribution, TCS is frequently reported at low background levels even in analytical method blanks (e.g. Allmyr et al., 2006; Chu and Metcalfe, 2007; Geens et al., 2012) and the low concentrations of TCS found in my controls are consistent with these findings. Despite having overall exposure concentrations below the target value, I feel that the exposure system employed simulates natural exposure scenarios more so than static non-renewal or static renewal studies. The simulation of natural conditions was furthered through the use of a sand substrate and nutrient levels reflective of levels found in North Central Texas watersheds. I further believe that my study provides a more conservative assessment of TCS effects on AM colonization compared to the results that would have been obtained with exposure concentrations closer to the target values. It should be noted that the measured concentration

of approximately 0.6 µg/L in the 0.4 µg/L targeted treatment is still within the range of TCS concentrations found in North American streams (Halden and Paull, 2005; Kolpin et al., 2002) while the measured concentration of approximately 6 µg/L in the 4 µg/L targeted treatment is well below the upper range of TCS found in sediment and soil pore water (Chalew and Halden, 2009).

Colonization differed among species and harvest, hyphae and arbuscules was affected by the interaction of species and harvest. These results are not at all unexpected. That AM colonization differs among wetland plant species is well established (Cornwell et al., 2001; de Marins et al., 2009; Kandalepas et al., 2010; Stevens et al., 2010). The effect of time on root colonization is equally well established (Smith and Read, 2008). Following spore germination and recognition of a suitable host the fungal hypha grows towards the root, and develops an appressorium when physical contact between the fungal hypha and the plant root is made. The appressorium serves as a point of entry of the fungus into the cortex of the host plant. Once within the cortex, arbuscules develop within host cortical cells and a hyphal network develops outside of the root. Vesicle production follows arbuscule development. Consequently, the low levels of vesicular colonization relative to arbuscular and hyphal colonization are reflective of the developmental stages of the AM association. The reduction in arbuscular colonization after day 20 in *H. laevis* and *S. herbacea* is very likely a function of differential rates of root development over the course of time rather than toxicity due to TCS to the AM fungi. Had the latter been the case, it would be expected that colonization levels in *E. prostrata* would also be reduced after day 20 as would hyphal and vesicular colonization; on the contrary, all increased. It is more likely that the reduction in arbuscular colonization was related to the root systems

entering an exponential growth phase and that arbuscular colonization was not able to keep pace with the more rapidly growing root system.

Despite the effects of species, time and the interaction of species × time on hyphal and arbuscular colonization, the effects of TCS on hyphal and arbuscular colonization were consistent among species and harvesting dates. Significant reductions in hyphal and arbuscular colonization were detected in my lowest exposure concentration, 0.4 µg/L. Since this concentration is within the range of concentrations found in North American streams (Halden and Paull, 2005; Kolpin et al., 2002; Morrall et al., 2004) it is plausible that AM colonization has been impacted in streams receiving WWTP effluent. Arbuscules are considered major sites for the exchange of nutrients and photosynthates between the fungus and the plant, while hyphae function in nutrient and photosynthate transport, colonizing new areas of the root, and foraging for water nutrients (Smith and Read, 2008). Reductions in levels of arbuscular and hyphal colonization may impair these functions thereby limiting the benefits obtained by both plant and fungal partners. While it is recognized that AM inoculated plants often outperform non-inoculated plants (Smith and Read, 2008), correlations between performance and colonization levels per se are less prevalent in the literature (but see Blanke et al., 2011) and absent for wetland plant species. Hyphal and arbuscular colonization were reduced in all three species, yet this does not imply that plant performance would be impacted or impacted equally in all species. The degree to which plants benefit from the AM association and the degree of dependency on the AM association are well known to differ among terrestrial species (Smith and Read, 2008) and are recently shown to differ among wetland species (Stevens et al., 2010).

Consequently, species with greater mycorrhizal dependency and those that derive a greater benefit from the association may be more affected by reductions in colonization levels.

At present, assessments of AM fungi are not a part of routine ecotoxicological testing. In fact, there are no standard methods for the assessment of any fungal taxa to perceived aquatic or terrestrial toxicants. Given the unique roles that AM play in terrestrial ecosystems; an assessment of potential contaminant impacts is warranted. Relative to endpoints in taxa commonly assessed in ecotoxicological studies, hyphal and arbuscular colonization are particularly sensitive to TCS exposure. In a comprehensive review of TCS exposure and toxicity, Dann and Hontela (2011) state that the aquatic organisms most sensitive to TCS exposure are algal species, and cite EC50 for 96 h biomass studies of *S. subspicatus* and *A. flos-aquae* of 1.4 µg/L and 1.6 µg/L respectively (Orvos et al., 2002), $EC_{50}$ for 72 h growth studies of *Dunaliella tertiolecta* of 3.5 µg/L (De Lorenzo et al., 2008) and EC 50 for 96 h biomass study of *S. capricornutum* of 4.7 µg/L (Tatarazako et al., 2004). A no observable effect concentration (NOEC) for 96 h biomass studies of *S. subspicatus* is stated as 0.69 µg/L (Orvos et al., 2002) (Table 3.1). In contrast, hyphal and arbsucular colonization differed from controls at my lowest exposure concentration, 0.4 µg/L indicating that these endpoints are more sensitive than current United States Environmental Protection Agency and Environment Canada published bioassays. My results were similar in sensitivity to root morphological endpoints assessed by Stevens et al. (2009) in an examination of TCS exposure on seedling development of three wetland plant species (*B. frondosa, S. herbacea*, and *E. prostrata*). For *B. frondosa* and *S. herbacea* root length was reduced at concentrations of 0.6 µg/L TCS.

## 4.6 Conclusions

Triclosan is widely found throughout North America in watersheds receiving WWTP effluent. At Environmentally relevant concentrations, between 1.4 µg/L and 0.6 µg/L TCS have been suggested to impact algal growth (Dann and Hontela, 2011), wetland plant growth (Stevens et al., 2009) and in this study, arbuscular mycorrhizal colonization. Given the potential for AM to influence wetland plant community structure, TCS exposure may indirectly affect valuable ecosystem services including nutrient cycling, carbon sequestration, and maintenance of soil structure and adversely affect biodiversity. Recent studies have linked a reduction in AM soil propagules with the incursion of non-native plant species. This process may be exacerbated by additional stressors, which negatively impact AM fungi, including eutrophication (Stevens et al., 2002), altered hydrology (Carvalho et al., 2003, Stevens and Peterson, 1996) and TCS exposure. In addition to quantifying the impact of TCS exposure on AM colonization, this study has shown that AM fungal colonization is a sensitive endpoint that can readily be included in ecotoxicological assessments. These results also highlight the need for additional studies to further elucidate the role of AM in aquatic ecosystems and the impacts of urban contaminants on AM associations under laboratory and field conditions.

# CHAPTER 5

# THE EFFECTS OF TRICLOSAN ON SPORE GERMINATION AND HYPHAL GROWTH OF THE ARBUSCULAR MYCORRHIZAL FUNGUS2 (*Glomus intraradices*)

## 5.1 Abstract

The effect of triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol; TCS), on spore germination, hyphal growth, and hyphal branching of the arbuscular mycorrhizal (AM) fungus, *Glomus intraradices* spores was evaluated at exposure concentrations of 0.4 and 4.0 µg/L in a static renewal exposure system. To determine if potential effects were mycotoxic or a consequence of impaired signaling between a host plant and the fungal symbiont, spores were incubated with and without the addition of a root exudate. Exposed spores were harvested at days 7, 14, and 21. AM spore germination, hyphal growth, and hyphal branching were significantly lower in both TCS concentrations compared to controls in non-root exudate treatments suggesting direct mycotoxic effects of TCS on AM development. Greater hyphal growth and hyphal branching in controls and 0.4 µg/L TCS treatments with root exudate compared to non-root exudate treatments demonstrated growth stimulation by signaling chemicals present in the root exudate. This stimulatory effect was absent in the 4.0 µg/L TCS treatments indicating a direct effect on plant signaling compounds or plant signal response.

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## 5.2 Introduction

Triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol; TCS) has been used as a bactericide for over 30 years, and is one of the most common biocides found in pharmaceuticals and personal care products (Glaser, 2004). Triclosan-containing products range from antibacterial mouthwash and toothpaste to household items such as cutting boards, furniture, textiles and sports equipment (Chalew and Halden, 2009). Triclosan enters municipal wastewater streams following disposal of TCS-containing consumer products. While much reduced, TCS is not completely removed during wastewater treatment plant (WWTP) processing (Singer et al., 2002) and consequently, WWTP effluent is a major source of TCS entry into the environment. Whereas TCS has been measured in WWTP effluents at levels from 0.1 to 3.1 µg/L, surface water concentrations range from 50 ng/L to 2.3 µg/L (Dann and Hontela, 2011). The application of biosolids and wastewater effluent from WWTPs as a soil amendment on agricultural land represents a further source of TCS entry to the environment (Kwon et al., 2010; Lozano et al., 2010).

Triclosan bioaccumulation and effects have been noted in a number of terrestrial and aquatic species. Triclosan accumulates in algae (*Cladophora* spp.) (Coogan et al., 2007), snails (*Helisoma trivolvis*) (Coogan and La Point, 2008), zebra fish (*Danio rerio*) (Orvos et al., 2002), roots, shoots, and rhizomes of emergent wetland macrophytes (*Typha latifolia*, *Pontederia cordata*, and *Sagittaria graminea*) (Zarate et al., 2012), roots and shoots of the crop plants radish (*Raphanus sativus*) and lettuce (*Lactuca sativa*) (Pannu et al., 2012), bean (*Phaseolus vulgaris*) (Karnjanapiboonwong et al., 2011), soybean (*Glycine max*) (Wu et al., 2010) and also detected in human blood plasma, breast milk, and urine (Calafat et al., 2008; Dayan, 2007;

Hovander et al., 2002). Toxic effects have been noted in aquatic organisms exposed to TCS such as crustaceans (*Daphnia* sp.) (Tatarazako et al., 2004), young Japanese medaka (*Oryzias latipes*) (Ishibashi et al., 2004), algal communities (Wilson et al., 2003), duckweed (*Lemna gibba*) (Fulton et al., 2009), and wetland macrophytes (*Bidens frondosa, Sesbania herbacea, and Eclipta prostrata*) (Stevens et al., 2009). Despite their ecological importance, effects on soil fungi have seldom been assessed.

One group of soil fungi, mycorrhizal fungi, is widespread and found in all terrestrial ecosystems (Smith and Read, 2008). These fungi develop mutualistic associations with most species of vascular plants and in exchange for host-derived photosynthates, provide increased access to soil nutrients and water. Arbuscular mycorrhiza (AM) is the most common mycorrhizal association estimated to occur in over 80% of angiosperm species (Strack et al., 2003). Arbuscular mycorrhizal fungi are endophytic obligate symbionts characterized by the formation of either arbuscules or hyphal coils within root cortical cells. These structures are specialized for exchange of materials between the plant and fungus. In addition, some AM species produce vesicles that function in lipid storage. Following root colonization, a fine network of hyphae extends from the host root that functions in nutrient and water acquisition. Due to their impact on plant nutrient status, arbuscular mycorrhizae influence plant community composition (Wolfe et al., 2006), and nutrient cycling in terrestrial ecosystems (Brundrett et al., 1996; Escudero and Mendoza, 2005; van der Heijden et al., 1998). Factors that negatively impact AM associations could, therefore, impair valued ecosystem functions.

Arbuscular mycorrhizal associations of terrestrial plant species are affected by a number of anthropogenic pollutants including polyaromatic hydrocarbons (e.g. anthracene), diesel fuel,

pesticides (e.g. benomyl, chlorothalonil, dimethoate), toxic metals (e.g. Al, Ni), and PPCPs (e.g. doxycyclin, carbamazepine, and 17-α-ethynylestradiol) including antibiotics (Cairney and Meharg, 1999; Hillis et al., 2008; Kirk et al., 2005; Tommerup and Kidby, 1980; Verdin et al., 2006; Wan et al., 1998; Wang et al., 2006). Few studies have examined the effects of pollutants on AM associations in wetland plants and This study found hyphal and arbuscular colonization levels in roots of *E. prostrata*, *Hibiscus laevis* and *S. herbaceae* were depressed at concentrations of TCS as low as 0.4 μg/L (Chapter 3), a value within the range of concentrations found in North American surface waters. This study did not, however, determine which stage(s) in the colonization process were affected by TCS exposure.

There are several stages in the sequence of events that follows AM spore germination and culminates with root colonization, each being regulated by chemical communication between the plant and fungus (Harrison, 2005). Spore germination results in the formation of hyphae with limited growth. However, in the presence of root signaling compounds such as $CO_2$ and strigolactones (Akiyama et al., 2005; Bécard and Piché, 1989), the rate of hyphal growth increases and extensive hyphal branching occurs increasing the probability of contacting a suitable host root (Akiyama et al., 2005; Harrison, 2005). Upon contact, a fungal hypha attaches to the root epidermis and forms an appressorium, a specialized structure that facilitates entry of the fungus into the host root. Fungal hyphae formed from the appressorium grow through the epidermal and exodermal/hypodermal layers, into cortical cells, the sites of arbuscule formation. Colonization can be altered at several stages during initial contact between AM fungi and host roots and subsequent colonization, if environmental conditions change (Fitter et al., 2004). The reduced colonization in seedlings exposed to TCS noted in former study may have

been limited either due to an inability of spores to germinate and detect a suitable host or at later stages in the colonization process after host contact. To understand the effects of TCS exposure on events occurring prior to hyphal contact with the epidermal surface of the host root, this study examined spore germination and hyphal morphology of AM fungi exposed to TCS. Specifically, I sought to distinguish between impairment of spore germination and hyphal growth which could be attributed to mycotoxic effects of TCS (Patel and Coogan, 2008) and impediment of plant signaling recognition by including treatments with and without a root wash containing water soluble root exudates.

## 5.3 Methods and Materials

### 5.3.1 Chemicals

Neat TCS was purchased from Fluka Laboratories (Buchs, Switzerland). The internal standard, $^{13}C_{12}$ TCS was purchased from Wellington Laboratories (Guelph, ON, Canada). Analytical grade Hexane (HEX), ethyl acetate (ETAC), chloroform (CHLF), and derivatizing chemical N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Fisher Scientific (Houston, TX, USA).

### 5.3.2 AM Species

*Glomus intraradices* AM spores were purchased from BioSyneterra Solutions Inc. (L'Assomption, Québec, Canada). Spores were stored at 4 °C for longer than 14 days before use (Juge et al., 2002).

### 5.3.3 Root Exudates

One hundred field-collected seeds of *S. herbaceae* (Mill.) McVaugh were germinated on filter paper moistened with de-ionized water inside sealed petri dishes. Within 72 h following germination, seedlings were transferred to 1 L glass beaker containing 500 mL of moderately hard reconstituted fresh water (pH = 7.0) (Rice et al., 2012) and grown under growth room conditions (12/12 light dark cycle; constant temperature of 25 °C; average PAR of 460 µmols/$m^2$). Two hundred mL of root wash was collected every 48 h and the same amount of reconstituted fresh water was added back to the container. Regular collection was undertaken to minimize any potential time-dependent degradation of root exudates. To avoid microbial contamination and exclude any organisms, root wash was filtered through Whatman Number P8 filters (Fisher Scientific, Houston, TX, USA), then through 0.22 µm mesh Fisher brand Wall Mount Dispenser Syringe ultra filter (Lab Supplies Outlaws, Cleveland, OH). The filtered root wash was stored at 4 °C until use.

### 5.3.4 Exposure Solutions

Stock solutions of 0.8 µg/L and 8.0 µg/L TCS were prepared in moderately hard, reconstituted fresh water (Rice et al., 2012). Stock solutions were mixed with either reconstituted fresh water or root wash at 1:1 ratio to prepare the 0.4 and 4.0 µg/L TCS exposure solutions. Exposure controls consisted of reconstituted water and root wash mixed at 1:1 ratio and 100% reconstituted fresh water.

### 5.3.5 Exposure System

Microscope slides, each overlaid with a rectangular strip of coarse porosity Fisherbrand Whatman P8-creped filter paper (75 mm × 25 mm, Fisher Scientific, Houston, TX), moistened with exposure solution were placed into 100 mL polypropylene coplin jars (Cole Parmer, Vernon Hills, IL) containing 10 mL of exposure solution. Because of the difficulties in trying to obtain images of spore hyphal growth on the heterogeneous texture of the coarse filter paper, twenty *G. intraradices* spores were placed equidistantly on the surface of a 47 mm diameter Gelman Sciences 0.45 µm gridded sterile filter membrane that was cut in half and placed on top of the moistened coarse porosity filter paper. The coarse porosity filter paper served as a wick, delivering the exposure solution to the spores, while the filter membrane provided a matrix for the growth and two-dimensional visualization of hyphal growth. Treatments consisted of a control (0 TCS) and two levels of TCS exposure (0.4 and 4.0 µg/L) and presence or absence of root exudates exposure (+, −). For each treatment combination there were five replicate Coplin jars. To allow for the examination of time dependent effects, three spore-containing slides were placed in each Coplin jar. Prior to the introduction of spores, the entire system was allowed to equilibrate with TCS. Exposures were not initiated until measured exposure concentrations were within the acceptable range of targeted concentrations for two consecutive measurements spanning 7-days. To confirm exposure concentrations, samples were collected and analyzed for TCS concentration (Section 4.3.9). Once the targeted concentrations had been obtained, spores were introduced into the exposure system. Coplin jars were incubated in the dark at 27 ± 2 °C (Daniels and Trappe, 1980) for 21 days. At 24-h intervals, the Coplin jars were removed from the incubator and the exposure solution in each Coplin jar was withdrawn and

replaced with fresh solution while working under low ambient light conditions. Exposure concentrations were again verified at the final harvest.

### 5.3.6 Assessment of Germination and Hyphal Morphology

To quantify the effects of TCS over time, one slide per jar was harvested on days 7, 14, and 21. To visualize hyphal growth, filter membranes were removed from the slides, placed on a new slide and 0.2 mL 0.05% trypan blue was applied to the underside of the membrane on the slide (Brundrett et al., 1996). Application of the stain to the underside of the membrane prevented the spores from floating off. After 5 min., slides were destained with tap water. Germination and hyphal growth were observed with a Zeiss Stemi 2000-C dissection scope (Carl Zeiss Inc. Germany) at 50 × magnification (Brundrett et al., 1996) and images (Fig. 4.1) were obtained with a Zeiss Axiocam MRC-5 camera (Carl Zeiss Inc. Germany). Images of spores with subtending hyphae were obtained prior to treatment initiation. Comparing hyphal growth in images obtained prior to and after treatment initiation provided a means for verification of spore germination. The spores displaying the growth of new hyphae were considered germinated (Bartolome-Esteban and Schenck, 1994). Quantification of hyphal morphology was conducted using WinRHIZO PRO (version 2007c, Regent Instruments, Quebec, Canada). To obtain the high contrast images needed for quantification, images were imported into Adobe Photoshop CS2 Version 9.0 (Adobe System Incorporated, US), a new layer was created and the hyphal network traced using the magnetic pencil tool. The layer containing the traced image was then imported into WinRHIZO for analysis.

### 5.3.7 Exposure TCS Preparation and Verification of TCS Concentration

All TCS exposure concentrations were verified by instrumental analysis prior to the exposure of the AM spores after equilibration of the exposure jars and at the end of the study. Aliquots of 20 mL of exposure water from five 0.4 µg/L TCS replicate jars were mixed and then divided into two 50 mL water samples in 100 mL conical flasks. For 4.0 µg/L TCS treatments, 10 mL aliquots of exposure solution from each jar were collected in 50 mL Teflon cap centrifuge vials. Water samples collected from exposure jars were extracted immediately after collection. Triclosan internal standard ($^{13}C_{12}$ TCS; 5 µL at 10 µg/mL) was added to each sample before extraction. Each sample was extracted three times by liquid–liquid extraction with 1:1 HEX:ETAC (10 mL for each extraction) and solvent was evaporated under nitrogen. Evaporated extracts were transferred in 1 mL CHLF to 2 mL auto-sampler vials where they were re-evaporated under nitrogen and derivatized with 50 µL of MSTFA for 30 min at 60 °C. After derivatization, each sample was re-evaporated to dryness, re-solubilized in 100 µL CHLF and transferred to a 200 µL auto sampler vial insert for final analysis.

### 5.3.8 Quality Control

Quality control samples were included with each sampling. The analysis included two replicate method blanks (laboratory DI water spiked with internal standards only), and two replicates of blank analyte spikes (DI water spiked with internal standards and TCS). Two additional root wash samples were also spiked with internal standards and TCS to provide matrix spikes. All quality control samples received the same extraction preparation as experimental samples.

### 5.3.9 Instrumental Analysis

Instrumental analysis of TCS was conducted by isotope dilution gas chromatography (GC)–mass spectrometry (MS) on an Agilent 6890 GC couple with a 5973 mass selective detector (70 eV). An eight point standard calibration curve was established with TCS analyte concentrations ranged from 5 pg/µL to 1,000 pg/µL with internal standard concentrations at 500 pg/µL. The MS was operated in the single ion monitoring mode (SIM) with 3 confirmatory masses monitored (50 msec dwell time) for quantification. A helium gas at 480 hPa was used as carrier gas in GC with inlet temperature at 260 °C (2µl, pulsed pressure at 1,700 hPa for 0.5 min, splitless injection). The GC column (Alltech, Deerfield, IL, USA; EC-5 30 m, 0.25 mm i.d., 0.25 µm film) temperature was programmed initially at 40 °C with a 1-min hold followed by a 50 °C per min ramp to 140 °C with a 5- min hold followed by a 10 °C per min ramp to 300 °C with a final 17-min bakeout. Transfer line temperature was maintained at 265 °C (Coogan, 2007).

### 5.3.10 Data Analysis

A generalized linear mixed model (GLMM) using Proc MIXED (SAS 9.2) was used to fit the data. The design was nested under two levels root wash (present/absent), each with three levels of TCS exposure. The split factor was the fixed-effect of harvest time. The model included sub-sampling within harvests and an interaction between concentration and harvest. Random effects consisted of jars nested within concentrations, jars nested within root wash treatments, and harvest within jars. If random effects were not significant at p-values > 0.25 they were removed from the model. Although data were collected over time (harvest), this is not repeated measures as samples were taken destructively; hence the harvest times are independent.

To assess the ANOVA assumptions, comprehensive residual analyses were conducted. This included formally testing the residuals for normality using the four tests offered by SAS (Kolmogorov-Smirnov, Shapiro-Wilk, Cramer-von Mises, Anderson-Darling). The residuals were plotted against the predicted values and explanatory variables used in the model (including most random effectsvariables). Such analyses may reveal outliers or other problems with the data set. To meet ANOVA assumptions, total hyphal length, number of branches, and average branch length were square root transformed, cube root transformed, and log transformed respectively. Although the analysis was conducted on transformed data, graphs and means presented in the results are raw means and ± one standard error. Since I was interested only in specific comparisons among treatment means and not in all possible pair-wise comparisons, multiple comparisons were conducted using the lsmeans statement in SAS without specifying adjustment.

To identify mycotoxic effects of TCS, comparisons between TCS treatments and controls were made separately for root wash and non-root wash receiving treatments over each sampling period. To identify impediments of plant signal reception and response, comparisons were made between root wash and non-root wash receiving treatments at each level of TCS exposure. While a significant difference between root wash and non-root wash treatments in the controls suggests an effect of plant root exudates on fungal endpoints, an absence of a significant difference between root wash and non-root wash treatments at either TCS exposure concentration accompanied by a difference between root wash and non-root wash treatments in the controls is indicative of a TCS-related impairment of plant signal reception or response.

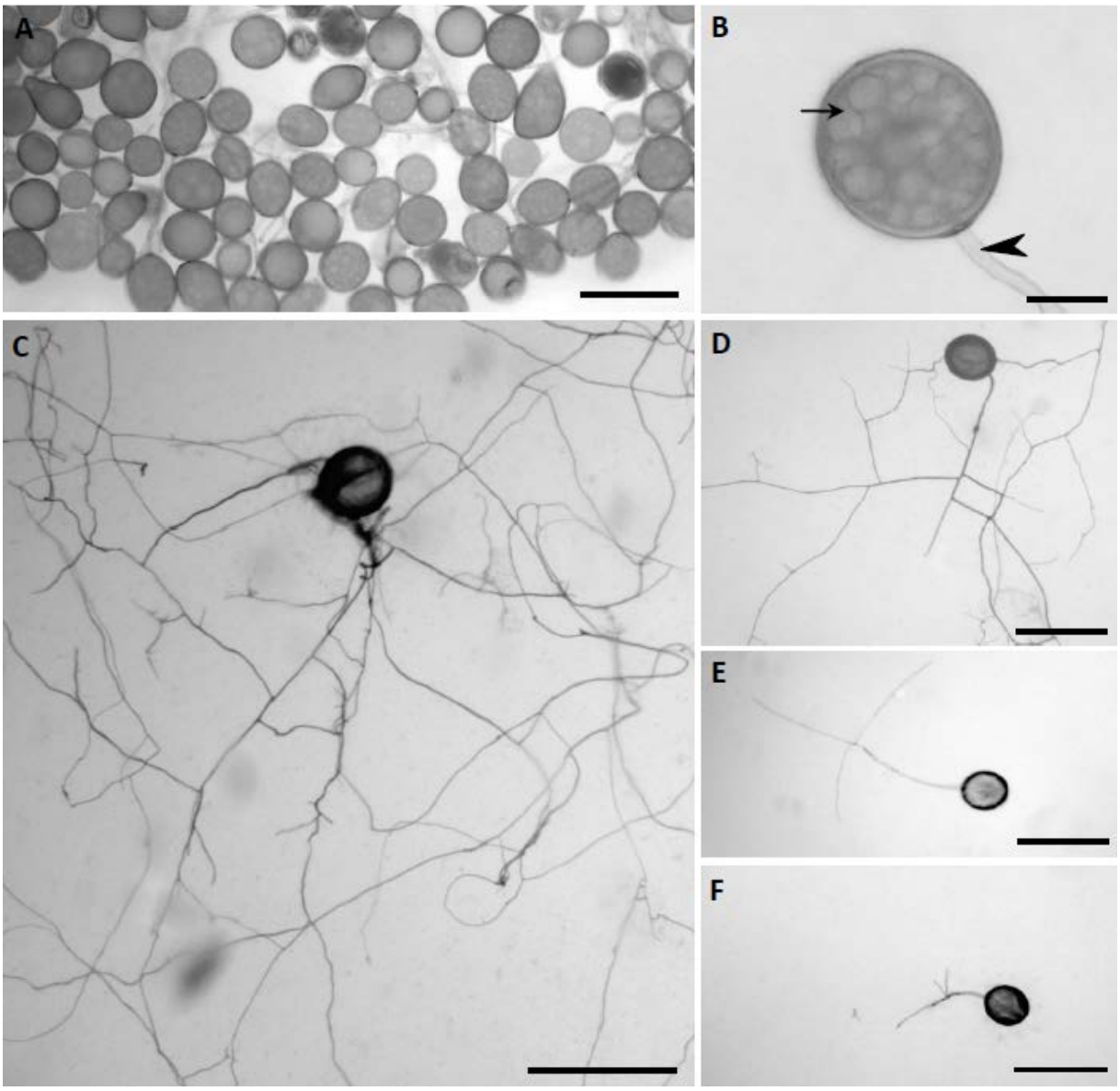


Figure 4.1 G. intraradices spores grown in a static renewal exposure system and exposed to two levels of Triclosan (TCS) exposure with and without the presence of root exudates. Germinated spores were stained with 0.05% trypan blue. A: G. intraradices spores prior incubation, Scale bar=200 m. B: A viable spore with lipid droplets (arrow) and subtending hypha (arrow head), Scale bar=20 m. C-F: Spores germinated at different exposure solutions for 21 days, Scale bar=200 m. C: Spore germinated in reconstituted fresh water (RCFW) with root wash. D: Spore germinated in RCFW (control), E: Spore germinated in 0.4 g/L TCS in RCFW, and F: Spore germinated in 4.0 g/L TCS in RCFW.

## 5.4 Results

### 5.4.1 TCS Exposure Concentrations

Following an equilibration period, pre and post-exposure measured concentrations of jar waters were close to the target exposure concentrations. The water controls and root wash controls were consistently below Practical quantitation limits (PQL) at 0.05 μg/L (Stevens et al., 2009). PQL was calculated at approximately 10X the instrument detection limit, which was estimated as 3X S.D. of background noise levels for quantitation ions. Recovery in the blank spike and matrix spike samples was 106.94 ± 10.6% (Table 4.1).

Table 4.1 Measured Triclosan (TCS) concentrations (μg/L) in the exposure chambers (Coplin jars). Data shown are means ± one standard deviation.

| **TCS** | **n** | **Day 0** | **Day 21** | **Average** |
|---|---|---|---|---|
| Blank | 2 | <0.05 | <0.05 | <0.05 |
| 0 TCS | 4 | <0.05 | <0.05 | <0.05 |
| 0.4 μg/L TCS | 4 | 0.455±0.042 | 0.375±0.028 | 0.415 |
| 4.0 μg/L TCS | 10 | 4.411±0.841 | 3.88±1.419 | 4.145 |
| Blk+MS Recovery (%) | 4 | 114.42±17.08 | 99.45±7.79 | 106.94 |

Blk+MS = Blank + Matrix Spike
Practical quantification limit (PQL) = 0.05 μg/L (Stevens et al., 2009)

### 5.4.2 Spore Germination

By the final harvest, spores had germinated on all slides and treatments with germination ranging from a low 43% in the 0.4 μg/L TCS treatments to 86.9% in the controls (Fig. 4.2a). Germination was affected by the main effect of harvest and the interaction of TCS exposure and root wash (Table 4.2). Overall, germination percentage was significantly lower at day 7 (30.83 ± 3.59%) compared to day 14 and 21 (58.67 ± 4.99 % and 67.41 ± 5.12% respectively), while there was no significant difference in germination between day 14 and day

21. There were no significant differences in spore germination between root wash and non-root wash treatments in the absence of TCS (Fig. 4.2b). However, consistent with a mycotoxic effect, in the non-root wash treatments, germination was significantly lower at 0.4 and 4.0 μg/L TCS

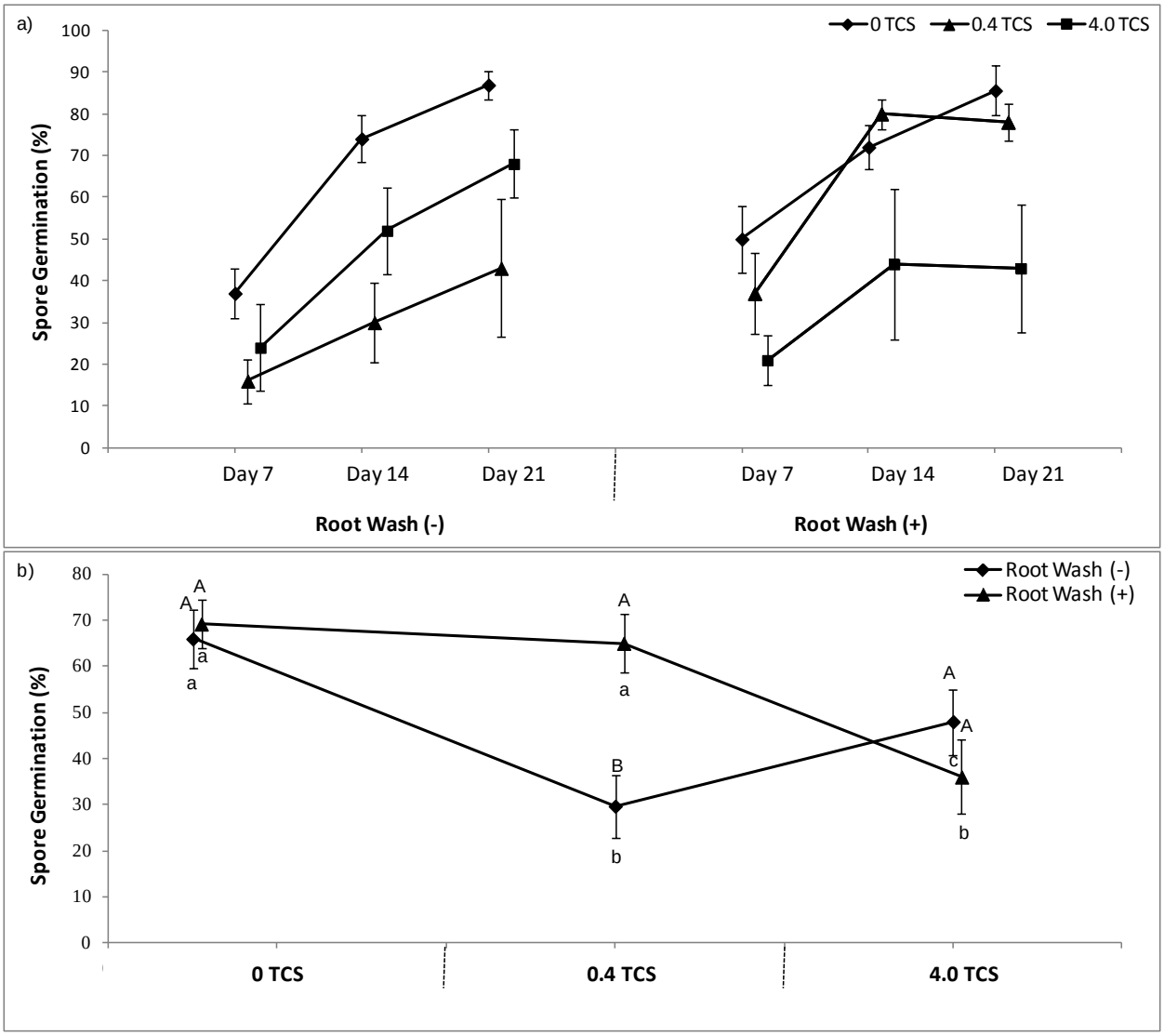


Figure 4.1 Effects of Triclosan (TCS) and root exudates on G. intraradices spore germination. (a) Effects of time of harvest, TCS exposure and root exudates on spore germination. Note: since there was not a significant three-way interaction term in the ANOVA multiple comparisons were not conducted at this level. (b) Effects of TCS exposure and root exudates on spore germination in G. intraradices. Different upper case letters indicate significant differences between root wash treatments within a given level of TCS exposure. Different lower case letters indicate significant differences among TCS treatments within root wash treatments. Raw means are presented with ± one standard error. ($p < 0.05$; TCS = μg/L)

compared to controls. In the root wash treatments, there were no significant differences in spore germination between the controls and 0.4 µg/L TCS treatments, while germination at 4.0 µg/L TCS treatments was significantly lower than both indicating an inhibitory effect on plant signal response.

### 5.4.3 Total Hyphal Length

Total hyphal length was highly significantly affected by the three-way interaction of TCS × root wash × harvest time (Table 4.2). Total hyphal length increased over time in all treatments and was significantly greater in the controls receiving a root wash at day 21 compared to all other treatments (Fig. 4.3ab). In treatments lacking a root wash, total hyphal length was significantly lower in 0.4 and 4.0 µg/L TCS treatments compared to controls at the final harvest indicating a mycotoxic effect (Fig. 4.3a). In treatments receiving a root wash, a reduction in total hyphal length as a result of TCS exposure was evident seven days following exposure.

A stimulatory effect of root wash exposure on total hyphal length was evident after 7 days in the control treatments and after 14 days in the 0.4 µg/L TCS treatments. There was, however, no significant effect of root wash exposure found in the 4.0 µg/L TCS treatment (Fig. 4.3b) indicating an inhibition of plant signaling.

Table 4.2 Summary table of three-way ANOVA assessing the effects of triclosan (TCS), root wash (RtWash), and harvest time (Harv) on *Glomus intraradices* spore germination, hyphal growth, hyphal branching and average hyphal branch length. Significant effects are in bold ($p$ <0.05)

| | **Spore Germination** | | | **Total Hyphal Length** | | | **Number of Hyphal Branches** | | | **Average Branch Length** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Effect | ndf/ddf | F | Pr>F | ndf/ddf | F | Pr>F | ndf/ddf | F | Pr>F | ndf/ddf | F | Pr>F |
| TCS | 2/69 | 16.35 | **<0.001** | 2/11.4 | 1.32 | 0.3047 | 2/11.7 | 17.48 | **0.0003** | 2/11.4 | 11.27 | **0.002** |
| RtWash | 1/69 | 5.28 | **0.0246** | 1/804 | 41.16 | **<0.0001** | 1/913 | 125.21 | **<0.0001** | 1/790 | 43.30 | **<0.0001** |
| Harv | 2/66 | 3.57 | **0.0338** | 2/807 | 43.78 | **<0.0001** | 2/904 | 44.41 | **<0.0001** | 2/787 | 92.99 | **<0.0001** |
| TCS × RtWash | 2/69 | 13.11 | **<0.001** | 2/801 | 5.47 | **0.0044** | 2/913 | 12.24 | **<0.0001** | 2/786 | 4.39 | **0.0127** |
| TCS × Harv | 4/69 | 0.24 | 0.9121 | 4/805 | 1.66 | 0.1569 | 4/904 | 2.94 | **0.0193** | 4/785 | 4.15 | **0.0025** |
| RtWash × Harv | 2/69 | 0.65 | 0.5255 | 2/755 | 2.82 | 0.0602 | 2/906 | 0.82 | 0.4397 | 2/730 | 2.26 | 0.1056 |
| TCS×RtWash×Harv | 4/69 | 1.23 | 0.3066 | 4/755 | 3.45 | **0.0084** | 4/906 | 4.68 | **0.0010** | 4/729 | 3.11 | **0.0148** |

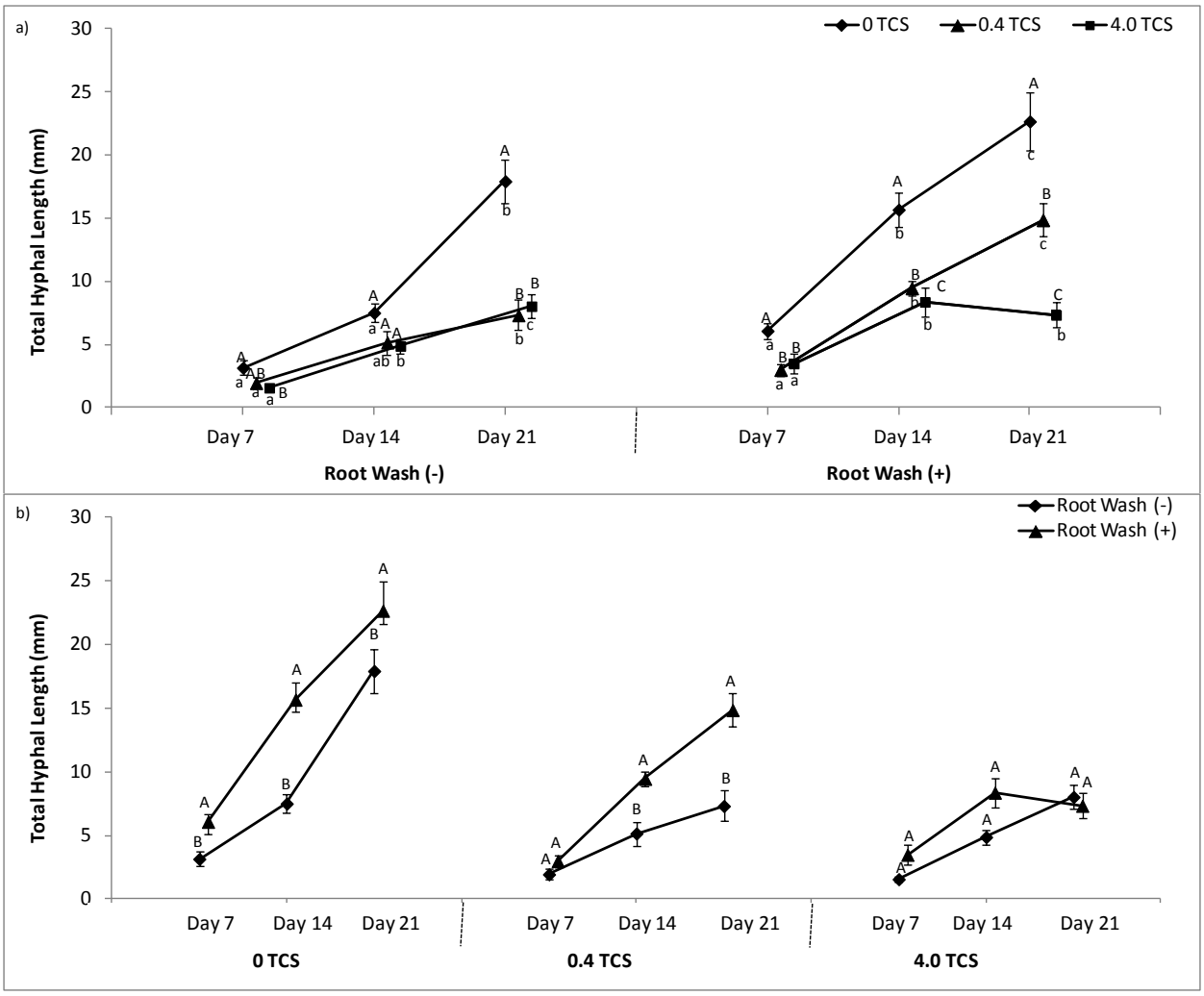


Figure 4.2 Effects of Triclosan (TCS), root exudates, and time of harvest on cumulative hyphal length of *G. intraradices*. (a) Effects of time of harvest and TCS exposure on cumulative hyphal length. Comparisons were made within each level of root wash. Different *upper case* letters indicate significant differences among TCS treatments at specific harvest time. Different *lower case* letters indicate significant differences among harvest times within a level of TCS exposure. (b) Effects of root exudates and time of harvest on cumulative hyphal length. Different *upper case* letters indicate significant differences among root wash treatments at each same time. Raw means are presented with ± one standard error. ($p < 0.05$; TCS = μg/L)

### 5.4.4 Hyphal Branching

The number of hyphal branches was affected by the three-way interaction of TCS × root wash × harvest (Table 4.2). In both root wash and non-root wash treatments the number of hyphal branches tended to increase over time in the controls and 0.4 μg/L TCS treatments,

however, there was no significant change in the number of hyphal branches over time in spores exposed to 4.0 µg/L TCS (Fig. 4.4a). After 14 days, in treatments lacking a root wash, the number of hyphal branches was significantly higher in controls compared to 0.4 and 4.0 µg/L

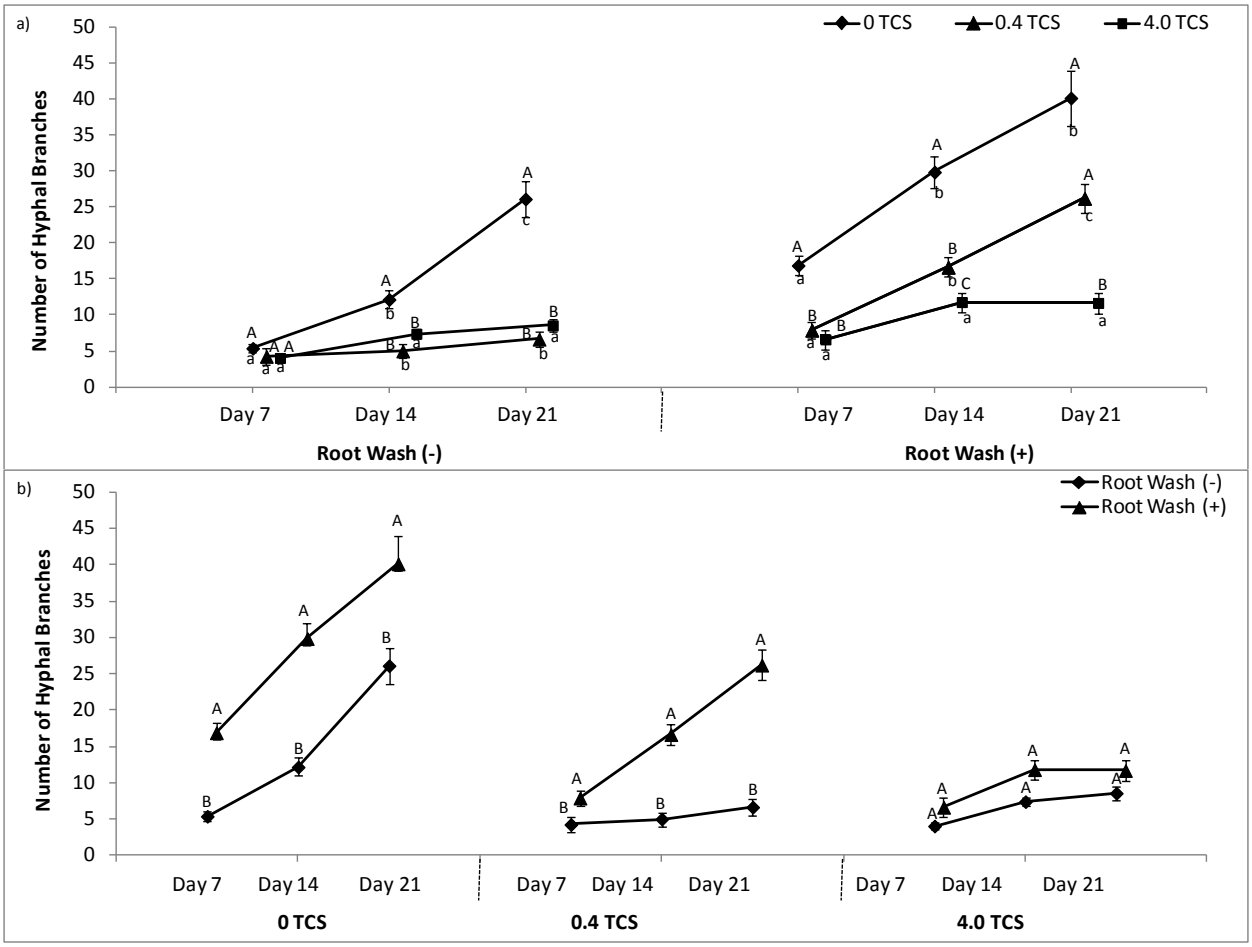


Figure 4.3 Effects of Triclosan (TCS), root exudates, and time of harvest on the number of hyphal branches of *G. intraradices*. (a) Effects of time of harvest and TCS exposure on number of hyphal branches. Comparisons were made within each level of root wash. Different *upper case* letters indicate significant differences among TCS treatments at specific harvest time. Different *lower case* letters indicate significant differences among harvest times within a level of TCS exposure. (b) Effects of root exudates and time of harvest on number of hyphal branches. Different *upper case* letters indicate significant differences among root wash treatments at each same time. Raw means are presented with ± one standard error. ($p < 0.05$; TCS = µg/L)

TCS treatments consistent with a mycotoxic response. In treatments receiving a root wash, a significant reduction in the number of hyphal branches as a result of TCS exposure was evident

after 7 days, however, after 21 days there was no longer a significant difference in hyphal branches in the control and 0.4 µg/L TCS treatments although both were significantly greater than the number of hyphal branches in the 4.0 µg/L TCS treatment (Fig. 4.4a).

A stimulation of hyphal branching by the addition of a root wash was evident within 7 days of exposure in the controls and 0.4 µg/L TCS treatments, however in the 4.0 µg/L TCS treatments there were no significant differences detected in the number of hyphal branches between treatments receiving a root wash and those that did not (Fig. 4.4b) indicating an inhibitory effect on plant signaling.

### 5.4.5 Average Branch Length

Average branch length was significantly affected by the three-way interaction of TCS × root wash × harvest time (Table 4.2). In treatments not receiving a root wash, average branch length increased over time in the 0.4 and 4.0 µg/L TCS treatments but did not increase in the control treatments (Fig. 4.5a). At day 7, average branch length was significantly greater in the controls and 0.4 µg/L TCS treatments compared to 4.0 µg/L TCS however, by day 21 average branch length was lowest in the controls compared to the 0.4 and 4.0 µg/L TCS treatments. In treatments receiving a root wash, average branch length increased at day 14 and 21 in the controls and 0.4 µg/L TCS treatments compared to day 7 but did not significantly change over time in the 4.0 µg/L TCS treatments (Fig. 4.5a). Within sampling periods, there were no significant differences in average branch length detected among treatments.

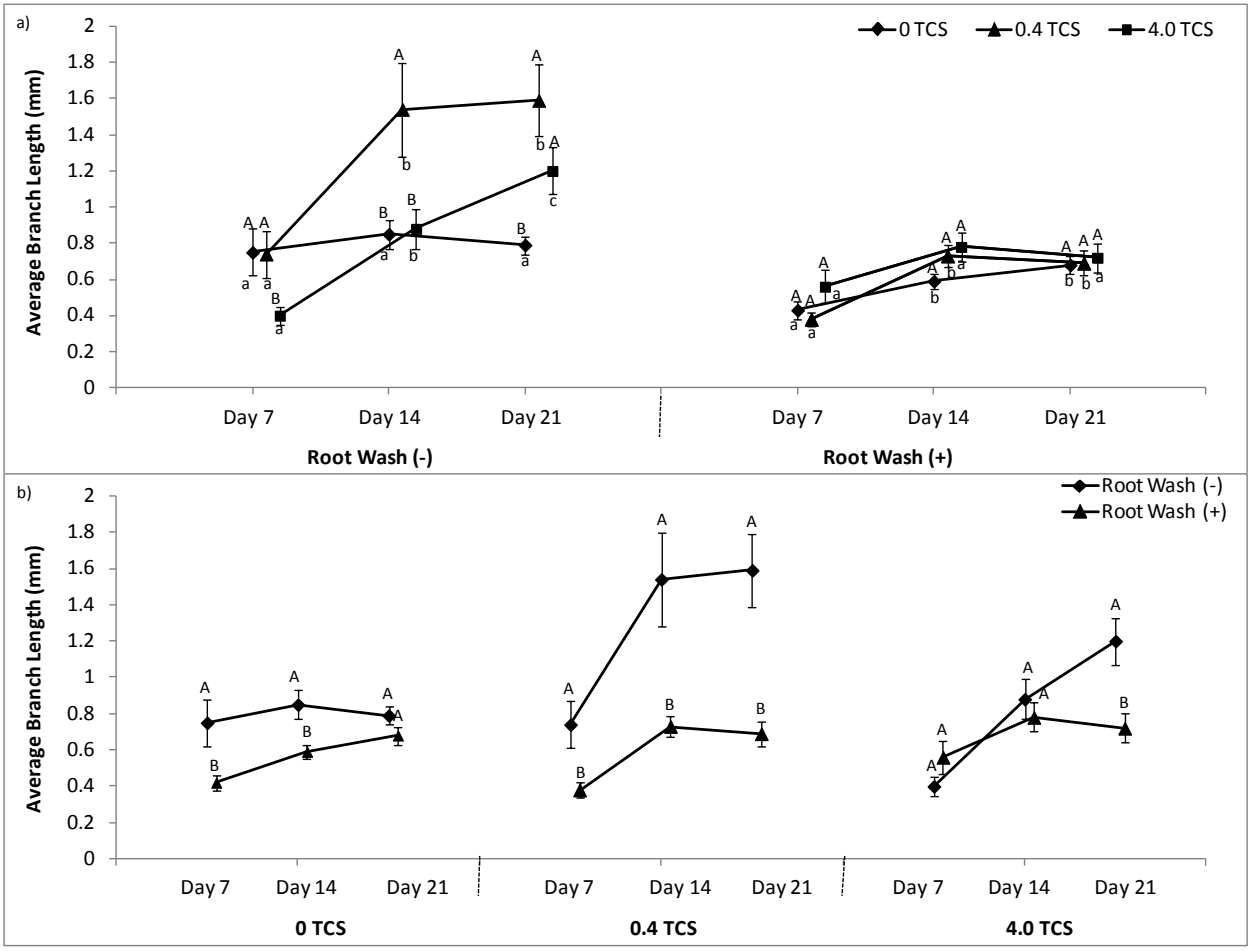


Figure 4.4 Effects of Triclosan (TCS), root exudates, and time of harvest on average branch length of *G. intraradices*. (a) Effects of time of harvest and TCS exposure on average branch length. Comparisons were made within each level of root wash. Different *upper case* letters indicate significant differences among TCS treatments at specific harvest time. Different *lower case* letters indicate significant differences among harvest times within a level of TCS exposure. (b) Effects of root exudates and time of harvest on average branch length. Different *upper case* letters indicate significant differences among root wash treatments at each same time. Raw means are presented with ± one standard error. ($p < 0.05$; TCS = μg/L)

Spores receiving a root wash had a significantly lower average branch length at day 7 and 14 in the controls compared to the non-root wash treatments, however by day 21 there were no longer any significant differences between root wash treatments (Fig. 4.5b). In contrast, in the 4.0 μg/L TCS treatments there were no significant differences in average branch length detected at day 7 and 14, while by day 21, root wash treatments displayed a significantly

reduced average branch length compared to the non-root wash treatments. Average branch length was consistently lower at all sampling periods in the root wash treatments compared to non-root wash treatments in spores exposed to 0.4 µg/L TCS suggesting inhibition of root signaling.

## 5.5 Discussion

The static renewal exposure system required minimal set up compared to continuous flow through exposure systems (i.e. Stevens et al., 2009) and provided measured exposure concentrations very close to the targeted levels. Because the system was very sensitive to slight changes in TCS concentrations in the exposure solution, and took considerable time to equilibrate following changes in TCS concentrations, I chose to proceed with spore exposure when the measured exposure concentration was within 15% of the target concentrations for two consecutive samplings. The resulting measured exposure concentrations in the 0.4 µg/L TCS treatments were within the range of TCS concentrations detected in North American streams (Kolpin et al., 2002; Halden and Paull, 2005), while measured concentrations in the 4.0 µg/L TCS treatment were below the maximum TCS concentrations found in sediment and estimated pore water (Chalew and Halden, 2009). Given the frequency of exposure solution replacement, TCS delivery using reconstituted freshwater, a widely accepted and established freshwater substitute (Rice et al., 2012), and constancy of TCS concentrations maintained in the exposure system, I believe this system reasonably approximated exposure conditions that would be experienced under field conditions.

Sporulation allows AM fungi to persist during adverse conditions and provides a means for colonization of new areas (Smith and Read, 2008). In disturbed areas or agricultural lands that have been planted with non-mycorrhizal crops, AM propagules in soils may be depleted or lacking. The influx of air, water or animal dispersed spores is the principal mechanisms for AM establishment in these areas (Peterson et al., 2004). Germination of dormant spores is induced by favorable environmental conditions, and while it may be enhanced by the presence of root exudates (see Miller and Oldroyd, 2012), the high germination of spores in my non-root wash receiving treatments further affirms that a plant-derived signal is not required for spore germination (Smith and Read, 2008). Relative to the controls, spore germination was significantly reduced by TCS exposure in both the root wash and non-root wash receiving treatments at 4.0 µg/L TCS, and in the non-root wash treatment at 0.4 µg/L TCS. The significant reduction in spore germination in the non-root wash treatments at 0.4 µg/L TCS and lack of a significant effect in the root wash treatment suggests that a compound(s) present in the root wash may overcome the inhibitory effects of TCS, however, further study is necessary to elucidate the mechanisms involved. While an increase in germination in the non-root wash treatments at 4.0 µg/L TCS compared to 0.4 µg/L TCS suggests a non-characteristic dose response, it is notable that both concentrations exhibited reduced germination compared to controls.

Following spore germination, and in the presence of plant signaling compounds, fungal hyphae will proliferate, undergo extensive branching and exhibit directional growth towards a suitable host (Giovannetti et al., 1994). In the absence of host photosynthates, axenic hyphal growth will cease due to limited storage reserves in the spores (Smith and Read, 2008). The

constant rate of increase in total hyphal length during this study showed hyphal growth was not limited by spore lipid reserves during the 21-day exposure period. On the contrary, in the controls, hyphal growth exhibited a linear increase over the 3-week period without indication of a rate reduction. In the control treatments and 0.4 µg/L TCS treatment, spores receiving a root wash exhibited more vigorous growth than those lacking a root wash, supporting the presence of a root signaling compound(s) in the root wash. Although reductions in hyphal length were observed within the first week in treatments receiving a root wash, by day 21, hyphal growth was significantly lower in all TCS treatments compared to controls. The sole study to this date that examined effects of pharmaceutical compounds on AM did not find an effect of TCS on hyphal growth at nominal concentrations up to 1000 µg/L (Hillis et al., 2008). In contrast to my study, Hillis et al. (2008) utilized a static, non-renewal design with nominal TCS concentrations delivered in an agar-based media (Bécard and Fortin, 1988). Since TCS concentrations were not monitored, bioavailability cannot be compared. In this study, the utilization of a 24-hour renewal of the exposure solution and delivery in artificial freshwater more accurately reflect exposure scenarios in wetland plants growing in water systems receiving wastewater treatment plant effluent.

The onset of extensive branching as hyphae approach a compatible host is well documented (Smith and Read, 2008). A fan-shaped complex forms from numerous small diameter lateral branches developing from the primary hyphae (Giovannetti et al., 1993). Root colonization is often effected by the lateral branches (Smith and Read, 2008), although, colonization from unbranched, thick walled hyphae has been noted (Nicolson, 1959). Strigolactone, derived from the carotenoid biosynthesis pathway (Matusova et al., 2005) has

been shown to induce hyphal branching in the AM fungus *Gigaspora margarita* (Akiyama et al., 2005). The mode of action of TCS on hyphal morphology is unclear, however, TCS induced reduction in root branching and reduced hyphal length in treatments lacking a root wash suggests direct mycotoxic effects; anti-fungal properties of TCS have been documented (Patel and Coogan, 2008), although the mechanism(s) for toxicity have not been identified. Interestingly, mycotoxic effects are exhibited at low concentrations of TCS (0.4 µg/L) and do not increase with increasing TCS concentrations. An effect of TCS exposure either directly on the signaling compounds present in the root wash, or on signal perception is evidenced by the lack of a significant root wash effect at 4.0 µg/L TCS, while in controls and 0.4 µg/L TCS, hyphal length and branching were significantly higher in the root wash treatments. The higher average branch length in the non-root wash treatments at all levels of TCS exposure is a reflection of the enhanced hyphal branching induced by the branching factors present in the root wash (Akiyama et al., 2005; Harrison, 2005). The increase in hyphal branches in the presence of root exudates led to a reduction in overall hyphal branch length.

Triclosan is widespread throughout US rivers and streams (Kolpin et al., 2002). At concentrations present in the water column, sediments and pore-water, TCS has been shown to affect plant morphology (Stevens et al., 2009), while field based studies have shown species specific differences in tissue locations and degree of bioaccumulation (Zarate et al., 2012). While Twanabasu et al. (2013) found a significant reduction in arbuscular mycorrhizal colonization in three wetland plant species at environmentally relevant concentrations; they did not identify which stage(s) in the colonization process was affected by TCS exposure. In this study, I have shown that at 0.4 µg/L TCS affects several aspects of the colonization process from

reducing spore germination to impacting hyphal growth and development. Impacts to fungal growth included mycotoxic effects as well as a reduced response to plant signaling compounds. AM fungi have been found in many major wetland habitats (Kandalepas et al., 2010; Stevens et al., 2010) and although their role is less well understood compared to terrestrial ecosystems, species specific differences in AM dependency have been found (Stevens et al., 2011). In terrestrial habitats arbuscular mycorrhizal fungi exert a significant influence over plant community composition and the ecosystem services provided by plant communities (Hartnett and Wilson, 1999). If they perform an equally substantial role in affecting wetland plant communities and the services they provide this role may be impaired by TCS exposure.

## 5.6 Conclusions

Triclosan has previously been shown to exert toxic effects on aquatic organisms including wetland macrophytes (Stevens et al., 2009), and inhibits AM colonization in wetland plants (Twanabasu et al., 2013) at concentrations within the range of those found in North American surface waters. Using exposure concentrations with and without a root wash treatment, this study has shown mycotoxic inhibition of the earliest stages of AM colonization, as well as a reduced response to root signaling compounds. Inhibition of spore germination, hyphal growth, and hyphal branching in non-root wash treatments indicates direct antifungal properties of TCS (Patel and Coogan, 2008). Reduced hyphal growth and branching in root wash treatments is indicative of an inhibitory interaction of TCS with signaling chemicals present in the root wash or interference of signal perception by AM. If these effects lead to reduced levels of AM colonization in wetland plants this could affect plant community structure

and ecosystem function. The detailed mechanism of TCS toxicity, interactions with ecological signaling mechanisms and resulting effects on plant communities and ecosystem functions require further study.

# CHAPTER 6

# SUMMARY

## 6.1 General Discussion and Conclusions

Mycorrhizal fungi are widespread throughout the plant kingdom, and are found to colonize more than 90% of the plants from all terrestrial habitats (Strack et al., 2003). They have been found to influence plant performance, community composition, and ecosystem services via nutrients and water uptake, increased photosynthesis, plant biomass production, increased resistance to plant diseases, and ameliorate climatic stresses (e.g. drought, salinity, and toxic metals) (Allen et al., 1981; Gildon and Tinker, 1983; Hartnett and Wilson, 1999; Rodriguez et al., 2003; Sheng et al., 2008). Extensive studies of mycorrhizal fungi in wetlands in past few decades have revealed that plants from many wetlands including degraded wetlands of Louisiana and bottomland hardwood forest in north central Texas harbor arbuscular mycorrhiza and dark septate endophytes (Radhika and Rodrigues, 2006; Kandalepas et al., 2010; Stevens et al., 2011). Although they are recognized as components in wetland ecosystems, their roles and the factors affecting them are poorly understood. Given the increased emphasis on wetland conservation and management, understanding the effects of natural and anthropogenic stressors on mycorrhizae in wetland plants may provide needed insight into the factors shaping wetland plant community structure and the ecosystem services they provide.

To explore the importance of AM in structuring and maintaining wetland ecosystem services and given the lack of information regarding the effects of natural and anthropogenic stresses on AM associations, this dissertation has focused on the effects of water quality,

hydrology, sedimentation, and a hurricane on AM fungal and DSE colonization in plant communities of degraded wetlands in Southeast Louisiana and the effects of an urban contaminant, triclosan, on pre- and post-colonization stages of AM fungi in three fresh water wetland plant species common in bottomland hardwood forest in north central Texas.

In agreement with previous studies (Rickerl et al., 1994; Stevens and Peterson, 1996; Miller, 2000; Escurado and Mendoza, 2005), hydrology was negatively correlated with levels of AM and DSE colonization; however, AM hyphal colonization in flooded treatments was increased by sedimentation. The results suggest that myccorhizal colonization and spore production are regulated by a more complex set of factors than simply water availability or nutrient availability alone as had been suggested by previous studies. Among the four environmental factors, hurricane exposure had the most dramatic and unexpected effects on AM and DSE colonization, which might be because, several other variables were manipulated during hurricanes such as flooding, increased salinity, sediment deposition, and plants exposure to hurricane wind resulting above ground vegetation damage. There was a clear indication of reduced spore density by sedimentation, which might be due to reduced spores in sediment added, germination of remaining spores, and reduced sporulation by AM fungi (Anderson et al., 1983; Harner et al., 2009). Higher DSE colonization in high salinity may be indicative of a greater role in high saline environments where AM fungi may severely be affected. Relatively high levels of AM and DSE colonization of *T. distichum* suggest a potential role of myccorhizae in the restoration of this commercially and environmentally important tree species.

One of the most frequently detected urban contaminants, triclosan, exhibited direct mycotoxic inhibition on spore germination at concentrations comparable to those found in

North American surface waters. Reduced hyphal growth and hyphal branching with and without root exudates have indicated mycotoxic effects as well as impairment in root fungal signaling. Therefore, the reduced hyphal and arbuscular colonization observed in three fresh water wetland plant species (Chapter III) might be due to mycotoxic as well as signaling inhibition of TCS on pre- and post-colonization stages. In support of the study by Hillis et al. (2010), this study indicates that AM colonization in natural freshwater wetlands receiving WWTP effluents is affected by the urban contaminants. The reduced AM and DSE colonization in wetland plants due to these natural and anthropogenic stresses including triclosan contamination could affect plant community structure and ecosystem functions in wetlands. The detailed mechanism of reduced colonization and its effects on plant community and ecosystem services, however, require further studies.

Although insightful, these studies are not without limitations. The mesocosm experiment was successful in creating varying habitats of Louisiana wetlands; however, a hurricane was simulated two and half years before samples were collected, the effects of which might not be fully reflected in the results. Hurricanes may have immediate effects on the above and below ground biotic communities (Hasselquist et al., 2010; Vargas et al., 2010) and/or long term effects on AM colonization as observed by Vargas et al. (2010) after two years of hurricane. Furthermore, samples from mesocosms were collected in January 2009, just before the growing season in Louisiana, which may limit our ability to compare to other studies collected at differing times throughout the growing season (i.e. Oliveira and Oliveira, 2005; Sivakumar, 2012). The flow-through exposure (chapter III) and static renewal exposure (chapter IV) systems exposing AM spores and colonization in three wetland plants were designed to

simulate exposure conditions in Trinity River in north central Texas. These systems may not reflect organic matter concentrations present in the sediments of natural freshwater wetlands, or TCS concentrations in the sediment pore water which can be an order of magnitude higher than the column water (Chalew and Halden, 2009). Given these limitations the results do suggest an impact of anthropogenic activity on mycorrhizal associations in wetlands that demands further studies. In addition to quantifying the impacts of TCS exposure on AM development and colonization in wetland plant species, my studies have shown that AM fungal colonization is a sensitive endpoint that can readily be included in ecotoxicological assessments of chemical contaminants (Hillis, 2009). Additional field and laboratory based studies with a more extensive array of wetland plants, fungal species, and anthropogenic stressors is required to fully elucidate the role of mycorrhizae in wetlands and the impacts human activities are having in this association.

## 6.2 Future Direction

More studies need to be conducted to further validate and implement the results of these experiments. This dissertation provides an insight to the effects of natural and anthropogenic stressors on AM and DSE colonization in plants from Louisiana swamps and Trinity River in controlled mesocosms and flow-through studies. To support these results, field based studies need to be conducted which provide a level of extrapolation for the effects upon a real ecological setting. Similarly, further impacts on the wetland plant communities and ecosystem services resulted by this reduced AM and DSE colonization require more investigations. To further elucidate the toxicity of TCS, more wetland plants species should be

included in similar studies with AM colonization. Likewise, effects of more PPCPs and other organic pollutants should be tested to explore AM response to these emerging contaminants. Simultaneously, molecular and genetic aspects of AM inhibition by TCS and other pollutants can be explored in order to understand the real mechanism of inhibition.

# APPENDIX A

# MESOCOSM EXPERIMENTAL DESIGN

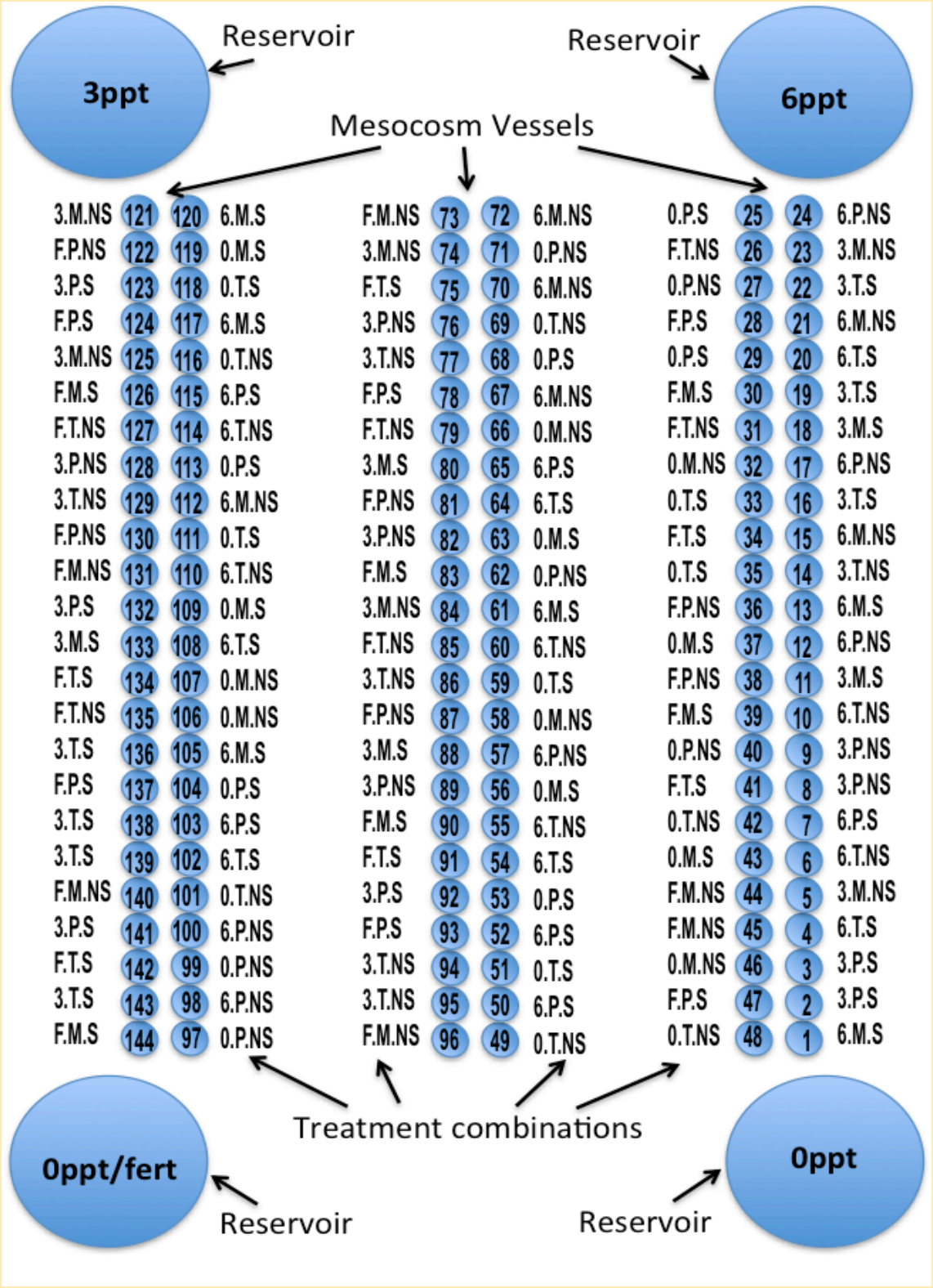


Figure A.1 Mesocosm experimental setup shematic


Figure A.2 Mesocosm experimental vessels

# APPENDIX B

# SIMULATION OF HURRICANE DEMETRA

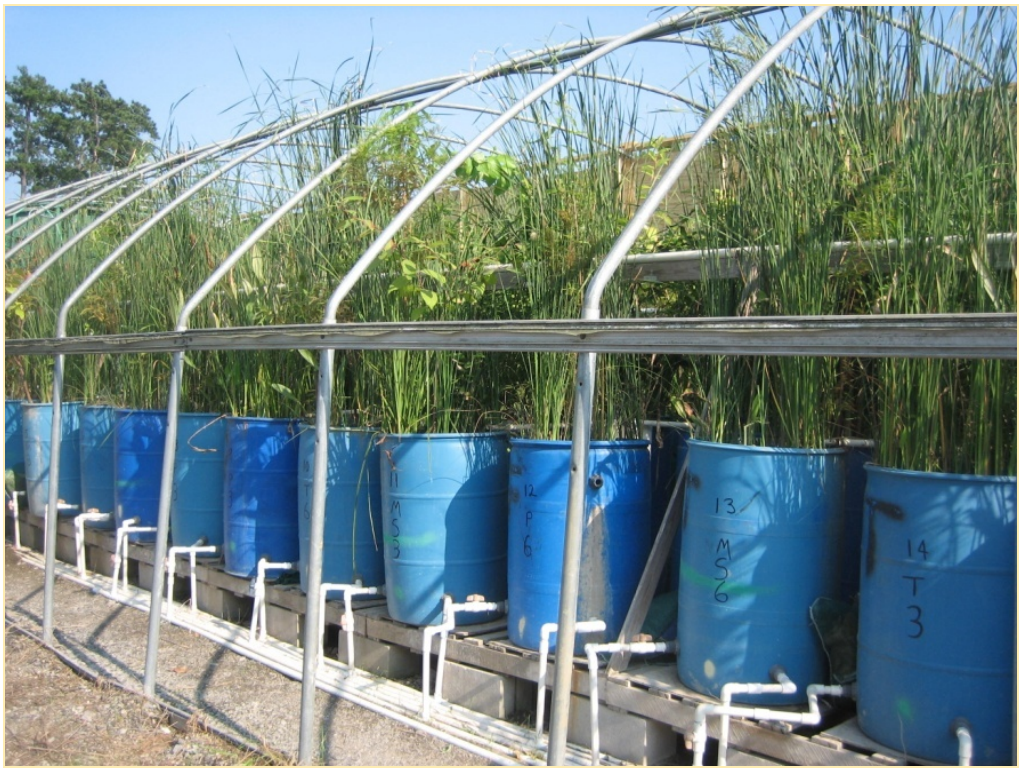


Figure B.1 Pre-hurricane mesocosms

Figure B.2 Hurricane simulation

Figure B.3 Post hurricane mesocosms

Figure B.4 Post hurricane one month later

# APPENDIX C

# THREE WETLAND PLANT SPECIES STUDIED FROM MESOCOSMS FOR QUANTIFICATION OF MYCORRHIZAL COLONIZATION

Figure C.1 a) *Typha domingensis*; b) *Panicum hemitomon*

Figure C.2 *Taxodium distichum*

# APPENDIX D

# THREE FRESHWATER WETLAND PLANT SPECIES INOCULATED WITH AM SPORES AND EXPOSED TO TCS IN FLOW-THROUGH EXPOSURE SYSTEM

Figure D.1 *Eclipta prostrata* (a) and *Hibiscus laevis* (b)

Figure D.2 *Sesbania herbacea*

# APPENDIX E

# FLOW-THROUGH EXPOSURE DESIGN SYSTEM

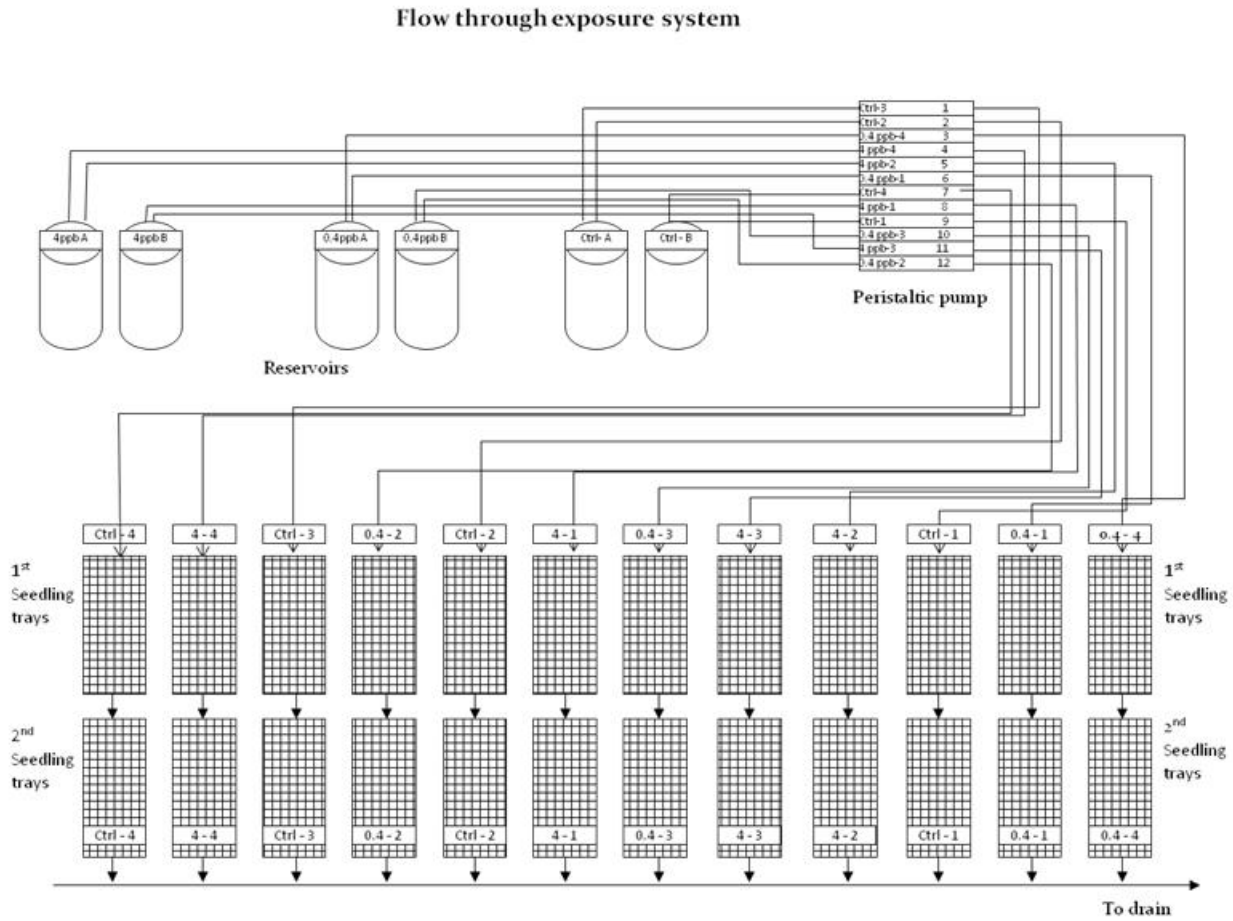


Figure E.1 Flow-through exposure system schematic design

Figure E.2 Seedling exposure trays before seedling transplant

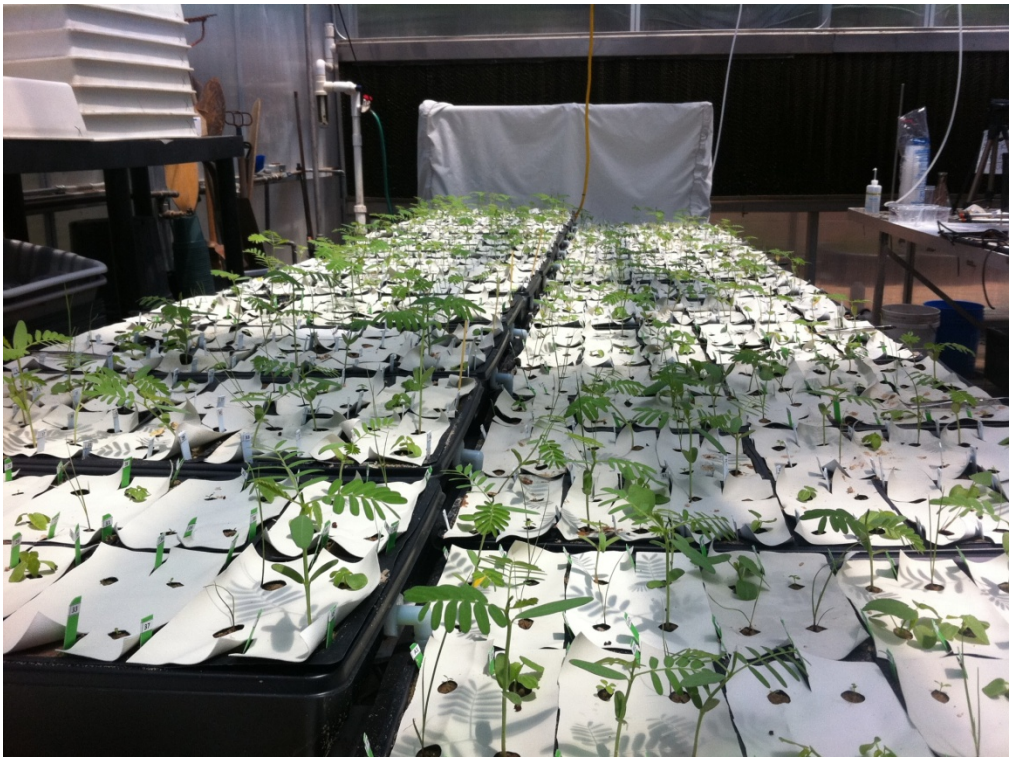

Figure E.3 Flow-through experimental setup after 15 days of seedling transplant

# APPENDIX F

# STATIC RENEWAL EXPOSURE SYSTEM

Figure F.1 Static-renewal exposure coplin jars with spore slides

Figure F.2 Spores in coplin jars in incubator

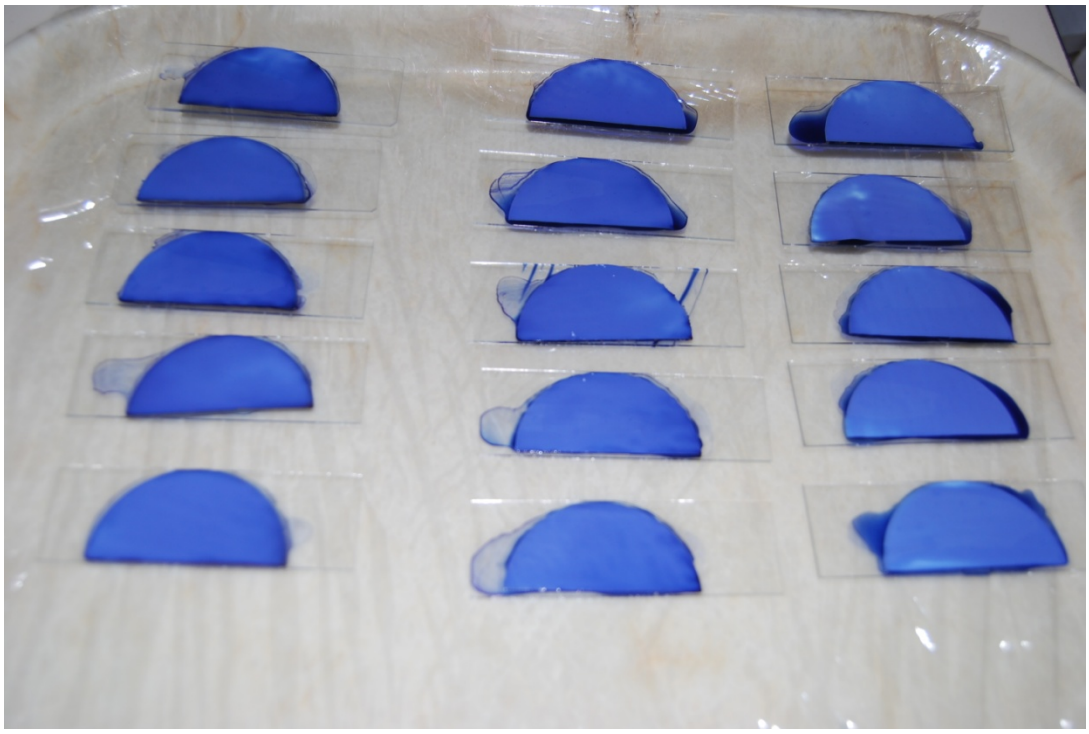

Figure F.3 Spores on filter membrane stained with trypan blue

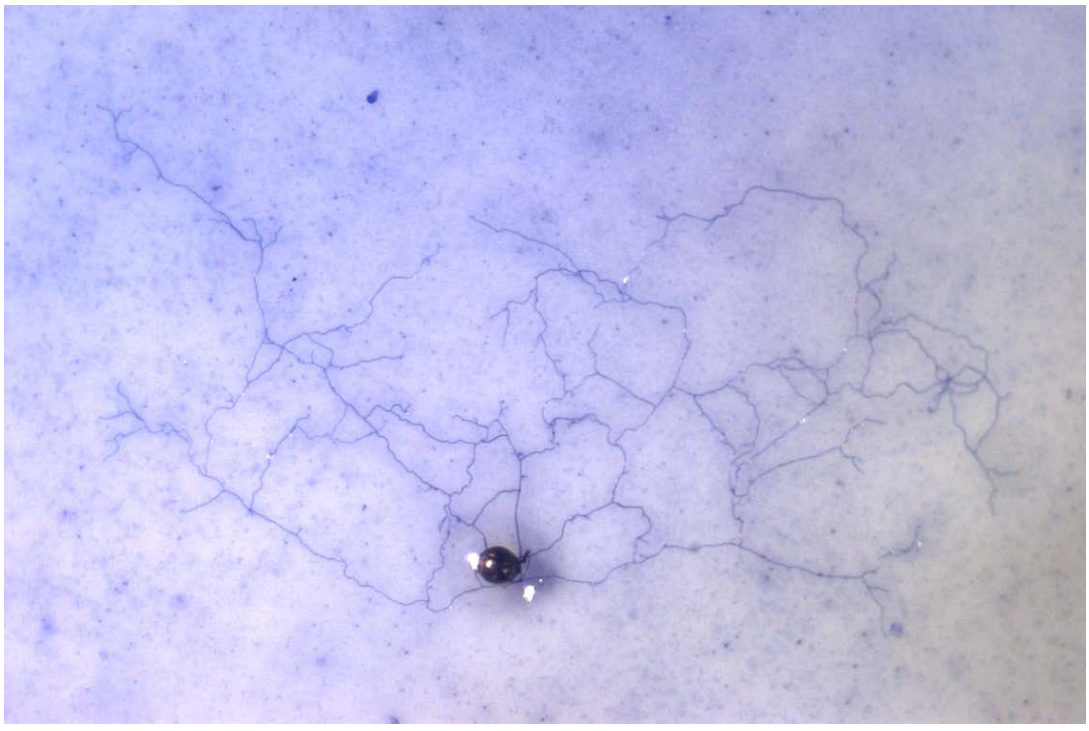

Figure F.4 A germinated spore photograph with hyphal growth and branching

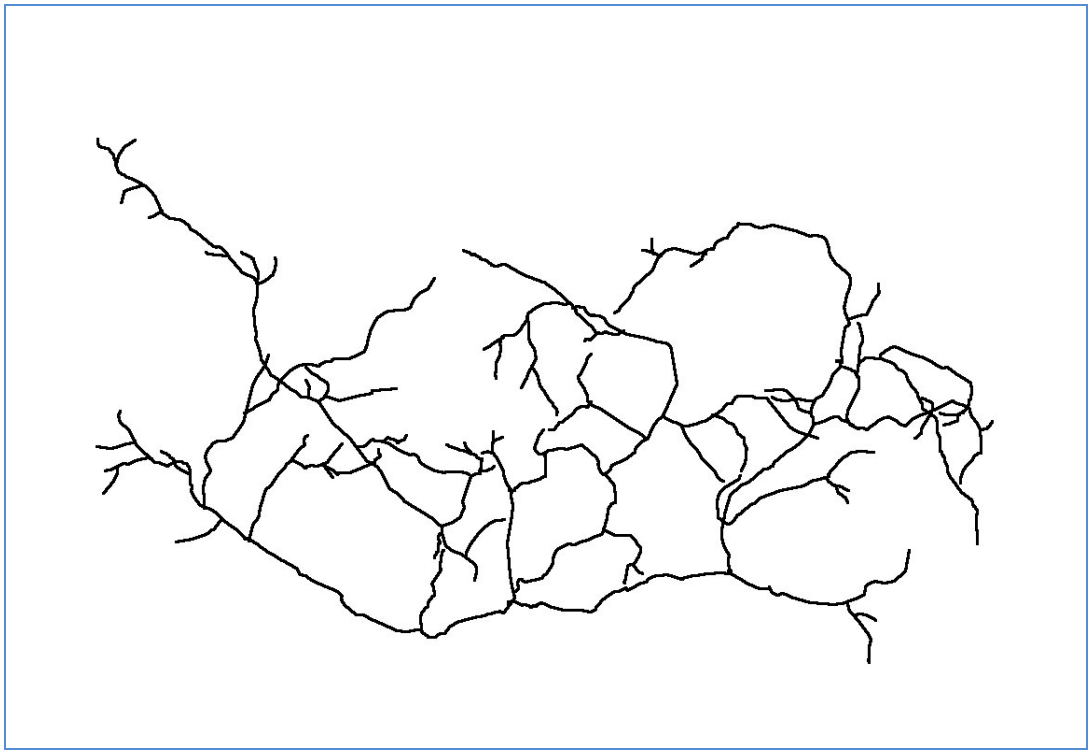

Figure F.5 Tracing of hyphal growth and branching by using magnetic tool in photoshop 2

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