RECYCLING NURSERY RUNOFF: UNDERSTANDING PLANT SENSITIVITY TO NUTRIENTS AND RESIDUAL PESTICIDES

By

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ABSTRACT

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Runoff generated from landscape nursery operations contains agrochemicals such as pesticides and fertilizers, which, if released off-site, may pollute the environment. Nursery producers are increasingly interested in alternatives to using freshwater for irrigation due to increased environmental awareness and reduced water availability. As a result, some progressive nursery growers are already adopting the practice of retaining and recycling nursery runoff water for irrigation. While retaining and recycling runoff may be a practical solution, growers' concerns about the potential negative impact of residual pesticides on crop growth and quality still impede its adoption. Therefore the objectives of my studies were to reduce the concentration of nutrients in runoff water and to evaluate the impact of irrigating with recycled runoff water on growth and physiology of nursery crops.

The first study was to identify minimum phosphorus concentration required for the optimum morphological and physiological performance in three common woody ornamental taxa; *Hydrangea quercifolia* (Queen of hearts), *Cornus obliqua* (Redtwig dogwood) and *Physocarpus opulifolius* (Seward). The optimum phosphorus concentrations for growth and photosynthetic biochemistry ranged between 4 and 7 $mg \cdot L^{-1}$, depending on taxa. For the second study, I investigated the response of common landscape nursery plants to residual pesticide commonly found in nursery runoff. *Hydrangea paniculata* (Limelight), *Cornus obliqua* (Powell Gardens), *Hosta* (Gold Standard) were exposed to low residual concentrations of isoxaben, chlorpyrifos and oxyfluorfen, simulating irrigation with nursery runoff. Exposure to oxyfluorfen produced

phytotoxicity symptoms (visual leaf damage), while chlorpyrifos and isoxaben did not produce phytotoxicity. Among the three taxa, *H. paniculata* was the most sensitive species, and *C. obliqua* was the most resistant. Therefore the effects of pesticides were pesticide-specific and taxa-specific. For the third study, I investigated whether phytotoxicity in response to residual herbicide exposure was dependent on the growth stage of plants. In this study, *H. paniculata* plants were exposed to a low residual concentration of oryzalin and oxyfluorfen at the various growth stages, starting shorty after bud-break. Residual herbicide exposure had more impact on growth and photosynthetic physiology at early growth stages; however, the recovery rate of those plants was also rapid. For my final study, I conducted three-year field research replicating an actual nursery grower practice of recycling nursery water. Six ornamental species were irrigated with recycled water obtained from a nursery bed receiving ten different pesticides. In addition, the efficacy of woodchip bioreactors to reduced pesticides in water was also tested. Results from this study established the possibility of using recycled water to irrigate ornamentals plants such as *Hydrangea macrophylla* (Let's dance blue jangles), *Hydrangea paniculata* (Limelight), *Thuja occidentalis* (American Pillar), *Juniperus horizontalis* (Blue rug), *Hydrangea arborescens* (Invincibelle Spirit II®) and *Rosa sp*. (Oso Easy Double Red®) without impacting the growth and physiology of those plants. Woodchips bioreactor was also found to be effective in remediating pesticides from water. The results of three greenhouse studies and a field study together provide new information on reducing the concentration of nutrients and pesticides in nursery runoff water and demonstrate the possibility of recycling nursery runoff. The findings of this dissertation are vital in solving the emerging problem of agrochemical pollution and water scarcity that is currently faced by nursery growers.

Dedicated to Suzzen for her relentless support and to my altruistic mom

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Irrigating Nursery Crops with Recycled Run-Off: A Review of Potential Impacts of Pesticides on Plant Growth and Physiology

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Abstract

Interest in capturing and reusing runoff from irrigation and rainfall in container nurseries is increasing due to water scarcity and water use regulations. However, grower concerns related to contaminants in runoff water and other issues related to water safety are potential barriers to the adoption of water capture and reuse technologies. In this review, we discuss some of the key concerns associated with potential phytotoxicity from irrigating container nursery crops with recycled runoff. The concentration of pesticides in runoff water and retention ponds is orders of magnitude lower than typical crop application rates, therefore the risk of pesticide phytotoxicity from irrigation with runoff water is relatively low. Nonetheless, some pesticides, particularly certain herbicides and insecticides, can potentially affect crops due to prolonged chronic exposure. Pesticides with high solubility, low organic adsorption coefficients, and long persistence have the greatest potential for crop impact as they are the most likely to be transported with runoff from container pads. Potential impact on plant growth or disruption of physiological processes differs among pesticides and sensitivity of individual crop plants. Growers can reduce risks associated with residual pesticides in recycled irrigation water by adopting best management practices (e.g., managing irrigation to reduce pesticide runoff, reducing pots spacing during pesticide application, use of vegetative filter strips) that reduce the contaminant load reaching containment basins as well as adopting remediation strategies that can reduce pesticide concentration in recycled water.

Keywords: Herbicides, Insecticides, Phytotoxicity, Nursery management

1. Introduction

Increasing water demand is exacerbating water scarcity worldwide. In a list of top 10 global risk factors published by World Economic Forum, inadequate water supply was the top risk factor in terms of impact and the eighth risk factor in terms of likelihood (World Economic Forum, 2015). Agriculture is the major use of freshwater withdrawals and accounts for 69% of global water withdrawal (FAO AQUASTAT, 2014). Fischer et al. (2007) estimates water requirements for irrgiation to increase by 45% globally in next 60 years.

Many parts of the United States are facing increasing drought severity and frequency. As a result, surface water sources are declining in many areas. For example, water flow in the Colorado River, which supplies water to around 40 million people, could diminish by 35% by 2050 (Johnson, 2013; Rajagopalan et al., 2009; Wines, 2014). With increasing drought and possible decline in water availability, horticultural producers need to find ways to improve irrigation efficiency including reusing runoff for irrigation. In 2015, irrigation accounted for 37% of total water use in the U.S. (Maupin, 2018). Horticulture is a major sector of U.S. agriculture and is expanding. In 2014, the sales of all horticultural crops in U.S. was estimated to be $13.8 billion, a $2 billion increase from 2009 (U.S. Department of Agriculture, National Agricultural Statistics Service, 2016). Ample irrigation is a crucial requirement for many horticultural crops, which has led to a continuous increase in water consumption since 2004 (Fulcher et al., 2016). In 2013, 556,490 acres out of 661,862 acres (84%) of horticulture operations were irrigated. These operations consumed 223 billion gallons of water, 65% of which was applied as overhead sprinkler irrigation (Vilsack and Reilly, 2013). Irrigation efficiency can be particularly low in container nursery production. In overhead irrigation systems, up to 80% of water applied may be lost as runoff, depending on container spacing and container size (Beeson and Knox, 1991; Mathers et

al., 2005). However, overhead or sprinkler irrigation remains the most common, reliable, and economical method of irrigating plants. A common nursery recommendation is to schedule irrigation to achieve a 15% leaching fraction (i.e., 15% of applied irrigation is leached through the bottom of containers). Allowing a portion of irrigation water to flow out of containers prevents the buildup of soluble salts in the container substrate. However, growers' application practices are often more liberal, such as applying 0.75 inch of irrigation per day, resulting in significant over-watering and runoff (Bailey et al., 1999; Warsaw et al., 2009).

1.1. Increase interest in water capture and reuse in nurseries

Container nursery production is one of the most intensive agricultural systems in terms of resource inputs. Nurseries produce high-value specialty crops in a relatively short period. To produce marketable plants and reduce the risk of crop failure, growers rely heavily on irrigation, fertilizers and pesticides. This can lead to significant losses of nitrogen (N), phosphorus (P) and potassium (K) through runoff (Andersen and Hansen, 2000; Broschat, 1995; Colangelo and Brand, 2001). Elevated concentration of N and P in water can cause eutrophication, dead zones and algal blooms (Conley et al., 2009; National Oceanic and Atmospheric Administration, 2017). Pesticide runoff in nursery production is also a common concern (Mangiafico et al., 2008, 2009). A 10-year survey of major streams and ground water found 97% of stream water and 61% of shallow ground water near agricultural areas had one or more pesticides present (Gilliom et al., 2006). Pesticides, even at low concentrations, may be detrimental to aquatic and terrestrial life. With rising water scarcity and increasing water pollution, the U.S. Environmental Protection Agency along with state and local regulatory agencies are limiting groundwater withdrawals for agriculture and, in some cases, mandating runoff capture and reuse (Beeson et al., 2004; Fulcher et al., 2016). Moreover, regulations aimed at reducing fertilizer and pesticide runoff will likely increase (Lin et

al., 2009). With this changing scenario, the nursery industry is obliged to consume less fresh water (Fulcher et al., 2016) and look for ways to capture and reuse nursery runoff.

1.2. Examples of water capture and recycle in nurseries

Capturing runoff water on-site in a containment or retention pond is often the best way to prevent potential environmental problems associated with nursery runoff (Fain et al., 2000). In nurseries that capture runoff, collected water may be recycled to irrigate plants, either with or without treatment. Capturing and recycling nursery runoff water protects water sources, reduces water costs and provides a constant water supply (Wilson and Broembsen, 2015). Initial investment cost to build recycled water systems can be high but are often subsidized by various agencies, which can offset the initial investment cost in a few years. For example, a major nursery in California was able to recover the cost of a water recycling system within one year based on savings associated with purchasing less water (Pitton et al., 2018). Therefore, more nurseries are capturing and recycling runoff water. In a survey of 24 greenhouses and nurseries across 11 states, nurseries met 33% of their daily water requirement during peak irrigation demand using recycled water (Meador et al., 2012). In a survey of 65 nurseries in Ventura County, California, the number of nurseries collecting runoff doubled in just 2 years (from 2004 to 2006), indicating a rapid adoption rate of runoff capture (Mangiafico et al., 2010). In a recent survey of 60 nursery and greenhouse producers in Virginia, 51 (77%) said that they would capture and collect runoff water (Mack et al., 2017). Similarly, in a 1998 survey of 24 nurseries on the Alabama coastline, 75% of the nurseries captured runoff in some way (Fain et al., 2000). Larger nurseries recycled 68% of total water applied while smaller nurseries, if they had a recycling pond, recycled 98% of their water (Fain et al., 2000). Out of 58 west-central Florida nurseries surveyed in a workshop in 2000, 20 reported collecting runoff (Gisele et al., 2006). Likewise, in a survey of 192 nursery growers

across the U.S., 43% said they used water from retention ponds as source of irrigation but were still concerned about the water quality (White et al., 2013).

1.3. Concerns about crop safety

Capturing and reusing nursery runoff can assure water security for nurseries and protect water resources, but its safety in terms of crop health is a concern for growers. This hinders adoption of water capturing and recycling technologies. Potential problems with irrigating nursery crops with runoff include water quality, introduction (or re-introduction) of fungal pathogens and potential damage to crops from contaminants, particularly pesticides, that may be present in runoff. Issues associated with pathogens in recycled water are discussed elsewhere in this issue (Parke et al., 2019). In this review, we will consider the potential for pesticides in runoff to impact the growth and physiology of nursery crops.

1.3.1. Water quality

Maintaining water quality is crucial for nursery producers. Electrical conductivity (EC), pH and alkalinity are major factors in determining irrigation water quality, but these factors may fluctuate in containment ponds. Nursery runoff water may have higher pH, EC and alkalinity than recommended (Lu et al., 2006a; Zhang and Hong, 2017). This may be due to leaching of soluble salts from containers or microbial activities in the pond. In a study on evaluating water quality of runoff flowing to nine different containment basins, pH of runoff was usually higher than the recommended pH of 6.8 (Copes et al., 2017). In a study on nutrients leaching at different irrigation and fertilizer rates, EC levels often fluctuated in leachate and were occasionally above 1 $dS \cdot m^{-1}$ which is slightly higher than recommended rate (< 1 $dS \cdot m^{-1}$) for irrigation water (Million et al., 2007; Will and Faust, 2010).

1.3.2. Pathogens (diseases)

Pathogens are usually the main concern when managing nursery runoff water. The presence of pathogens even in one production area can infest an entire collection pond; if water from the infected ponds is reused, inoculum can spread over an entire nursery. Plant pathogens are frequently detected in nursery runoff water and collection ponds (Bush et al., 2003; Ghimire et al., 2011; Junker et al., 2016; MacDonald, 1994; Pottorff and Panter, 1997; Werres et al., 2007), but this does not always translate to infection of plant material. Along with reusing contaminated nursery runoff water, reuse of dirty pots, lack of proper drainage and contact of pots with contaminated ground are the most common reasons behind the spread of pathogens such as *Phytophthora* sp. and *Pythium* sp. (Kong and Richardson, 2004; Parke et al., 2008).

1.3.3. Pesticides

Apart from water quality and diseases, growers are also concerned about pesticides when considering reuse of runoff water. Parween et al. (2016) extensively evaluated the effect of pesticides on grasses and agronomic crops. In this review, we evaluate how nursery crops are impacted by presence of residual pesticides in irrigation water. Most of the research reviewed relates to nursery production, but where information was limited, we reviewed other, related agricultural production systems.

When pesticides are applied in container nurseries, wide plant spacing may result in pesticide deposition to non-target areas between the plant containers. For example, in a study on methiocarb application efficiency on weeping fig (*Ficus benjamina*) and lady palm (*Rhapis excelsa*), 16% to 30% of pesticide granules landed in the spaces between containers (Wilson et al., 2005). Subsequent overhead irrigation can carry pesticides in runoff that ultimately is captured in

retention ponds. Pesticide runoff has been reported in various nursery operations (Keese et al., 1994; Riley, 2003; Wilson et al., 1996;). In a study by Gilliam et al. (1992), 80% of applied granular herbicide landed off-target when empty 2.8-L containers were spaced at 30 cm apart. Typically, only a small fraction of pesticides leach out of containers because of high pesticide retention properties, particularly adsorption, of most soilless substrates. Hence, the largest portion of pesticide runoff is due to pesticide application to non-target areas (Roseth and Haarstad, 2010). Properties of pesticides such as solubility, volatility and adsorption as well as nursery management practices such as irrigation method and timing, crop spacing and ground cover determine the quantity of pesticides in runoff water that eventually reach retention ponds. Briggs et al. (1998a), found isoxaben, thiophanate-methyl, trifluralin and chlorpyrifos in runoff water from container production systems. Species vary in their sensitivity to pesticides (i.e., a pesticide safe for one species may be potentially detrimental for other species), therefore plant sensitivity should be considered when decisions related to water recycling are made (Baz and Fernandez, 2002; Fernandez et al. 1999; Lu et al., 2006b; Moorman, 2011; Straw et al., 1996). Some of the common pesticides found in runoff water and retention ponds, along with their concentrations, are listed in Table 1.

2. Pesticides used in nursery production and potential for crop damage

In general, pesticides are compounds designed to control pests that damage crops. In nursery crops, the most widely used pesticides are herbicides, insecticides and fungicides. Nematicides and rodenticides may also be applied in certain cases. Pesticides are usually carbon based compounds and have functional groups that target specific sites in animal and/or plant metabolism to kill or inhibit their performance.

2.1. Herbicides

Herbicides are often the greatest concern among contaminants in nursery runoff because the pesticide target and crop are the same type of organism - plants. Herbicides can be pre-emergence or post-emergence. Pre-emergence herbicides are commonly used in container nurseries and are applied to prevent seed germination and weed emergence. Post-emergence herbicides are used to kill established weeds. If used on container plants, many post-emergent herbicides may injure established crop plants along with target weeds hence, they are rarely applied in containers except to control weeds in non-crop areas - walkways, aisles and ditches (Atland, 2014; Robbins and Boyd, 2011).

Mode of action refers to the specific mechanisms by which herbicides kill or suppress weeds. More than 20 different modes of action have been documented for commercially available herbicides (Duke and Dayan, 2015); out of those, herbicides with six different modes of action are commonly used in nurseries (Table 2) (Robbins and Boyd, 2011). The term site of action is more specific and defines where specifically an herbicide makes an impact (Table 2). For example, mode of action of oxyfluorfen is cell membrane disruption and its site of action is protoporphyrinogen oxidase (PPO) inhibition (Ross and Childs, 1996; University of Wisconsin-Extension, 2013).

2.2. Insecticides

Although the mode of action of insecticides targets insect metabolism, some insecticides can affect plants as well. Insecticides can reduce plant growth, mainly by inhibiting chlorophyll formation and interfering with photosynthetic reactions (Mishra et al., 2008; Parween et al., (2011a, 2011b, 2012). Chlorpyrifos, a commonly used insecticide, can alter chlorophyll concentration, affect leaf sugar content, inhibit chlorophyll formation and degrade chlorophyll

(Parween et al., 2011a, 2011b; Prasad et al., 2015). Insecticides belonging to the pyrethroid family and organophosphate family can stress plants, triggering production of free radicals (Bashir et al., 2007; Parween et al., 2012). Free radicals are highly reactive molecules that can damage cell membranes. Carotenoid content increased when plants were exposed to pyrethroid and organophosphate, as a response to an increase in free radicals (Prasad et al., 2015). Pyrethroid and organophosphate also produce reactive oxygen species and cause lipid/membrane peroxidation (Prasad et al., 2015). Superoxide dismutase, an antioxidant enzyme in plants, increased when plants were exposed to these pesticides (Parween et al., 2012; Prasad et al., 2015). Deltamethrin, a pyrethroid, inhibits formation of spindle fibers during cell division thus producing abnormal cells (Chauhan et al., 1986). Pyrethroids may also reduce photosynthetic light use (Rózsavölgyi and Horváth, 2008). Insecticides from organophosphate and carbamate families can inhibit nitrification, as nitrifying bacteria are sensitive to these insecticides (Lin et al., 1972). Imidacloprid can reduce seed germination (Dubey and Fulekar, 2011; Stevens et al., 2008) and deltamethrin can extend the vegetative cycle of plants (Fidalgo et al., 1993). Pyriproxyfen, fipronil, imidacloprid and thiamethoxam reduce the phosphorus solubilization activity of rhizosphere bacteria causing reduction in phosphorus available for plant uptake (Ahemad and Khan, 2011). Indole acetic acid (IAA) production from rhizosphere bacteria is also reduced by these pesticides, that may lead to reduction in cell elongation and growth (Ahemad and Khan, 2012). Oxydemeton-methyl and pirimicarb when combined with fungicides, mancozeb and flusilazol, reduced photosynthesis by inhibiting phosphorylation and adenosine triphosphate (ATP) formation (Untiedt and Blanke, 2004). Hence, insecticide presence in retention ponds has the potential to induce phytotoxicity.

2.3. Fungicides

Nursery managers often apply various fungicides to protect crops from a wide range of fungal diseases. While fungicides may be effective at controlling fungal diseases, they may also be phytotoxic to sensitive plants. (Chase, 2010; Getter, 2015). Fungicides may contain inorganic ingredients, such as copper and sulfur, or organic compounds, like metalaxyl or triflumizole. Both inorganic and organic compounds can induce phytotoxic effects on plants (Petit et al., 2012; Tjosvold et al., 2005). Fungicides may affect plant growth and performance by directly inhibiting photosynthesis or by degradation of ribulose 1,5-bisphosphate (RuBP) carboxylase (Van Assche and Clijsters, 1990). They also may slow regeneration of RuBP, reduce stomatal conductance, lessen stomatal opening and degrade photosystems (Nason et al., 2007; Xia et al., 2006). Fungicides can also oxidize and destroy membranes. Destroying membranes leads to reduction of electron transport reactions, altered source sink relations and reduction in pigments such as chlorophyll a, chlorophyll b and carotenoids (Benton and Cobb, 1997; Saladin et al., 2003; Vinit-Dunand et al., 2002). Fungicides have also been reported to reduce photochemical efficiency through the reduction in photochemical quenching (Dias, 2012). There are a few instances of increased photosynthesis and growth in response to relatively low doses of fungicide treatment (Saladin et al., 2003; Untiedt and Blanke, 2004).

3. Properties of pesticides that may affect phytotoxicity of runoff

Pesticides differ in their physical and chemical properties. These properties, ultimately, determine the potential for compounds to move with runoff water and cause phytotoxicity. Some of the basic properties of pesticides and how those properties determine the fate and potential

phytotoxicity of various pesticide compounds are considered below (adapted from National Pesticide Center).

3.1. Solubility

Solubility is the ability of a pesticide compound to dissolve in a solvent (water). It is measured in milligram of a compound per liter ($mg \cdot L^{-1}$) of solvent or parts per million (ppm). Pesticides with solubility lower than 10 $mg \cdot L^{-1}$ are considered to have low water solubility, while pesticides with solubility higher than 1000 $mg \cdot L^{-1}$ are highly water soluble. Highly soluble pesticides are likely to dissolve and move with runoff water to containment ponds where their concentration may build up. Pesticides like acephate (818,000 $mg \cdot L^{-1}$), glyphosate (1,050,000 $mg \cdot L^{-1}$) and mefenoxam (8400 $mg \cdot L^{-1}$) are highly water-soluble and may accumulate in retention ponds.

3.2. Adsorption

Adsorption or sorption coefficient (K_d) is a measure of how well compounds bind to soil particles. However, K_d does not take into consideration soil organic matter, which is the main sorbent of pesticides in container substrates. Therefore, the organic carbon-water coefficient (K_{oc}) is used to estimate pesticide adsorption of container media (Wauchope et al., 2002). Pesticides with a higher adsorption coefficient adsorb to substrate particles and ground surface. Hence, they are less likely to move with runoff water compared to compounds with a low K_{oc}. Within retention ponds, pesticides with high K_{oc} may also bind to sediment and are less likely to move with recycled irrigation. Bifenthrin (K_{oc} = 131,000 to 302,000), chlorpyrifos (K_{oc} = 7,000 to 25,000) and oxyfluorfen (K_{oc} = 8900) have high adsorption coefficients and are less likely to move with runoff

water. Regardless, some pesticides such as oxyfluorfen can cause crop damage even at a very low concentration, 0.01 $mg \cdot L^{-1}$ (Poudyal et al., 2018).

3.3. Persistence

Pesticide degradation occurs via various factors such as light, water, chemicals, microbes or plants. The half-life (DT_{50}) refers to the time required for a pesticide to reduce to half of the concentration initially applied. The half-life of pesticides varies depending on environmental conditions. Based on half-life, pesticides are classified as non-persistent (DT_{50} < 30 d), moderately persistent (DT_{50} = 31-90 d), and persistent (DT_{50} > 90 d) (Deer, 2004). Persistent pesticides have a greater potential to remain longer in retention ponds. With other factors remaining constant, a pesticide with a longer half-life (persistent) has a higher potential to cause phytotoxic effect on plants; although pesticides with short half-lives (non-persistent) can require frequent re-application that can increase their concentration in retention ponds. Pesticides such as isoxaben (2 to 6.6 months), oryzalin (1.4 to 4.4 months) and oxyfluorfen (1 to 6 months) may have long half-lives so, even after months they can still be present in retention ponds.

3.4. Volatility

Volatility is a measure of the potential of a compound to evaporate and is usually measured in millimeters of mercury (mm Hg). Pesticides with vapor pressure < 0.000001 mm Hg are less volatile whereas, pesticides with vapor pressure > 0.01 mm Hg are highly volatile. During pesticide applications, vapor from volatile pesticides may quickly drift to nearby non-target plants and may cause immediate phytotoxicity on sensitive species. Highly volatile pesticides are less likely to end up in runoff and be transported to a containment pond. Pesticides like chlorpyrifos (0.00002 mm Hg) and triflumizole (0.0000014 mm Hg) are volatile and may drift to nearby areas causing

injury to sensitive species. The volatility of pesticides, while important, is not constant and differs with environmental conditions and interactions with other chemicals. For example, volatilization may reduce the half-life of chlorpyrifos in surface water to as short as 0.3 d to 3.2 d. However, in a retention pond, this may extend to 1 to 2 months due to slower microbial transformation and lower pH of collected water (Meikle et al., 1983; Leistra et al., 2006; Lu et al., 2006a).

4. Factors affecting the potential for crop injury

Pesticide concentrations are low in nursery runoff water and even lower in retention ponds compared to labeled application rates. For comparison, the recommended rate of isoxaben application is around 4500 $mg \cdot L^{-1}$ (calculated using label rate), and the concentration found in retention pond is around 0.055 $mg \cdot L^{-1}$ (Wilson et al., 1996). A similar pattern is true for most pesticides, but irrigating with runoff can result in chronic plant exposure to pesticides. Long-term exposure to low concentrations of residual pesticides has potential to cause crop injury. This potential for damage depends on a series of factors including plant sensitivity, pesticide type, pesticide dose, length of pesticide exposure, and growth stage when plants are exposed.

4.1. Pesticide

Certain pesticides may be more likely to cause plant injury than others. Baz and Fernandez (2002) observed that isoxaben (4 ppm) was more damaging to semi-aquatic woody nursery plants than oryzalin (4 ppm), both in terms of growth and photosynthetic responses. Similarly, isoxaben (5 ppm) had a greater impact on growth and photosynthetic parameters of semi-aquatic herbaceous perennials compared to oryzalin (5 ppm) (Fernandez et al., 1999). In a large phytotoxicity trial by Mathers et al. (2012), phytotoxic damage was observed on rose (*Rosa* sp.) when sprayed with isoxaben (2.25 lb/acre) + oryzalin (0.8 qt/acre) but exposure to indaziflam (38.1 lb/acre) did not

cause damage. In the same study, combined application of flumioxazin (0.612 fl oz/acre) and oryzalin (25.85 fl oz/acre) injured compact euonymus (*Euonymus alatus*) and common purple lilac (*Syringa* sp.), but dimethenamid-P (13.4 fl oz/acre) + pendimethalin (0.04 qt/acre) did not induce phytotoxicity on compact euonymus (Mathers et al., 2012). In a fungicide study, ghent azalea (*Rhododendron daviesi*) exhibited phytotoxicity resembling sunburn and leaf lesions after 1 week of treatment with a sulfur-based fungicide (19 ppm) but other fungicides, propiconazole (0.088 $mL \cdot L^{-1}$) and trifloxystrobin (0.15 $mL \cdot L^{-1}$), did not produce phytotoxicity (Vea and Palmer, 2017a). In the same study, copper sulfate pentahydrate (0.655 $mL \cdot L^{-1}$) produced phytotoxic damage on flowering dogwood (*Cornus florida*) but chlorothalonil (0.36 $mL \cdot L^{-1}$) was completely safe (Vea and Palmer, 2017a). In a dose-response study including isoxaben (0 to 1.4 ppm), oxyfluorfen (0 to 0.02 ppm) and chlorpyrifos (0 to 0.4 ppm), panicle hydrangea (*Hydrangea paniculata*), silky dogwood (*Cornus obliqua*) and hosta (*Hosta* sp.'Gold Standard') were exposed to low doses of each pesticides. Out of three pesticides, only oxyfluorfen (0.005 to 0.02 ppm) caused phytotoxic damage (Poudyal et al., 2018). Neem oil extract (7 $mL \cdot L^{-1}$) reduced photosynthesis and growth of gerbera daisy (*Gerbera sp*), but insecticide abamectin (1.51 ppm) had no effect (Spiers et al., 2008). Insecticides such as cinnamaldehyde (1.494 $mL \cdot L^{-1}$) and pyrethrin (1.164 $mL \cdot L^{-1}$) were phytotoxic on spanish lavender (*Lavandula stoechas*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), st. john's wort (*Hypericum perforatum*), woolly thyme (*Thymus pseudolanuginosus*) and nutmeg thyme (*Thymus praecox*), but insecticides such as capsaicin (62.25 $mL \cdot L^{-1}$) and azadirachtin (3.75 ppm) were safe on those species (Cloyd and Cycholl, 2002).

4.2. Plant sensitivity

Plants often differ in their sensitivity to pesticides. In an evaluation trial of isoxaben (0.045 lb/acre) and pendimethalin (2 lb/acre), both compounds reduced plant height for winter

creeper (*Euonymus fortune* 'Emerald n Gold') but did not affect heller's japanese holly (*Ilex crenata* 'Helleri') (Regan and Ticknor, 1987). In a study by Bhandary et al. (1997a), oryzalin, oxyfluorfen and isoxaben were applied separately with irrigation water at either 1 or 10 ppm. Isoxaben reduced fresh root mass for heller's japanease holly at 10 ppm but did not affect dwarf gardenia (*Gardenia jasminoides radicans*). Oryzalin (10 ppm) and oxyfluorfen (10 ppm) reduced shoot fresh weight of fountain grass (*Pennisetum rupeli*) but did not affect shoot fresh weight of daylily (*Hemerocallis hybrid*), dwarf gardenia or heller's japanese holly. In a similar study, when dwarf gardenia and fountain grass were irrigated with 0.01, 0.1 and 1 ppm of oryzalin; 1 ppm reduced root and shoot weight for fountain grass but did not affect dwarf gardenia (Bhandary et al., 1997b). Isoxaben, even at the label recommended rate, caused serious phytotoxic damage on foxglove (*Digitalis purpurea*), purple coneflower (*Echinacea purpurea*), lamb's ears (*Stachys byzantine*) and false spirea (*Astilbe* sp.) but was safe on statice (*Limonium latifolium*), little bluestem (*Schizachyrium scoparium*) and red maple (*Acer rubrum*) (Vea and Palmer, 2017b). Application of oxyfluorfen + oryzalin (12 lb/acre) on woolly yarrow (*Achillea tomentosa*) cause stunted growth (70% to 80% growth reduction) but woolly thyme (*Thymus pseudolanuginosus*) was not affected (Staats et al., 1998).

4.3. Pesticide dose and exposure

Although pesticide concentrations in retention ponds are low compared to application rates, continuous irrigation even with low doses may build up and cause phytotoxic responses. Miticides such as phosmet (applied at 0.6, and 1.2 $g \cdot L^{-1}$) and vinyl dimethyl phosphate (applied at 0.6, 1.2, and 2.4 $g \cdot L^{-1}$) had dose-dependent effects on flowers of various cultivars of chrysanthemum (*Chrysanthemum* sp.) damage was only seen with at higher doses (Poe, 1970). In a study by Bhandary et al. (1997a), 1 ppm of oryzalin reduced fresh shoot weight of fountain grass by 13.5%

while 10 ppm reduced growth by 92.7%. Similarly, 1 ppm of oxyfluorfen reduced fresh shoot weight of fountain grass by 11.4% compared to 31.2% by 10 ppm of oxyfluorfen. In the same study, isoxaben at 1 ppm and 10 ppm increased root phytotoxicity (based on a 0 to 10 rating) by 37% and 56%, respectively. Indaziflam (herbicide) was applied at 200, 400, and 800 lb/acre to different ornamental plants and the application was repeated after 4 weeks. In smooth hydrangea (*Hydrangea arborescens* 'Invincibelle'), big leaf hydrangea (*Hydrangea macrophylla* 'Endless summer') and judd viburnum (*Viburnum X Juddii*) increasing the dose of pesticide increased phytotoxic damage, and repeated applications further exacerbated the damage (Mathers et al., 2012). Isoxaben application at the recommended rate (0.5 to 1 lb/acre) had no phytotoxic effect on butterfly bush (*Buddleia davidii*) or hairawn muhly (*Muhlenbergia capillaris*), but Isoxaben application produced phytotoxic symptoms when the dose was increased to 2 lb/acre (Vea and Palmer, 2017b).

4.4. Growth stages and plant parts

Sensitivity of nursery crops to pesticides is likely to vary depending on the growth stage of the plants at the time of application. However, research detailing the phytotoxic response of plants to pesticide application at various growth stages is limited and results have been variable. Generally, pesticide labels suggest avoiding pesticide application at the seedling or younger stage. In a study by Richardson (1972), phytotoxicity in sugarcane was higher when 2,4-D (3.3 kg/ha) was applied at later stages of growth. However, in sunflower, application of the herbicide (flumioxazin, 30 g/ha) at early stages of growth caused greater photosynthesis reduction and slower recovery from damage compared to application at later stages (Jursik et al., 2013). Chlordimeform (1.2 $g \cdot L^{-1}$), an insecticide, produced phytotoxic damage on younger leaves of

chrysanthemum but had no effect on older leaves. Cyhexatin (0.14 $g \cdot L^{-1}$), a miticide, produced phytotoxic damage on flowers of chrysanthemum, but foliage was not affected (Poe, 1970).

5. Mitigating risks from residual pesticides in recycled runoff

As indicated in the foregoing discussion, residual pesticides in recycled runoff can potentially damage nursery crops under certain conditions. Fortunately, there are several ways to reduce the risk of phytotoxicity arising from irrigation with recycled runoff. A complete discussion of mitigation technologies is beyond the scope of this review. Below we present a few examples of techniques to reduced or remediate pesticides in runoff. For a more complete review of mitigation technologies, readers are referred to a review paper by Majsztrik et al. (2017) and to the Clean WateR^3 website (www.cleanwater3.org/).

5.1. Pesticide dependent reduction

The potential for a pesticide to be present in runoff depends on the formulation of the pesticide. In a study on the effect of formulation on runoff of isoxaben and trifluralin, concentration of isoxaben in runoff was 25% to 61% greater when the product was applied as a granular formulation compared to a spray application (Briggs et al., 2002a). For trifluralin, formulation did not affect runoff concentration (Briggs et al., 2002a). The type of nursery bed liner can also affect pesticide runoff. When comparing nursery bed flooring, plastic ground cover had the greatest isoxaben runoff, followed by landscape fabric and then gravel. Runoff loss from both granular and sprayable formulation was higher for plastic and fabric compared to gravel (Wilson et al., 1995). Irrigation design is also effective in controlling pesticide runoff. Cyclic irrigation, where total water for each day is applied in short intermittent cycles, usually has less pesticide runoff compared to continuous irrigation (Briggs et al., 1998b). Using pesticides with low solubility, whenever

possible, can also reduce the concentration of pesticides in runoff (Riley, 2003). Plant shape and size, container size and spacing can also influence pesticide runoff. Minimizing plant spacing when applying pesticides can reduce non-target application. Herbicide application loss was 23%, 51% and 80% when spacing was 0, 8 and 12 inches between containers, respectively (Gilliam et al., 1992). Highly soluble and less volatile pesticides such as isoxaben and thiophanate-methyl have greater runoff potential compared to less soluble pesticides such as chlorpyrifos and trifluralin (Briggs et al., 1998a).

5.2. Constructed wetlands and vegetative buffer

A constructed wetland is a marsh designed to hold and treat runoff, while vegetative buffers are usually narrow strips of land established with plants in the path of runoff. Both of these systems reduce pesticide concentration through adsorption, microbial degradation, volatilization, infiltration and plant uptake (Newman, 2010). In a review of pesticide removal using constructed wetlands, pesticides such as organochlorine (97% removal) and organophosphate (94% removal) were almost completely removed while pyrethroid removal was around 80% (Vymazal and Březinová, 2015). Pesticide removal efficiency of constructed wetlands and vegetative buffer increases with pesticide K_{oc} value and runoff retention time (Stearman et al., 2003; Vymazal and Březinová, 2015). Both vegetative buffers and constructed wetland systems can reduce pesticide concentration in runoff anywhere from 50% to 99% (Otto et al., 2016) and are particularly effective at removing pyrethroids (Bennett et al., 2005; Budd et al., 2009). Runoff retention time for pesticide removal may vary from few hours to days depending upon the properties of pesticides (Vymazal and Březinová, 2015).

5.3. Sand Filters

Well-engineered sand filter systems have great potential to remove runoff pesticides (Hedegaard and Albrechtsen, 2015). A rapid sand filter can remove pesticides like mecoprop (MCPP), bentazone and glyphosate with a success rates varying from 7 to 85% (Hedegaard and Albrechtsen, 2014). Removal of pesticides such as trifluralin, fenitrothion and endosulfan have also been achieved using sand filters (Aslan, 2005). The microbial community on the top layer of slow sand filter can degrade pesticides, thus, creating an effective pesticide removal system (Escolà Casas and Bester, 2015; Samuelsen et al., 2017).

5.4. Activated carbon filters (ACF) and filter socks

Activated carbon is a positively charged substrate that can adsorb polar or negatively charged pesticides. Efficacy of carbon filters depends on the type of carbon filter material used, temperature and flow rate of the filtration system. Activated carbon filters effectively remove agricultural pesticides (Hetrick et al., 2011; Jusoh et al., 2011; Kabashima et al., 2004; Martín-Gullón and Font, 2001) as runoff water passes through the carbon filter. Removal efficiency may be as high as 99.5% for organic compounds and negatively charged ions (Majsztrick et al., 2017). Carbon filters with granular activated carbon completely removed acephate and paclobutrazol when the contact time was 64 s. The same system also removed bifenthrin, chlorpyrifos, imidacloprid and glyphosate by 72.2%, 89%, 85.3% and 99% respectively (Grant et al., 2019).

Filter socks are long tubes made of mesh material that are commonly employed to intercept sediment carried in runoff. In a low-flow system, fill material such as wood chips, can be used to remove pesticides from runoff water, but in a high-flow system, filter socks may not be very effective (Majsztrick et al., 2017; Roseth and Haarstad, 2010). Different substrates like pine bark,

sphagnum moss, peat, sand and compost are effective filler material for pesticide removal. In a study testing the efficacy of substrate for removal of 21 different pesticides, pine bark was the most efficient and removed nearly 100% for 20 different pesticides. Peat removed nearly 100% of 16 different pesticides (Roseth and Haarstad, 2010). Substrates with high adsorption coefficient such as pine bark will have higher removal efficiency (Roseth and Haarstad, 2010). In a study by Shipitalo et al. (2010), filter socks filled with compost were effective in removing sediments and agrochemicals such as alachlor (18% removal) and glyphosate (5% removal) from surface runoff.

Along with above listed strategies, additional best management practices can help manage pesticides in nursery runoff (Southern Nursery Association, 2013).

6. Conclusion

Managing water resources is a major concern for nursery growers throughout the U.S. Growers in many regions face the prospect of increasing scrutiny and regulation by environmental agencies. Therefore, nursery growers are looking for alternative ways to meet crop water demand. Capturing and reusing runoff water may be an option to cope with water shortages and environmental regulations, but the risk associated with pesticide phytotoxicity may hinder grower adoption of recycle and reuse technologies. Pesticides can cause phytotoxic responses by interfering with metabolic processes of plants including inhibiting tubulin formation, inhibiting chlorophyll formation, penetrating lipid membranes and more. This interference leads to reduction in plant photosynthesis and growth. However, pesticide concentrations in remediation ponds is typically several orders of magnitude lower than the application rate, due to dilution from irrigation and rainwater (Camper et al., 1994), greatly reducing potential phytotoxicity to nursery crops. Nursery growers need to be cautious when irrigating with captured runoff that may contain

herbicides and certain insecticides. Herbicides such as oxyfluorfen may be phytotoxic at very low concentration. Plants, such as rose, may be sensitive to numerous pesticides. Even with low pesticide concentrations, repeated application may build up pesticides and cause phytotoxic symptoms.

Among pesticides, herbicides typically pose the greatest risk because of the similarity between pest controlled (weeds) and the crop plant. Nonetheless, insecticides and fungicides may also affect nursery crops; fungicides containing copper sulfate may cause phytotoxic responses. Other factors to consider include pesticide solubility, adsorption potential and half-life of pesticides. Pesticides with high solubility, low adsorption to organic matter and a long half-life are more problematic as they have a higher tendency to be carried in runoff and also degrade very slowly. The growth stage of plants is also important when considering potential phytotoxicity. Exposure to pesticides at early stages of growth may have greater potential for injury but research with growth stage and phytotoxicity is very limited with nursery crops.

The best strategy to prevent pesticide phytotoxicity is to minimize pesticide movement to retention ponds. Reducing container spacing during pesticide application can reduce off-target pesticide loss. Using less soluble pesticides will lower the potential for pesticides to be in recycled irrigation water. Creating vegetative buffer zones and using filter socks to trap pesticides reduces pesticide movement and accelerates their degradation. All of these techniques lower the concentration of pesticides ending up in retention ponds. Therefore, capture and reuse of runoff for irrigation may be a viable and sustainable option for nursery growers, helping them deal with water scarcity and environmental issues.

APPENDIX

APPENDIX

Table I - 1. Pesticides detected in irrigation runoff and retention ponds from container nursery production sites. Reported concentrations are for the maximum amount detected and always occurred during first flush of runoff.

Pesticide detected	Concentration[z]	Water sampled	Citation
Metolachor	7.8 ± 3.6 ppm	Irrigation runoff	Mahnken et al., 1999
Simazine	2.2 ± 0.7 ppm	Irrigation runoff	
Trifluralin	0.08 ppm	Irrigation runoff	Wilson et al., 1996
Isoxaben	0.75 ppm	Irrigation runoff	
Trifluralin	5.00 ppb	Containment pond	
Isoxaben	55.0 ppb	Containment pond	
Oxyfluorfen	4.9 ppb	Irrigation runoff	Goodwin et al., 2001
Oryzalin	43 ppb	Irrigation runoff	
Oxyfluorfen	< 1.00 ppb	Containment pond	
Oryzalin	< 1.00 ppb	Containment pond	
Bifenthrin	10.6 ppb	Runoff near production area	Kabashima et al., 2004
Cis-permethrin	24.6 ppb	Runoff near production area	
Trans-permethrin	4.4 ppb	Runoff near production area	
Bifenthrin	0.03 ppb	Irrigation runoff	Mangiafico et al., 2009
Chlorpyrifos	0.12ppb	Irrigation runoff	
Diazinon	0.02 ppb	Irrigation runoff	
Trifluralin	0.17 ppm	Irrigation runoff	Warsaw et al., 2012
Metalaxyl	2.19 ppm	Irrigation runoff	
Oxyfluorfen	< 0.10 ppm	Containment pond	Riley et al., 1994
Pendimethalin	4 ppb	Containment pond	
Oxyfluorfen	< 0.01 ppm	Retention pond	Camper et al, 1994
Pendimethalin	4 ppb	Retention pond	
Oryzalin	0.16 ppm	Retention pond	
Chlorpyrifos	1.59 ppb	Water entering retention pond	Mangiafico et al., 2008

Table I - 1 (cont'd)

Diazinon	17.4 ppb	Water entering retention pond	
Bifenthrin	20.6 ppb	Water entering retention pond	
Cypermetherin	2.00 ppt	Water entering retention pond	
Thiophanate-methyl	1.64 ppm	Irrigation runoff	Briggs et al., 2002b
Metalaxyl	61.0 ppm	Irrigation runoff	
Chlorothalonil	0.95 ppm	Irrigation runoff	
Isoxaben	2.20 ppm	Irrigation runoff	Briggs et al., 2003
Oryzalin	3.80 ppm	Irrigation runoff	

[z] 1 ppm = 1 $mg \cdot L^{-1}$, 1 ppb = 1 $\mu g \cdot L^{-1}$, 1 ppt = 1 $pg \cdot L^{-1}$

Table I - 2. Common herbicides applied in nurseries and their effect on plants

Site of action	WSSA group[z]	Effect on plants	Common name	Citation
5-enolpyruvyl-shikimate-3-phosphate (ESPS) synthase inhibitors	9	These herbicides are absorbed through foliage and translocated through phloem. They inhibit synthesis of 5-enolpyruvylshikimate-3-phosphate (EPSP). EPSP is a key enzyme in shikimic acid pathway that produces amino acids; tryptophan, tyrosine and phenylalanine. Inhibition of this enzyme cease essential amino acid formation, causing plant death	Glyphosate	(Shaner, 2006)
TIR1 (transport inhibitor response 1) auxin receptor/ synthetic auxin	4	These herbicides are absorbed through foliage and root. They trigger production of 9-*cis*-epoxycarotenoid dioxygenase (NCED) which in turn up-regulates abscisic acid production. Abscisic acid causes senescence, inhibition of cell division and elongation and stomatal closure. These herbicides also trigger 1-aminocyclopropane-1-carboxylic acid (ACC) biosynthesis, which cause senescence related symptoms.	Dicamba, 2-4 D	(Grossmann, 2007, 2010)
Photosystem II inhibitors	5	In photosystem II, electron flows through the number of sites, including the movement from Q_A to Q_B (plastoquinones) which is mediated by D1 protein. Herbicides with this mode of action bind with D1 protein of thylakoid in electron transport chain blocking the movement of electron through plastoquinones (Q_A to Q_B). This ceases the production of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and (ATP) and reduces the rate of photosynthesis. Choked electron transport chain also cause oxidative stress leading to lipid membrane destruction and disintegration. These class of herbicides are absorbed through root and shoot.	Simazine, Atrazine	(Nakajima et al., 1996; Lambreva et al., 2014)

Table I - 2 (cont'd)

Protoporphyrinogen oxidase (PPO) inhibitors	14	Protoporphyrinogen oxidase (PPO) enzyme oxidizes protoporphyrinogen IX to protoporphyrin IX. Protoporphyrin IX is the precursor for chlorophyll and heme synthesis. PPO inhibitor blocks synthesis of PPO enzyme leading to reduction in chlorophyll formation. This ultimately reduces photosynthesis. Increased accumulation of protoporphyrinogen IX, in presence of light, reacts with molecular oxygen to produce reactive oxygen species (ROS). ROS are very unstable and destroy cell membranes causing cell leakage.	Oxyfluorfen	(Lee and Duke, 1994)
Long-chain fatty acid (LCFA) inhibitors	15	This class of herbicide inhibits formation of long-chain fatty acid (LCFA) by reducing incorporation of stearic acid, malonic and acetate in the chain. It also inhibits formation of enzymes required for elongation of LCFA. LCFA inhibitors also inhibit LCFA incorporation into cell wall. LCFA being essential component of plasma membrane, inhibiting its formation will kill cell and plants.	Acetochlor, Metolachlor	(Schmalfuß et al., 1998; Matthes et al., 1998)
Microtubule assembly inhibitors	3	This class of herbicide binds with free tubulin. Tubulin synthesizes microtubules, which is essential for cell division. When herbicide binds to tubulin, tubulin cannot synthesize microtubules; hence, the cell is arrested in dividing stage. Symptoms of injury include swollen root tips.	Pendimethalin, Trifluralin, Oryzalin	(Sandmann et al., 1980)

[Z]WSSA = Weed Science Society of America

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SECTION II

PHOSPHORUS REQUIREMENT FOR BIOMASS ACCUMULATION IS HIGHER COMPARED TO PHOTOSYNTHETIC BIOCHEMISTRY FOR THREE ORNAMENTAL SHRUBS

Phosphorus requirement for biomass accumulation is higher compared to photosynthetic biochemistry for three ornamental shrubs

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Abstract

Ornamental nursery producers grow a variety of plant species and rely heavily on water and nutrient applications to maximize plant growth and quality. We conducted the current study to understand the morpho-physiological basis of plant response to phosphorus (P) concentration and identify optimum phosphorus concentration required for three common woody ornamental taxa; *Hydrangea quercifolia* Bartr. 'Queen of Hearts', *Cornus obliqua* Raf. 'Powell Gardens' and *Physocarpus opulifolius* Maxim. 'Seward'. In a greenhouse experiment, all plants were watered with a complete nutrient solution that varied only in P concentration (0, 1, 2, 4, 6, 8 $mg \cdot L^{-1}$). For total dry biomass growth, the optimum P concentration was close to 7 $mg \cdot L^{-1}$ for all three taxa. However, *P. opulifolius* required less phosphorus for maximum growth index (plant height + plant width in two directions) compared to *H. quercifolia* and *C. obliqua*. Phosphorus concentration below 4 $mg \cdot L^{-1}$ reduced leaf size and resulted in greater partitioning of biomass and phosphorus to root growth. Analysis of responses of photosynthesis to intercellular carbon dioxide (*A/Ci* curves) indicated a continuous increase in photosynthetic parameters to increasing phosphorus concentrations. Rate of rubisco for carboxylation (V_{cmax}), RuBP regeneration rate *(J)* and rate of triose phosphate use (*TPU*) limited photosynthesis in phosphorus-deficient plants for all three taxa. However, P requirement for photosynthesis biochemistry was less compared to growth. Light-harvesting potential (Fv'/Fm') for all three taxa was least sensitive to P requirement. Optimum P concentrations for growth and photosynthetic biochemistry ranged between 4 and 7 $mg \cdot L^{-1}$, depending on taxa. These P concentrations are lower than common recommendations and less than the amounts provided by typical commercial fertilizers. Application of phosphorus above 7 $mg \cdot L^{-1}$ is above that needed for growth and physiological function and could contribute to phosphorus runoff from nurseries.

Keywords: A/Ci curves; gas exchange; nursery management; phosphorus partitioning

1. Introduction

Horticulture is a large and economically significant industry, both in the U.S. and around the world. In the U.S., the sale of horticultural crops was worth $13.8 billion in 2014 (United States Department of Agriculture National Agricultural Statistics Service, 2016). Landscape nursery production is one of the major sectors of horticulture and requires frequent, usually daily, irrigation and continuous additions of mineral nutrients when the crops are actively growing. For container-grown ornamentals, growers seek to optimize irrigation by applying enough water to meet evapotranspiration loss while leaching out deleterious accumulated soluble salts. A commonly cited best management practice (BMP) is to irrigate to the level that results in 10-20% leaching of applied water (leaching fraction) (Bilderback et al., 2013) although even this recommendation may lead to over-irrigation (Pershey et al., 2015). Nonetheless, in practice, application of water often exceeds the BMP, and a large portion of irrigation water may be lost as agrichemical laden irrigation return flow (Warsaw et al., 2009; Danelon et al., 2010). Container nursery crops in the U.S. are typically grown in soil-less media, composed primarily of softwood bark. Phosphorus leaching is higher in soil-less media (pine bark, sphagnum peat, vermiculite or sand) in comparison to regular soil (Broschat, 1995) due to inherent phosphorous load from the bark itself along with poor retention due to low anion exchange capacity and preferential flow through the porous substrate when irrigated (Owen et al., 2008; Fields et al., 2014). Thus, 30-60% of applied P is commonly leached when using bark based substrate (Newman, 2014). This leachate and resultant irrigation return flow can pollute surface and groundwater systems (United States Environmental Protection Agency, 2005, 2016).

Water quality concerns associated with nursery and greenhouse irrigation return flow, such as eutrophication and algal blooms, are primarily related to nitrogen and P present in runoff water

(Conley et al., 2009; Paerl, 2009; Fulcher et al., 2016). In 2014, P runoff was the primary cause of harmful algal blooms (HABs) in Lake Erie that left more than half a million people without drinking water (Michalak et al., 2013; Watson et al., 2016). Lowering P fertilization is an option for nursery growers to protect water resources, but lowering P below the sufficiency threshold will reduce plant growth and quality. For container-grown ornamentals, 5 to 10 mg·L-1 of substrate extractable P is often considered the target level for optimum plant growth (Broschat and Klock-Moore, 2000; Ristvey et al., 2004; Zhang et al., 2004; Bilderback et al., 2013) but many liquid and control release fertilizers commonly used in the landscape nursery trade provide 15 to 50 mg·L-1 of P when applied at labeled rates (Broschat, 1995; Soti et al., 2015). Phosphorus requirements of plants vary by taxa, and recent studies suggest the possibility of reducing P below 10 mg·L-1 without compromising plant growth and quality (Shreckhise et al., 2018, 2019b). For example, Hydrangea paniculata 'Limelight' and Rhododendron 'Karen' grown in pine bark substrate, achieved maximum shoot dry weight at 4.7 mg·L-1, and 2.9 mg·L-1 of P, respectively. For Lantana camara 'New Gold' grown in a mixture of perlite and vermiculite, P concentration of ≤ 10 mg·L-1 was sufficient for optimum growth at the reproductive stage of the plant (Kim and Li, 2016). In contrast, Impatiens hawkeri Bull. `Paradise Violet', and Catharanthus roseus `Pacifica Red' Vinca grown in soil-less substrate had maximum growth and shoot dry weight at 31 mg·L-1 and 23 mg·L-1 of P application (Whitcher et al., 2005). Increasing P also increases the shoot-to-root ratio by increasing shoot mass (Biddinger et al., 2019), as seen in L. camara, where increasing P from 3 mg·L-1 to 5 mg·L-1 increased shoot-root ratio (Kim and Li, 2016).

The impacts of P availability on growth reflect the integration of its effect on physiological processes, particularly photosynthetic biochemistry. Phosphorus is an essential plant nutrient that is present in plants in various membranes, nucleic acids, and energy compounds (Armstrong,

1999). The effects of P deficiency on growth may be due to both source and sink limitations on photosynthesis (Pessarakli, 2005). Phosphorus is required for numerous physiological processes, including light reactions and Calvin-Benson cycle of photosynthesis (Brooks, 1986; Poorter et al., 2010). Phosphorus deficiency reduces photosynthesis by limiting RuBP regeneration (Fredeen et al., 1990), inhibiting ATP synthesis (Carstensen et al., 2018), inhibiting enzymes required in the Calvin-Benson cycle (Rao and Terry, 1989) and reducing stomatal conductance (Martins et al., 2015). Phosphorus deficiency also reduces chlorophyll fluorescence (Nowak and Stroka, 2001) by lowering the efficiency of PSII, and damage the photosynthetic apparatus by increasing the production of free radicals (Xu et al., 2007). Physiological impacts in response to P are predominantly determined by the severity of P deficiency and the length of P starvation (Terry and Ulrich, 1973; Xu et al., 2007).

Improving our understanding of the interrelationships between P effects on photosynthetic biochemistry and plant productivity may provide additional insights in optimizing P fertilization to maximize growth while minimizing adverse environmental impacts. To date, holistic studies optimizing P fertilization for maximum physiological and morphological performance of plants, and quantifying benefits of lowering P in terms of P runoff are still lacking. Therefore, the goal of our study was to investigate the feasibility of reducing P fertilization without reducing crop growth or quality and linking the benefits of lowering P to water quality preservation. Our specific objectives were to (1) determine the effect of P on photosynthesis (A/Ci curves, and light-adapted fluorescence) and morphological responses (growth index, total dry weight, root-shoot ratio, leaf number, and leaf size) in three different ornamental plant taxa, (2) identify the type of photosynthetic limitation that may result from P deficiency, and (3) categorize P partitioning to plant growth and P runoff.

2. Materials and Methods

2.1. Experimental Setup

The experiment was conducted in a research greenhouse at Michigan State University, East Lansing, MI, USA . *H. quercifolia* Bartr. ('Queen of Hearts' oakleaf hydrangea), *C. obliqua* Raf. ('Powell Gardens' Red Rover® silky dogwood) and *P. opulifolius* Maxim. ('Seward' Summer Wine® ninebark) grown in 11.36 L containers were used for the study. All the plants were planted as liners from 10-cm diameter plug cells on May 6, 2016, in a mixture of aged pine bark (85% volume) and peat moss (15% volume) (Renewed Earth LLC, Otsego, MI, USA) without any amendments. Before the current study, plants were grown outdoors under typical nursery practices for the region; 19 mm of daily overhead irrigation and top-dressed with controlled-release fertilizer (19:1.75:6.65; N:P:K) with micronutrients (5-6 months release rate, Harrell's LLC, Lakeland, FL). Plants were brought into an unheated hoop-house covered with 0.15 mm poly film on the October 28, 2016, where they received partial chilling outdoors. Pots were carefully checked for residual fertilizer prills, which were removed even though it was already past the six months fertilizer release period. Plants were then moved to a walk-in cooler (6° C) for five weeks to complete their chilling requirement. Plants were brought into the greenhouse on January 11, 2017 and observed until bud-break. The temperature in the greenhouse was set to 22°C for 18 hours (6:00 to 24:00) and 20°C for the remaining 6 hours. Supplemental lighting was provided by high-pressure sodium lamps in the greenhouse that automatically turned on when the photosynthetic photon flux density was lower than 440 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and provided a 16-h photoperiod.

2.2. Phosphorus treatments

Fertigation started on January 26, 2017. Phosphorus was applied as potassium phosphate (potassium phosphate dibasic, Sigma-Aldrich, St. Louis, Missouri) mixed in irrigation water (fertigation). Plants received one of six P treatment concentrations (0, 1, 2, 4, 6, or 8 $mg \cdot L^{-1}$). Each treatment had six individual plant replications per taxa. After the emergence of leaves, each plant was hand watered with a solution consisting 100 $mg \cdot L^{-1}$ of nitrogen (urea), 60 $mg \cdot L^{-1}$ of potassium (muriate of potash), 80 $mg \cdot L^{-1}$ of micronutrients (Table 4 - Micromax® micronutrients, ICL Fertilizers, Dublin, Ohio) and the designated rate of P. The fertilizer solutions were precisely measured for each irrigation event and completely dissolved in irrigation water before application. After each fertigation event, leachate was collected in saucers placed under each container and measured for volume. Irrigation amounts were routinely adjusted to account for changes in plant water use during the study, targeting a 15-20% leaching fraction (leached volume/nutrient solution applied). During early growth (February to March 2017), fertigation was less frequent (weekly or two times a week), beginning April 2017, plants started growing vigorously; therefore, fertigation frequency was increased (once every other day to every day). The experiment was carried out for six months, until July 17, 2017.

2.3. Growth measurements

Growth index (GI; the average value of the sum of plant height and two perpendicular widths) of each plant was measured at the end of the study on July 10, 2017. Following the final growth measurements, all leaves were detached from the stems of each plant, counted, and leaf area was determined using a leaf area meter (LI-3100, LI-COR Inc., Lincoln, NE USA). Leaf size (cm^2 $leaf^{-1}$) was calculated as total plant leaf area / number of leaves per plant. Leaves, stems, and

roots were dried and weighed. Root and shoot dry weights were summed to calculate total dry biomass (TDB), and the root-to-shoot ratio was calculated by dividing root mass with shoot mass.

2.4. Phosphorus partitioning

Our experimental protocol allowed us to develop a P budget for each plant. To estimate the amount of actual P applied and P leached, fertilizer solutions and leachates were analyzed for total phosphorus content using flow injection analysis digestion method in Lachat (Model: QuikChem 8500 series 2 with Total Nitrogen and Total Phosphorus manifolds) at seven different times during the course of study. Tissue P concentration in leaves and stems was determined from each plant, while P concentration in roots was determined from three (out of six) subsamples for each treatment. Samples for tissue P concentration were sent to a commercial plant laboratory (Waters Agricultural Laboratories, Camilla, GA USA) for analysis where plant tissues were analyzed using the wet digestion method combined with the inductively coupled plasma (ICP) method (Cunniff and Association of Official Analytical Chemists International, 1995). This approach allowed us to formulate a P budget based on the amount applied, taken up by the plant, and the amount leaving the container as leachate.

$$P_{applied} = P_{uptake} + P_{leached} + P_{substrate}$$

Where: $P_{applied}$ = total phosphorus applied during the experiment

P_{uptake} = phosphorus taken up by each plant during the experiment, calculated as the sum of phosphorus in leaves, stems, and roots - initial phosphorus in stems and roots.

$P_{leached}$ = total phosphorus leached from each container

$P_{substrate}$= total phosphorus stored in the substrate at the end of the study calculated as; total phosphorus applied – (phosphorus taken up by plants + total phosphorus leached from the container).

2.5. Physiological measurements

A portable photosynthesis system (LI-6400 XT, LI-COR, Inc., Lincoln, NE) equipped with a fluorescence chamber head (LI-6400-40, LI-COR, Inc., Lincoln, NE) was used to measure photosynthesis and light-adapted fluorescence (Fv'/Fm') for all three taxa. Plants received daily watering during the entire course of physiological measurements; hence, they did not show any sign of water stress.

2.5.1. *A/Ci* curves

A section of a fully expanded healthy leaf was enclosed in the fluorescence chamber head at either the 3rd or 4th node from the top for *P. opulifolius* and *C. obliqua* and at the 1st or 2nd node for *H. quercifolia*. Photosynthetically active radiation (PAR) in the chamber was set to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The block temperature on the chamber head was set to 25°C and the reference CO_2 was varied, starting at 400 ppm, gradually decreased to 0 ppm and then increased to 800 ppm (400,300,200,100, 50,0, 300,400,600 and 800 ppm) (Singh et al., 2013). Photosynthesis values at various internal carbon dioxide concentration (*Ci*) were used to generate *A/Ci* curves, using the non-linear rectangular hyperbola model developed by Archontoulis and Miguez (2015),

$$y = \frac{a * x * Yasym}{\{Yasym + a * x\} - Rd}$$

Where y = photosynthesis, x = Intercellular CO_2 concentration, Yasym = Asymptotic value of Y, a = initial slope of curve at low x levels (<200 ppm) and Rd = dark respiration.

Data from *A/Ci* curves were used to estimate V_{cmax} (the maximum velocity of rubisco for carboxylation), *J* (rate of photosynthetic electron transfer for RuBP regeneration) and *TPU* (triose phosphate use) values, using the non-linear equation provided as an Excel spreadsheet by Sharkey (2016). Curves used to generate values for V_{cmax}, *J*, and *TPU* were visually observed for the best fit to minimize errors. In some cases, where the calculator estimated an unrealistic value of day respiration (Rd) and mesophyll conductance (gm), Rd was constrained to < 6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and gm was constrained to < 3 $\mu mol \cdot m^{-2} \cdot s^{-1} \cdot Pa^{-1}$.

2.5.2 Chlorophyll fluorescence

Quantifying light-adapted chlorophyll fluorescence (*Fv'/Fm'*) can determine the rate of electron transport or the efficiency of PSII (Murchie and Lawson, 2013); therefore, a section of a healthy leaf (similar as above), of each plant, was enclosed in the same chamber with 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR, 400 ppm CO_2, and 40-60% humidity at 25°C. After approximately 5 minutes of acclimatization, *Fv'/Fm'* was measured (LI-6400XT, LI-COR Inc., Lincoln, NE, USA) to determine the efficiency of PSII.

2.6. Statistical analysis

All data were analyzed separately for each taxa using SAS software (SAS Institute Inc., Cary, NC, USA). A logistic growth curve was used to determine optimum P fertilization for GI and TDB (Archontoulis and Miguez, 2015; Shreckhise et al., 2018),

$$y = \frac{c}{\{1 + \exp[-a(x - b)]\}}$$

Where, y = either GI or TDB, c = asymptote of the curve, a = growth rate and b = inflection point of maximum growth rate.

The equation for the logistic growth curve was differentiated to find the point of asymptotic deceleration for GI and TDB (Mischan et al., 2011) using the fourth order derivative adapting the process of Shreckhise et al. (2018).

For all other comparisons, data were analyzed using one-way ANOVA. Post-hoc mean comparisons were made using Fisher's least significance difference (LSD). Correlations among morpho-physiological variables were analyzed using Pearson correlation test.

3. Results

3.1. Morphological response to phosphorus concentration

Plant growth response to P concentration varied by taxa (Fig. 1a and 1b). Growth Index (GI) and Total Dry Biomass (TDB) were lowest for plants not receiving any P. The response of GI and TDB to P concentration followed a logistic growth curve model. Using a fourth-order derivative for each model we determined the optimum P concentration for each taxon. For *P. opulifolius* and *H. quercifolia*; optimum P for GI was achieved at 3.44 and 6.3 $mg \cdot L^{-1}$, respectively (Fig. 1a). Optimum P concentration for GI for *C. obliqua* could not be calculated within the range of phosphorus that we applied, using the logistic growth curve. Optimum P for TDB was achieved at 6.94, 6.76 and 6.54 $mg \cdot L^{-1}$ of phosphorus for *P. opulifolius*, *H. quercifolia*, and *C. obliqua*; respectively (Fig. 1b).

Leaf number (LN), leaf size (LS; total leaf area per plant /number of leaves per plant), and total leaf area per plant (TLA) increased with increasing P concentration (Fig. 2). *P. opulifolius* and *C. obliqua* had maximum leaf numbers at 8 $mg \cdot L^{-1}$ of P while *H. quercifolia* had maximum leaf number at 4 $mg \cdot L^{-1}$ of P. Phosphorus concentrations below 4 $mg \cdot L^{-1}$ reduced LS, while those

above 4 mg·L^{-1} did not affect LS on any of the taxa (Fig 2). Total leaf area per plant for *C. obliqua* and *H. quercifolia* reached a maximum at 6 mg·L^{-1} of phosphorus while that for *P. opulifolius* was maximum at 8 mg·L^{-1} of P. Visual symptoms of P deficiency, i.e., shorter internodes, purpling of leaves, and smaller leaf sizes were observed in all three taxa at 0 and 1 mg·L^{-1} of P (Fig. 3).

Increasing P concentration increased root and shoot growth for all three taxa. Roots had maximum dry weight at 6 mg·L^{-1} of P for all three taxa. Maximum shoot dry weight was achieved at 6 mg·L^{-1} of P for *P. opulifolius* and *H. quercifolia* and at 8 mg·L^{-1} of P for *C. obliqua* (Table 1).

Root-to-shoot ratio for *P. opulifolius* decreased from 0.93 to 0.52 when P concentration was increased from 0 mg·L^{-1} to 2 mg·L^{-1}, further increases in P concentration did not decrease root-to-shoot ratio. For *H. quercifolia* and *C. obliqua* root-to-shoot ratio decreased from 0.55 to 0.33 and 0.7 to 0.37, respectively, when the P concentration was increased from 0 mg·L^{-1} to 4 mg·L^{-1}, further increase in P concentration did not decrease root-to-shoot ratio (Fig 4).

3.2. Partitioning of applied phosphorus

In order to assess the fate of P applied, we compared the total amount of P in each fraction (P in leaves, stems, roots, and leachate). Non-substrate P (P in leaf, stem, root, and leachate) was higher than the total P applied for 0 mg·L^{-1} of P treatment for all three taxa. For all plants receiving 1 mg·L^{-1} of P or more, the non-substrate P was lower than total P applied; hence some P should have been stored in the substrate. Leaves accounted for the largest fraction of P taken up by plants, except for *P. opulifolius* at the 0 and 1 mg·L^{-1} concentrations, for which P content in roots accounted for > 50% of total plant P (Table 2).

For all three taxa, increasing P concentration increased the total amount of P output as leachate and P in the substrate (Table 2). Increasing P concentration from 0 to 2 $mg \cdot L^{-1}$ increased P allocation to leaves, but beyond 4 $mg \cdot L^{-1}$ of P, P allocation to leaves decreased (Table 2). Increasing P concentration increased P partition to leachate. For example, increasing P concentration from 1 $mg \cdot L^{-1}$ to 8 $mg \cdot L^{-1}$ increased P fraction into leachate from 10% to 17% respectively and total P leached increased by 91% for *P. opulifolius*, 59% for *H. quercifolia* and 70% for *C. obliqua* when P concentration was increased from 6 $mg \cdot L^{-1}$ to 8 $mg \cdot L^{-1}$ (Table 2).

3.3. Photosynthetic response to phosphorus concentration

A/Ci curves were modeled with a non-linear model of a rectangular hyperbola (R-squared > 0.96 for all three taxa). For all three taxa, increasing P increased net photosynthesis. Increases in photosynthesis associated with P concentration were consistently greater at higher values of *Ci* (> 300 ppm) (Fig 5).

For *P. opulifolius*, V_{cmax} was lowest at ≤ 1 $mg \cdot L^{-1}$ of P and reached a plateau at 2 $mg \cdot L^{-1}$ of P (Fig. 6a). For *H. quercifolia*, V_{cmax} was lowest at ≤ 2 $mg \cdot L^{-1}$ of P and highest at ≥ 4 mg L^{-1} of P. For *C. obliqua*, 0 $mg \cdot L^{-1}$ of P had the lowest V_{cmax} while P concentration at ≥ 4 mg L^{-1} of P did not show a significant difference in carboxylation efficiency (Fig 6a). Photosynthetic limitation by the rate of electron transport was also evident at lower P concentrations. *P. opulifolius* and *H. quercifolia* had lowest electron transport rate at ≤ 1 $mg \cdot L^{-1}$ of P, and the lowest electron transport rate for *C. obliqua* was at 0 mg L^{-1} of P. *P. opulifolius* and *C. obliqua* had maximum electron transport rate at ≥ 2 $mg \cdot L^{-1}$ of P, while *H. quercifolia* had maximum electron transport rate at ≥ 4 $mg \cdot L^{-1}$ of P (Fig 6b). Phosphorus concentration also affected photosynthesis limitation as a result of *TPU* but was less sensitive compared to V_{cmax} and *J*. For all three taxa, *TPU* was lowest at the

P concentrations ≤ 1 mg·L^{-1} and highest ≥ 2 mg·L^{-1} of P. Therefore, increasing phosphorus from 2 to 8 mg·L^{-1} did not increase *TPU* (Fig 6c).

Light-adapted fluorescence (*Fv'/Fm'*) reached maximum levels at relatively low P concentrations for all three taxa (Fig. 7). Increasing P to 1 mg·L^{-1} increased Fv'/Fm' to a maximum for *H. quercifolia* and *C. obliqua* and 2 mg·L^{-1} of P maximized fluorescence for *P. opulifolius*.

3.4. Correlation among morpho-physiological variables

For all three taxa, TDB correlated ($p<0.05$) with P percentage in leaf (r = 0.87 *P. opulifolius*; r= 0.44 *H. quercifolia*; r= 0.65 *C. obliqua*) and average leaf size (r = 0.85 *P. opulifolius*; r=0.91 *H. quercifolia*; r=0.81 *C. obliqua*) (Table 3). Biomass productivity (TDB) correlated well (r = > 0.56) with parameters related to photosynthetic biochemistry such as V_{cmax}, *J* and *TPU* for all taxa (Table 3). Correlation order of TDB with those physiological parameters for all three taxa were in the order V_{cmax} > *J* > *TPU*. Biomass productivity also correlated (r = > 0.45) with quantum efficiency of PS II (*Fv'/Fm'*) for all three taxa but was weaker compared to photosynthetic biochemistry (Table 3). Foliar P concentration was only correlated with parameters related to photosynthetic biochemistry (V_{cmax}, *J, TPU* and *Fv'/Fm'*) for *P. opulifolius* and *C. obliqua*. Root-to-shoot ratio was negatively correlated with TDB for all taxa (r = -0.73 *P. opulifolius*; r = -0.73 *H. quercifolia*; r = -0.62 *C. obliqua*) and with P concentration in leaf for *P. opulifolius* (r=-0.78) and *C. obliqua* (r = -0.66) (Table 3).

4. Discussion

Plant productivity is the integrated result of leaf surface area accretion, net photosynthetic activity, and allocation of photosynthate to plant organs. In the current study, P concentration affected all aspects of plant productivity.

4.1. Morphological response to phosphorus concentration

For all three taxa, optimum P concentration required for maximum GI and maximum dry mass accumulation varied but was always less than 7 $mg \cdot L^{-1}$. This is consistent with recent observations that growth of woody ornamentals may be maximized at 2.9 to 4.7 $mg \cdot L^{-1}$ (Shreckhise et al., 2018). Leaf size for all three taxa followed similar trend as GI and TDB, as leaf size increased with P up to 4 $mg \cdot L^{-1}$ with no further increase at higher P concentrations. Phosphorus concentration of 8 $mg \cdot L^{-1}$ for *P. opulifolius* and 6 $mg \cdot L^{-1}$ for *C. obliqua* and *H. quercifolia* produced maximum total leaf area per plant. Hence, P fertilization is required for leaf expansion and growth. Increases in P concentration was reported to increase leaf area in common bean, sunflower (*Helianthus annuus*) and white clover (*Trifolium repens*) (Lynch et al., 1991; Rodríguez et al., 1998; Høgh-Jensen et al., 2002). Root-to-shoot ratio was maximum at 1 $mg \cdot L^{-1}$ of P for all three taxa and minimum at 2 $mg \cdot L^{-1}$ of P for *P. opulifolius*, and at 4 $mg \cdot L^{-1}$ of P for *H. quercifolia* and *C. obliqua*. Thus, these results reveals the effect of phosphorus on carbon allocation; at critically low phosphorus rates ($\leq$ 2 $mg \cdot L^{-1}$), phosphorus will be utilized more for root growth but when P concentration increases ($\geq$ 4 $mg \cdot L^{-1}$) it will be more readily used for shoot growth, since P acquisition is easily obtained from the labile pool of P in the substrate. Root-to-shoot ratio had a negative correlation to TDB and P % in leaf. Therefore, decreasing root-to-shoot ratio was primarily because of increase in shoot growth.

4.2. Fate of applied phosphorus

Combined P in leachate and plant tissue was greater than total P applied for treatment receiving 0 $mg \cdot L^{-1}$ of P as plants used P stored in the substrate when external phosphorus was not supplied. Ristvey et al., (2007), observed a similar response in container-grown azalea (*Rhododendron* L. 'Karen') grown a P additions as low as 0 $mg \cdot week^{-1}$. The total amount of P in leaves and stems increased with increasing P concentration (to 8 $mg \cdot L^{-1}$ for *H. quercifolia* and *C. obliqua* and to 6 $mg \cdot L^{-1}$ for *P. opulifolius*), but according to our model, P application beyond 6.94 $mg \cdot L^{-1}$ did not increase GI, TDB or any physiological performance in any of the taxa. Therefore, the increase in tissue P content when P is applied above this concentration indicates luxury consumption; i.e, absorption and storage of P beyond the current plant requirement, which also has been observed in a wide range of plant species including container-grown plants (Ristvey et al., 2007) and forest trees (Lawrence, 2001). Increasing P concentration increased P loss in leachate and total amount of P in the substrate. Similar observation was made in other studies where increasing P application increased phosphorus loss from the system (Ristvey et al., 2007; Shreckhise et al., 2018, 2019a). Therefore, increasing P beyond the optimum requirement would have no benefit on growth and physiological processes but could increase the amount of phosphorus in runoff. Hence, we would not recommend application of phosphorus over the optimum requirement of 6.94 $mg \cdot L^{-1}$ *for P. opulifolius* 6.76 $mg \cdot L^{-1}$ for *H. quercifolia* and 6.54 $mg \cdot L^{-1}$ for *C. obliqua*.

4.3 Physiological performance in response to phosphorus concentration

To fix one molecule of CO_2, in Calvin-Benson cycle, three molecules of ortho-phosphate (PO_4) are required (Walker and Robinson, 1978); therefore P deficiency can limit photosynthesis.

In our study increasing P concentration, up to a point, increased net CO_2 assimilation for a wide range of intercellular CO_2 concentrations (0 to 600 ppm) in all three taxa. Similar increases in net assimilation with increasing P concentration was observed in sunflower (*Helianthus annuus* L. cv Asmer), maize (*Zea mays* L. cv Eta) (Jacob and Lawlor, 1991) and pine seedling (Loustau et al., 1999). Photosynthesis in light-saturated conditions can be rubisco limited, RuBP limited, or *TPU* limited; and a well-constructed carbon dioxide response (*A/Ci*) curve can be used to determine the type of limitation (Farquhar et al., 1980; Sharkey, 2016). For *H. quercifolia* and *C. obliqua*, P concentrations < 4 $mg \cdot L^{-1}$ reduced carboxylation rate. For those two taxa, photosynthesis at lower P concentrations (< 4 $mg \cdot L^{-1}$) was reduced partly because of the limited supply of rubisco enzyme. For *P. opulifolius*, rubisco restricted photosynthesis only at < 2 $mg \cdot L^{-1}$ of phosphorus. The rate of RuBP regeneration may also limit photosynthesis in phosphorus deficient plants. For *P. opulifolius* and *C. obliqua*, photosynthesis was limited by RuBP regeneration at phosphorus concentrations < 2 $mg \cdot L^{-1}$. For *H. quercifolia* RuBP regeneration limited photosynthesis at < 4 $mg \cdot L^{-1}$ of phosphorus. At higher rates of photosynthesis, export of carbon compounds from Calvin-Benson cycle slows down, causing a reduction in photosynthesis (Yang et al., 2016) also referred to as *TPU* limited photosynthesis. In our study, 2 $mg \cdot L^{-1}$ of P was sufficient to overcome the limitation caused by *TPU* for all three taxa. Therefore, photosynthesis limitations caused by rubisco and RuBP regeneration were more sensitive compared to the limitation caused by *TPU*. This sensitivity is also further verified by the correlation analysis of TDB with V_{cmax}, J and *TPU*. Phosphorus deficiency has also been observed to reduce V_{cmax} and J_{max} for several other taxa (Loustau et al., 1999; Lin et al., 2009; Singh et al., 2013). In contrast to the parameters of the Calvin-Benson cycle, light utilization by plants of all three taxa was less affected by P concentration. For all three taxa, plants that received no P had lower *Fv'/Fm'* compared to plants that received P, and 1 $mg \cdot L^{-1}$ of

P for *H. quercifolia* and *C. obliqua* and 2 $mg \cdot L^{-1}$ of P for *P. opulifolius* was sufficient to maximize *Fv'/Fm'*. Other studies have observed no reduction in chlorophyll content and light harvesting capacity at low phosphorus rates (Brooks, 1986; Campbell and Sage, 2006). In our study, light harvesting capacity was reduced when no P was supplied, but a low rate of P was sufficient for optimum functioning of photosystem II.

5. Conclusion

For all three taxa, GI and TDB were the parameters that were most sensitive to P application thus, needed higher P concentrations compared to other morphological parameters and physiological variables. Analysis of *A/Ci* curves indicated a broader response to P concentration compared to light-adapted chlorophyll fluorescence, thus, suggesting an overall photosynthetic response to be phosphorus driven more by photosynthetic biochemistry rather than light harvesting reactions. When compared among all three taxa, reduction in carboxylation rate (rubisco limited) was the main reason for reduction in photosynthesis followed by the rate of electron transport (RuBP regeneration) then by triose phosphate use (*TPU*).

Overall, GI and TDB were optimized at approximately 7 $mg \cdot L^{-1}$ of P for all three taxa, which is much lower than those in water-soluble fertilizers or P release rate of controlled-release fertilizers that are commonly available and used in the nursery industry. Therefore, nursery growers may be able to reduce P fertilization without reducing crop growth. Even a slight reduction in phosphorus rates over a long period can substantially reduce total phosphorus runoff. For example, if P concentration were lowered, from 8 $mg \cdot L^{-1}$ to 6 $mg \cdot L^{-1}$, leachate P concentration would be reduced by 59-91% depending on taxa grown. Reducing P in irrigation return flow can

ultimately lower growers' environmental footprint without affecting physiological or morphological processes across many ornamental taxa.

APPENDIX

APPENDIX

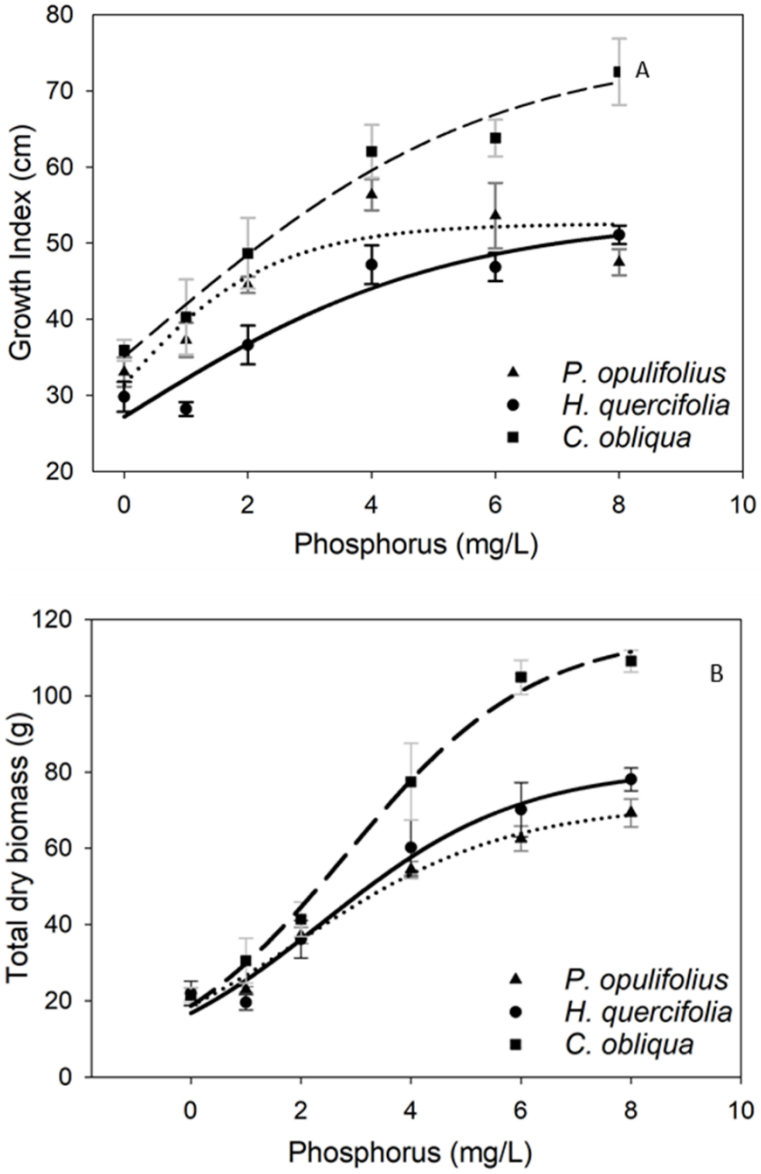

Figure II - 1. Growth index (A) and total dry biomass (B) of *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens' in response to increasing phosphorus concentration. Non-linear regression curves (logistic growth curves) are plotted for both GI and TDB. Standard errors of the means are denoted as vertical lines on the curves.

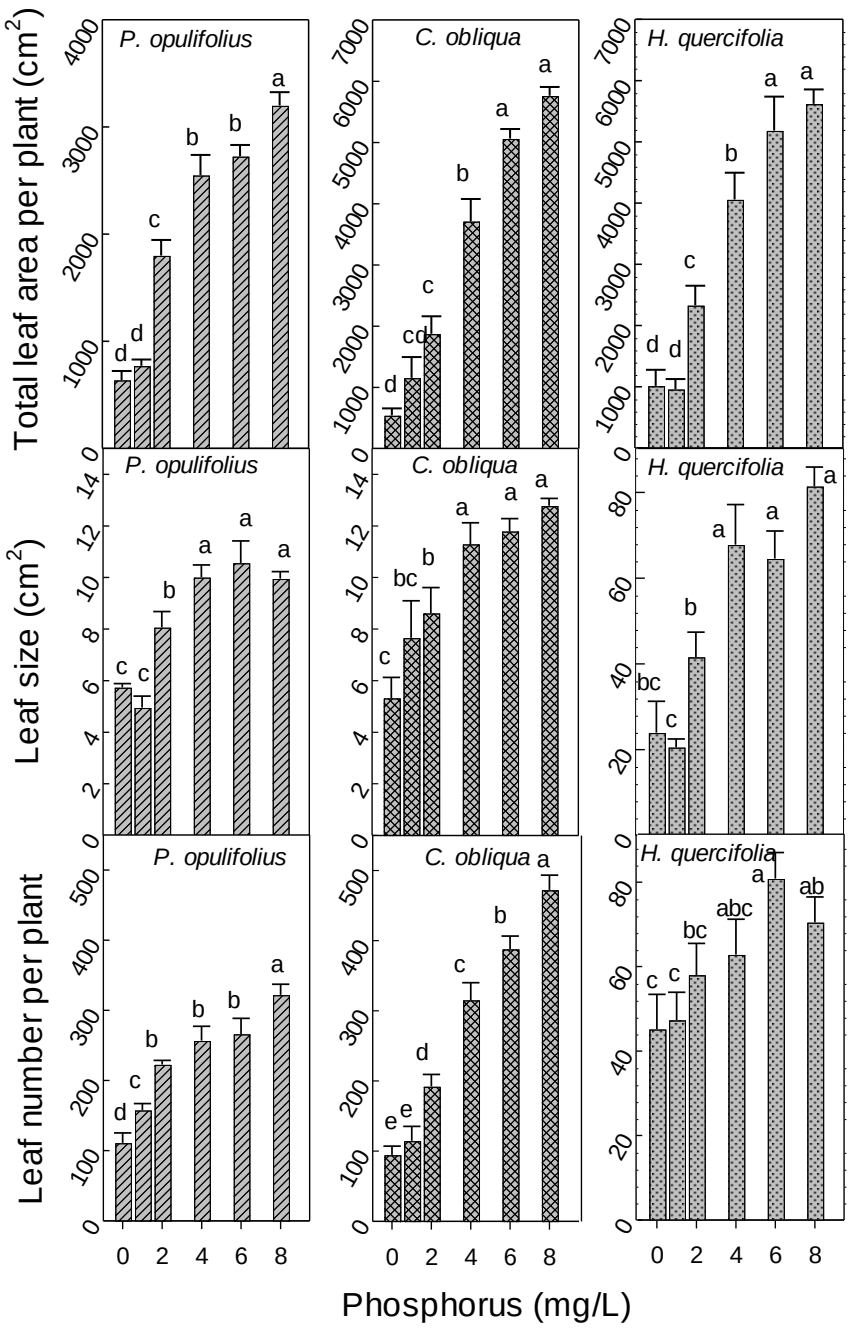

Figure II - 2. Leaf number per plant, leaf size, and total leaf area per plant for *P. opulifolius* 'Seward', *C. obliqua* 'Powell Gardens', and *H. quercifolia* 'Queen of hearts' in response to phosphorus concentration. Standard error of the means are denoted by vertical 'T' lines. Mean separations for each taxa were carried out using Least Significant Difference (LSD) post-hoc test. Means within a taxon that are followed by same letters are not significantly different at p=0.05.

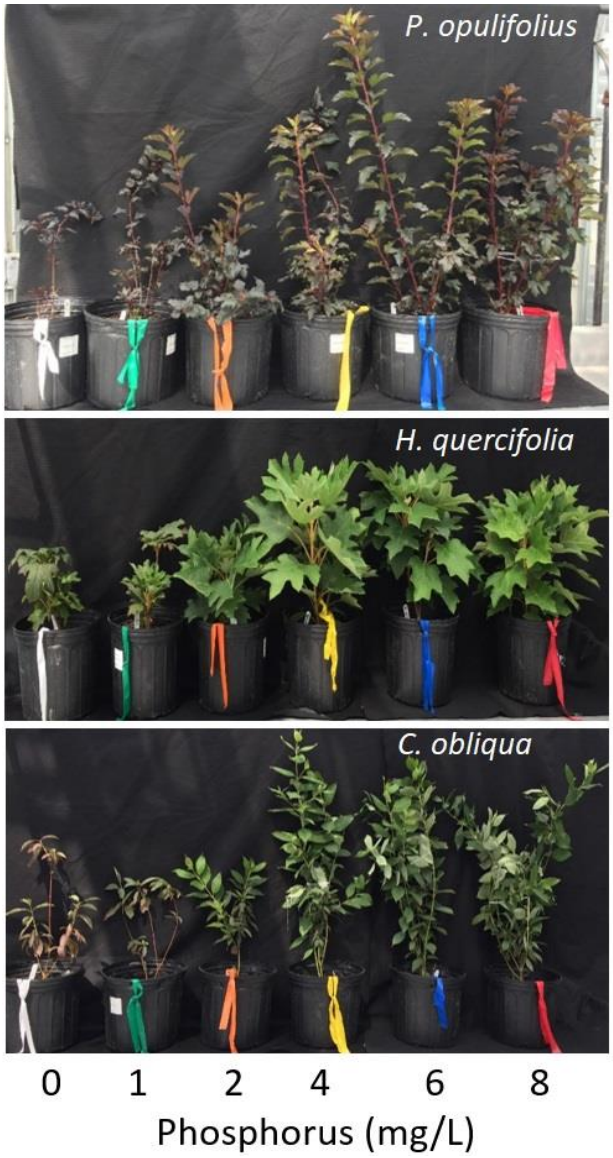

Figure II - 3. Representative plants for each P concentration of *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens', after receiving 0 to 8 $mg \cdot L^{-1}$ for 6 months in the greenhouse.

Table II - 1. Root and shoot dry weight (g) of *P. opulifolius* 'Seward' *H. quercifolia* 'Queen of heart', and *C. obliqua* 'Powell Gardens', and in response to phosphorus concentration. Mean separations were carried out using Fisher Least Significant Difference (LSD) post hoc test when appropriate. Means within a taxon that are followed by same letters are not significantly different at given p values.

	P. opulifolius		*H. quercifolia*		*C. obliqua*	
P ($mg \cdot L^{-1}$)	Root	Shoot	Root	Shoot	Root	Shoot
0	10.09c	10.94d	7.75c	14.22d	8.70e	12.8d
1	10.09c	12.78d	6.70c	12.86d	11.21de	19.28d
2	12.70c	24.39c	10.70c	25.44c	13.26cd	28.10d
4	17.59b	36.83b	15.01b	45.17b	21.49bc	55.98c
6	18.22ab	44.30a	17.31ab	52.84ab	30.87a	74.06b
8	21.88a	47.42a	19.39a	58.68a	25.9ab	83.19a
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table II - 2. Partitioning of applied phosphorus to leachate, leaf, stem and root, including the amount of P stored in the substrate for *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens'. Mean separation were carried out using Fisher Least Significant Difference (LSD) post-hoc test. Means that are followed by same letters are not significantly different given p value

.

P ($mg \cdot L^{-1}$)	Total-P input (mg)	=	Total P in leachate (mg)	Total P in leaf (mg)	Total P in stem (mg)	Total P in root (mg)	Total P in substrate (mg)	Percent of P in leachate	Percent of P in leaf	Percent of P in stem	Percent of P in root	Percent of P in substrate
						P. opulifolius 'Seward'						
0	0.58	=	1.44d	3.84d	1.31c	6.49b	-12.50d	-	-	-	-	-
1	22.53	=	2.31cd	5.53d	1.72c	4.44b	8.53c	10.25	24.55	7.63	19.71	37.86
2	40.31	=	3.62c	12.69c	5.06c	7.51b	11.43bc	8.98	31.48	12.55	18.63	28.36
4	78.99	=	11.71b	24.20b	11.57b	17.49a	14.02bc	14.82	30.64	14.65	22.14	17.75
6	119.04	=	18.43b	30.78a	18.80a	26.13a	24.9b	15.48	25.86	15.79	21.95	20.92
8	204.25	=	35.23a	36.70a	23.98a	29.37a	78.97a	17.25	17.97	11.74	14.38	38.66
p-value			<0.0001	<0.0001	<0.0001	<0.0005	<0.0001					
						H. quercifolia 'Queen of hearts'						
0	0.55	=	1.73f	5.45d	2.45bc	5.28bc	-14.36d	-	-	-	-	-
1	21.04	=	2.31e	8.52d	1.36c	3.22c	5.65cd	10.97	40.49	6.46	15.21	26.85
2	37.64	=	3.37d	18.20cd	2.64bc	6.1761bc	7.27cd	8.95	48.35	7.01	16.37	19.31
4	73.73	=	5.68c	30.56bc	5.84ab	10.29ab	21.36bc	7.7	41.45	7.92	13.95	28.97
6	111.13	=	18.77b	37.11ab	6.28ab	10.37ab	38.60b	16.89	33.39	5.65	9.33	34.73
8	192.69	=	29.76a	48.0a	6.98a	16.24a	89.71a	15.44	25.95	3.62	8.43	46.56
p-value			<0.0001	<0.0005	<0.05	<0.01	<0.0001					

Table II - 2 (cont'd)

							C. obliqua 'Powell Gardens'					
0	0.59	=	2.72d	4.87d	2.54d	0.69d	-10.23d	-	-	-	-	-
1	21.91	=	2.67d	8.59cd	3.53cd	1.17d	5.94cd	12.19	39.2	16.12	5.36	27.12
2	39.19	=	3.37d	17.77c	5.77bcd	2.98cd	9.30bc	8.59	45.33	14.73	7.59	23.74
4	76.75	=	9.59c	33.82b	6.48bc	7.12c	19.75bc	12.49	44.06	8.44	9.28	25.71
6	115.65	=	20.02b	41.03b	8.13b	21.59a	24.89b	17.31	35.48	7.03	18.67	21.52
8	198.45	=	34.10a	58.51a	13.26a	16.24b	76.36a	17.18	29.48	6.68	8.18	38.48
p-value			<0.0001	<0.0001	<0.0005	<0.0005	<0.0001					

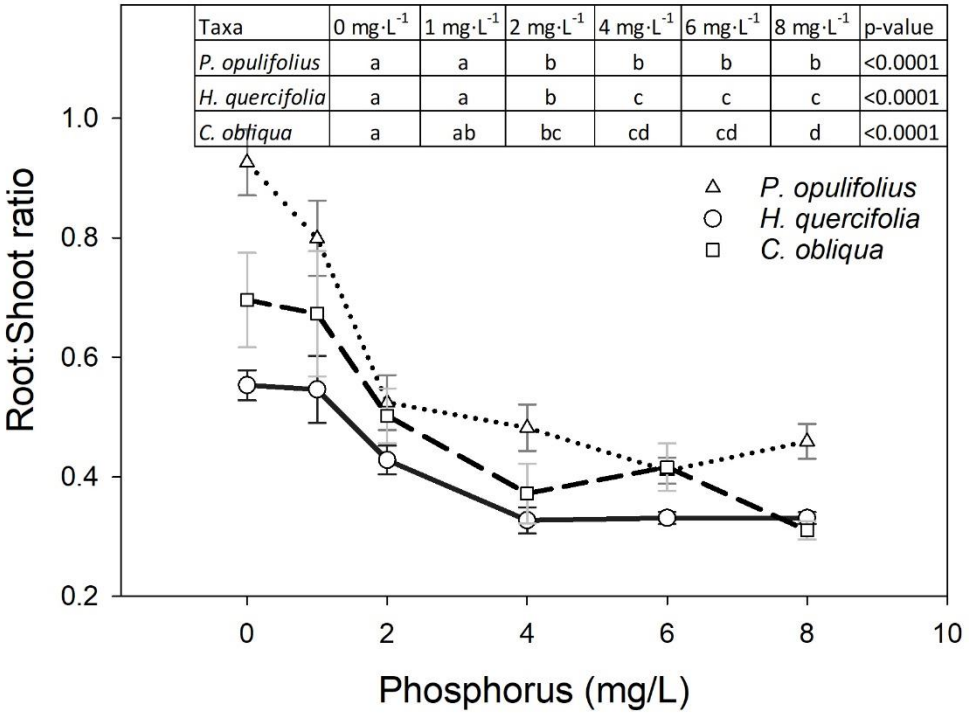

Figure II - 4. Root-to-Shoot (R:S) ratio of *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens', and in response to phosphorus concentration. Mean separations were carried out using Fisher Least Significant Difference (LSD) post-hoc test and presented as inset table. Means within a taxon indicated by the same letter are not different at the given p value. Standard errors are denoted as vertical lines on the curves.

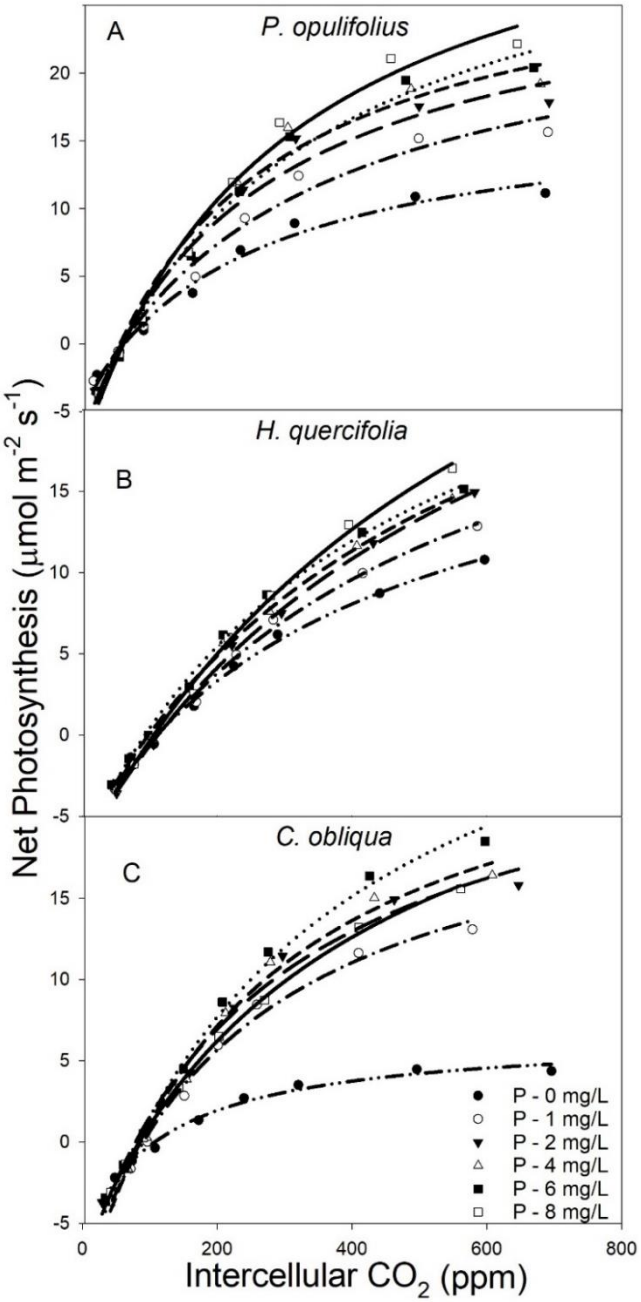

Figure II - 5. Response of photosynthesis to increasing internal carbon dioxide concentration (A/Ci Curve) for *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens'. Curves were generated as the mean of five replicates. All the curves followed non-linear model of rectangular hyperbola. R-squared values for all models for all three taxa were above 0.96.

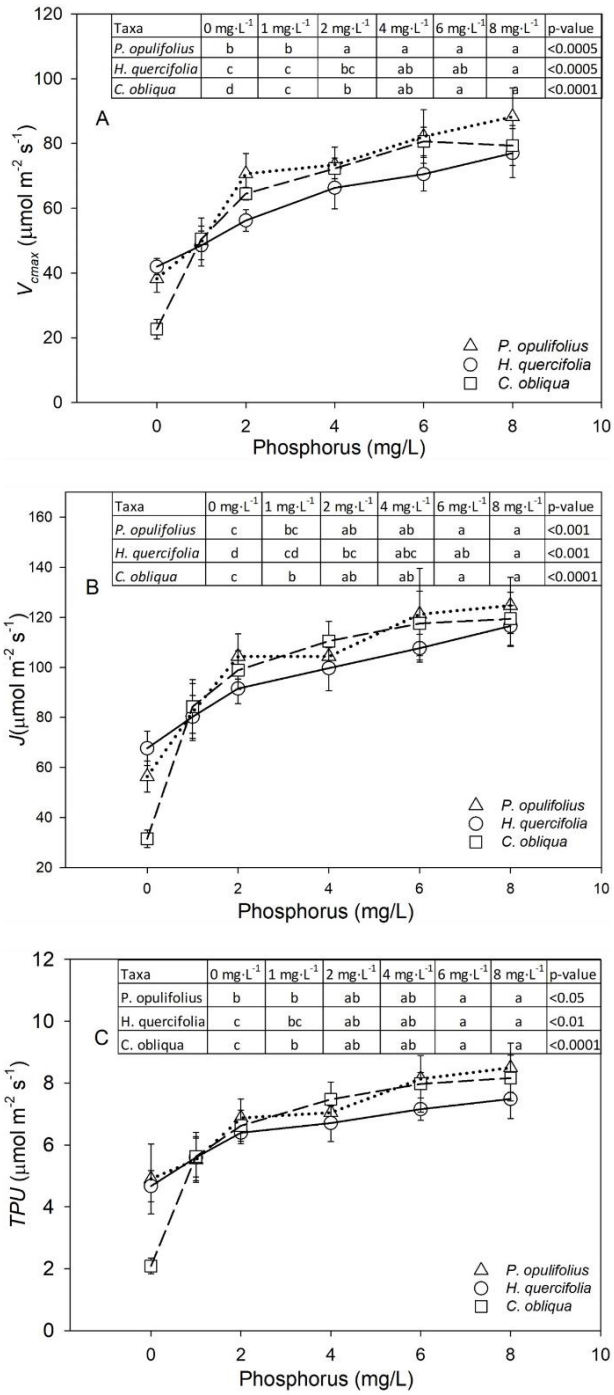

Figure II - 6. Maximum velocity of rubisco for carboxylation (V_{cmax}) (A); rate of photosynthetic electron transport for RuBP regeneration (*J*) (B), and triose phosphate use (*TPU*) (C) of *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens' in response to phosphorus concentration. Values of *A/Ci* Curves were analyzed based on equations provide by Sharkey (2016) to generate V_{cmax}, *J* and *TPU* for each replication. Fisher Least Significant Difference (LSD) was used to compare means among phosphorus fertilization levels and presented as inset table. Means within a taxon indicated by the same letter are not different at given p-value. Standard errors are denoted as vertical lines on the curves.

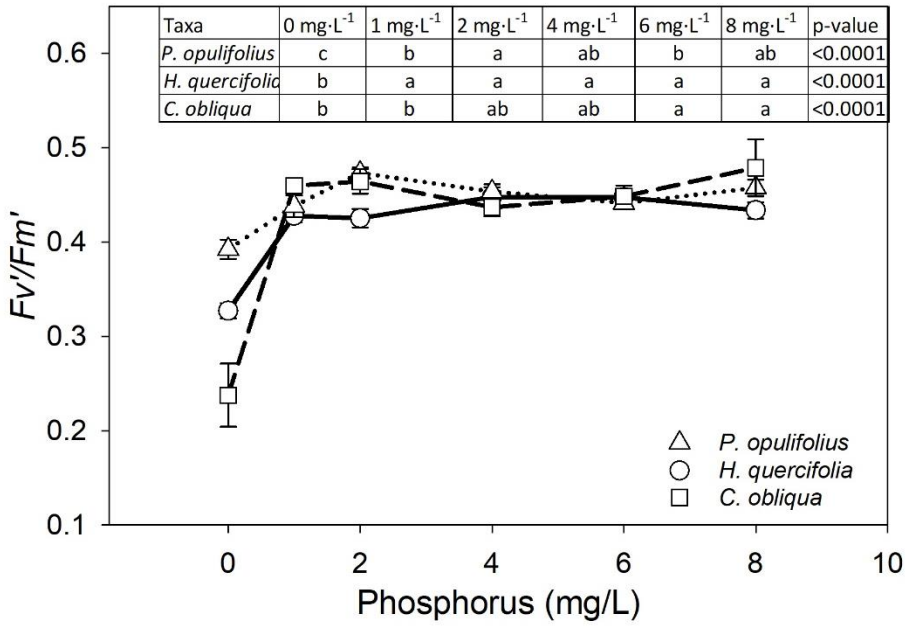

Figure II - 7. Light-adapted fluorescence (Fv'/Fm') response to increasing phosphorus concentration for *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens'. Fisher Least Significant Difference (LSD) was used to compare means among phosphorus fertilization levels and presented in as inset table. Means within a taxon indicated by the same letter are not different at given p value. Standard errors are denoted as vertical lines on the curves.

Table II - 3. Pearson's correlation coefficient for *P. opulifolius* 'Seward', *H. quercifolia* 'Queen of hearts', and *C. obliqua* 'Powell Gardens'. TDB is total dry biomass, R/S ratio is root to shoot ratio, Leaf size (total leaf area per plant/ leaf number per plant), P% in leaf is phosphorus percent in leaf by weight, V_{cmax} is maximum velocity of rubisco for carboxylation, *J* is the rate of photosynthetic electron transport for RuBP regeneration, *TPU* is triose phosphate use and *Fv'/Fm'* is the light-adapted fluorescence.

Pearson's correlation coefficient for *P. opulifolius*							
	R/S ratio	Leaf size	P% in leaf	V_{cmax}	*J*	*TPU*	*Fv'/Fm'*
TDB	-0.73***	0.85***	0.87***	0.69***	0.65***	0.56**	0.45*
R/S ratio		-0.74***	-0.78***	-0.60***	-0.50**	-0.44*	-0.55***
Leaf size			0.75***	0.62***	0.58**	0.51**	0.42*
P% in leaf				0.77***	0.63***	0.55**	0.48**
V_{cmax}					0.92***	0.81***	0.47*
J						0.99***	0.46*
TPU							0.34^{NS}
Pearson's correlation coefficient for *H. quercifolia*							
	R/S ratio	Leaf size	P% in leaf	V_{cmax}	*J*	*TPU*	*Fv'/Fm'*
TDB	-0.73***	0.91***	0.44*	0.77***	0.75***	0.68***	0.51**
R/S ratio		-0.68***	-0.23^{NS}	-0.60***	-0.56**	-0.52**	-0.52**
Leaf size			0.44*	0.70***	0.68***	0.61***	0.46**
P% in leaf				0.31^{NS}	0.30^{NS}	0.24^{NS}	0.04^{NS}
V_{cmax}					0.92***	0.89***	0.44*
J						0.98***	0.47*
TPU							0.47*
Pearson's correlation coefficient for *C. obliqua*							
	R/S ratio	Leaf size	P% in leaf	V_{cmax}	*J*	*TPU*	*Fv'/Fm'*
TDB	-0.62***	0.81***	0.65***	0.78***	0.7***	0.69***	0.51**
R/S ratio		-0.75***	-0.66***	-0.6**	-0.59**	-0.58**	-0.53**
Leaf size			0.53**	0.7***	0.61**	0.60**	0.61**
P% in leaf				0.65***	0.56**	0.57**	0.43*
V_{cmax}					0.91***	0.91***	0.70***
J						0.99***	0.68***
TPU							0.66***

*** p-value of ≤ 0.0005; ** p-value of ≤ 0.005 and; * p-value of ≤ 0.05, NS p-value > 0.05.

Table II - 4. Breakdown of micronutrients analysis with elements and concentration.

Micronutrients	Source	Amount in percentage
Calcium (Ca)	Calcium Carbonate	6.00%
Magnesium (Mg)	Magnesium carbonate	3.00%
Sulfur (S)	Copper, zinc ferrous and manganese sulphate	12.00%
Boron (B)	Sodium borate	0.10%
Copper (Cu)	Copper sulfate	1.00%
Iron (Fe)	Ferrous sulphate	17.00%
Manganese (Mn)	Manganese sulfate	2.50%
Molybdenum (Mo)	Sodium molybdate	0.05%
Zinc (Zn)	Zinc sulfate	1.00%

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SECTION III

DOSE-DEPENDENT PHYTOTOXICITY OF PESTICIDES IN SIMULATED NURSERY RUNOFF ON LANDSCAPE NURSERY PLANTS

Dose-dependent phytotoxicity of pesticides in simulated nursery runoff on landscape nursery plants

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Abstract

Managers of ornamental nurseries are increasingly reusing runoff water as an irrigation source, but residual pesticides in recycled water may result in plant phytotoxicity on crop plants. Our study focused on understanding the responses of container-grown landscape plants to residual pesticides in irrigation water. Hydrangea paniculata 'Limelight', Cornus obliqua 'Powell garden', and Hosta 'Gold standard' were exposed to various concentrations of isoxaben, chlorpyrifos, and oxyfluorfen (0, 0.15, 0.35, 0.7, and 1.4 mg/L of isoxaben; 0, 0.05, 0.1, 0.2, and 0.4 mg/L of chlorpyrifos; and 0, 0.005, 0.01, 0.015, and 0.02 mg/L of oxyfluorfen) applied as overhead irrigation. After three months of application, we assessed the dry weight biomass, growth, and parameters related to photosynthetic physiology (soil plant analysis development (SPAD) chlorophyll index, light-adapted chlorophyll fluorescence, and photosynthesis carbon dioxide response (A/Ci) curves. We also sampled the plant leaf, stem, and root tissues for residual pesticides. The effects of the pesticides were pesticide-specific and taxa-specific. Exposure to oxyfluorfen resulted in visible injury in all three taxa and reduced total biomass, chlorophyll index, and photosynthesis in Hydrangea and Hosta. All three taxa absorbed and retained pesticide in leaf and stem tissues. Growers should follow best management practices to reduce exposure from irrigation with runoff, particularly for herbicides with post-emergent activity.

Keywords: nursery runoff; isoxaben; oxyfluorfen; chlorpyrifos; photosynthesis; A/Ci curve

1. Introduction

Horticulture is a major industry in the U.S. In 2014, the sale of floriculture, nursery, and specialty crops were worth $13.8 billion, up by 18% since 2009 (United States Department of Agriculture National Agricultural Statistics Service, 2016). For container nurseries, irrigation is often applied based on general rules of thumb, such as 19 mm of water per day. These application rates often greatly exceed plant water needs and result in substantial runoff (Warsaw et al., 2009; Danelon et al., 2010). In a nursery with 4 L containers placed six inches apart, up to 80% of applied water may be lost as runoff (Mathers et al., 2005). Furthermore, frequent pesticide application is common among nursery producers. Therefore, runoff generated from container nurseries may contain various pesticides, and if released without treatment, surface water contamination and toxicity to aquatic life can occur (Keese et al., 1994; Lao et al., 2008; Warsaw et al., 2012). Due to the significant freshwater use by the nursery industry and the environmental problems associated with runoff, water regulations for nurseries are becoming more stringent. To cope with new regulations and ensure water security, the capture and reuse of runoff water is increasing among nursery growers (Brown, 2002; Schmitz et al., 2013; Wilson and Broembsen, 2015). While capturing and reusing runoff may be a practical solution to reduce contaminants in neighboring ecosystems, growers' concerns about potential negative impacts of residual pesticide on crop growth and quality may impede its adoption (Wilson and Broembsen, 2015) as nursery growers

report evidence of pesticide phytotoxicity when runoff water is used for irrigation (personal communication with growers).

In the current study, we examined the impacts of isoxaben, oxyfluorfen, and chlorpyrifos on three widely cultivated nursery crops. We selected these compounds for study because they are commonly used in the nursery trade and represent different modes of action. Moreover, all three pesticides may be found in nursery runoff, and if present at higher concentrations, can injure nursery plants (Bhandary et al., 1997; Briggs et al., 1998). Isoxaben (common tradenames Gallery®, Snapshot®) is a pre-emergence herbicide that works by inhibiting cell wall biosynthesis in dividing cells, causing stunted plants. Various nursery plants are susceptible to this herbicide. Isoxaben at 5 mg/L reduced plant height, leaf emergence, and photosynthesis in *Canna generalis* (canna), *Pontaderia cordata* (pickerel weed), and *Iris* (charjoys Jan) (Fernandez et al., 1999). Isoxaben at 10 mg/L also reduced the root visual appearance scale in *Pennisetum rupeli* (fountain grass) and *Hemerocallis hybrid* (daylily) as well as the fresh root weight in *Ilex crenata* ("Helleri" Hellers holly) (Bhandary et al., 1997), but isoxaben application at 1.1 kg a.i./ha alone did not injure six different container-grown ornamental grass species (Neal and Senesac, 1991), nor did it affect plant height in *Ilex crenata* (Japanese holly). Chlorpyrifos (common tradenames Dursban®, Lorsban™) is an insecticide that may sometimes affect plants by inhibiting the activity of enzymes for growth and development, causing smaller plants (Parween et al., 2011a). Chlorpyrifos (525 mg/L) induced membrane disintegration through lipid peroxidation and also increased

superdimutase (SOD) activity in *Vigna radiata* (Parween et al., 2012). Chlorpyrifos at 30 mg/L also reduced growth and biomass in *Azolla pinnata* (Prasad et al., 2015). Oxyfluorfen (common tradename Goal) is a widely used pre and post-emergence herbicide in container nursery production. It is mostly used for controlling broadleaf weeds and annual grasses (Dow AgroSciences, 2014). Oxyfluorfen acts by inhibiting the synthesis of protoporphyrinogen oxidase "PPO" enzymes leading to cell membrane disruption (Lee and Duke, 1994). Oxyfluorfen at 1 g/ha, when applied as a post-emergence herbicide to control weeds in sunflower, produced severe phytotoxicity on sunflower (Jursik et al., 2011). Oxyfluorfen is not recommended for use in sunflower, but has been found to be safe on eight different container-grown ornamental crops at 0.9 kg a.i./ha including red-osier dogwood (*Cornus sericea*), cranberry cotoneaster (*Cotoneaster apiculata*), European cranberry viburnum (*Viburnum opulus* 'Notcutt'), border forsythia (*Forsythia intermedia* 'Spectailis'), English ivy (*Redera helix*), green luster holly (*Ilex crenata* 'Green Luster'), Japanese pachysandra (*Pachysandra terminalis*), and Browni yew (*Taxus x media* "Brownii") (Vea and Palmer, 2009). Application of oxyfluorfen at 0.07 kg a.i./ha as a foliar spray produced severe phytotoxicity on *Euonymus fortunei* (Colorata) (Horowitz et al., 1989).

Pesticides in runoff water from nurseries are usually diluted with other water sources and therefore occur at relatively low concentrations. However, frequent, often daily, irrigation with nursery runoff creates the potential for chronic low-dose exposure to an array of pesticides that may have phytotoxic effects. Pesticides that cause phytotoxicity usually interfere with

physiological and biochemical processes in non-target plants. Many of these effects are related to photosynthetic function, and measuring these responses can indicate the extent of physiological damage caused by pesticides (Krugh and Miles, 1996; Spiers et al., 2008; Parween et al., 2011b; Vinet and Zhedanov, 2012). A novel aspect of our approach in the current study was to examine the potential impacts of chronic, low dose application of pesticides on physiological responses of nursery crops. Advances in portable photosynthesis systems have simplified the measurement of key photosynthetic responses such as A/Ci curves that can provide insights into photosynthetic reactions that may be early indicators of phytotoxic responses. For example, the maximum carboxylation rate of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) (Vcmax) may limit photosynthesis at lower (0 to 200 ppm) intercellular carbon dioxide concentrations, and the rate of electron transfer for ribulose 1,5-bisphosphate (RuBP) regeneration (J) may limit photosynthesis at higher (>300 ppm) intercellular carbon dioxide concentrations (Veeraswamy et al., 1993; Sharkey et al., 2007; Parween et al., 2011b; Sharkey, 2016)

A few studies have described the effects of isoxaben, chlorpyrifos, and oxyfluorfen on various plants (Lal et al., 1987; Neal and Senesac, 1991; Salihu et al., 1999; Parween et al., 2011a), but the impact of residual (low) concentrations of these compounds on common landscape nursery plants receiving pesticides for an entire growing season has not been documented. Phytotoxicity of isoxaben, oxyfluorfen, and chlorpyrifos may vary depending on the plant taxa irrigated, the concentration of pesticide in water, and the duration of the pesticide application and growers

particularly lack information on the long-term phytotoxicity caused by these pesticides in runoff water. They are also unaware of the severity of phytotoxicity caused by these pesticides on common landscape nursery plants. Therefore, understanding the impacts of prolonged exposure to low doses of these pesticides may provide insights into the safe use of recycled runoff for irrigation and ultimately encourage the reuse of runoff water among nursery growers. Therefore, the objectives of this study were to evaluate the morphological and physiological effects of various concentrations of isoxaben, chlorpyrifos, and oxyfluorfen on three commonly cultivated container-grown landscape nursery plants, Hydrangea paniculata 'Limelight', Hosta 'Gold Standard', and Cornus obliqua 'Powell Gardens'.

2. Materials and Methods

2.1. Plant Material and Treatments

This study was conducted in a greenhouse at the Michigan State University Horticulture Teaching and Research Center (HTRC) located in Holt, Michigan, USA. We used Limelight Hydrangea (*Hydrangea paniculata* 'Limelight'), Red Rover® silky dogwood (*Cornus obliqua* 'Powell Gardens'), and Gold Standard Hosta (*Hosta* 'Gold Standard') potted in 12 L black plastic containers for our study. We planted *Hydrangea* and *Cornus* plants as liners in spring 2017 in pine bark and peat moss substrate (80:20; volume: volume). These two plants were grown outdoors at the HTRC and received standard nursery culture including 19 mm of daily overhead irrigation and controlled release fertilizer (19:4:8 N:P_2O_5:K_2O with micronutrients, 5–6 months, Harrell's LLC,

Lakeland, FL, USA) applied as a top-dressing. In early December 2017, *Hydrangea* and *Cornus* plants, along with bulbs of *Hosta* plants, were placed in a walk-in cooler at 6 °C for five weeks to complete their chilling requirements before they were brought into the greenhouse on January 8, 2018. The temperature in the greenhouse was set to 22 °C, and a sodium lamp provided 16-h of photoperiod. Different concentrations of isoxaben, oxyfluorfen, or chlorpyrifos were applied as overhead irrigation mixed in irrigation water. We selected five different concentrations of each pesticide as treatments (0, 0.15, 0.35, 0.7, and 1.4 mg/L of isoxaben; 0, 0.05, 0.1, 0.2, and 0.4 mg/L of chlorpyrifos; and 0, 0.005, 0.01, 0.015, and 0.02 mg/L of oxyfluorfen). Pesticide rates were based on pesticide residues reported in nursery retention ponds (Riley et al., 1994; Briggs et al., 2003; Mangiafico et al., 2009) and the solubility of the pesticides in water. A black 100-L covered plastic tank was used as a stock tank for each treatment. A calculated amount of each pesticide was dissolved in 100 L of water to achieve the desired concentration. A sump pump was used to agitate the pesticide solution and apply the pesticide solution as overhead irrigation on plants. An irrigation distribution test for treatment zones had a distribution uniformity of 89.73%. Pesticide solutions were freshly prepared two to three times a week. Each pesticide treatment consisted of three taxa and six replications per taxa. Treatments began once all the plants had produced a new flush of growth (8 February 2018). We applied pesticide treatments with each irrigation event that varied from once every three days to every day, depending on plant water use. Pesticide treatments continued for three months.

2.2. Physiological Measurements and Growth

A portable photosynthesis system (LI-6400 XT, Li-Cor, Inc., Lincoln, NE, USA) mounted with a leaf chamber fluorometer (LI-6400-40, Li-Cor, Inc., Lincoln, NE, USA) was used to develop A/Ci curves and light-adapted fluorescence for all three taxa. A section of fully mature leaf on either the third or fourth node from the top for *Cornus* and *Hydrangea* and on the first or second node for *Hosta* was used for all physiological measurements. Photosynthetically active radiation (PAR) in the chamber was set to 1500 µmol m^{-2} s^{-1}. The block temperature was set to 25 °C, and the reference CO_2, supplied by a 12 g CO_2 cartridge (LI-6400 XT, Li-Cor, Inc., Lincoln, NE, USA), was varied, starting at 0 ppm to 800 ppm (0, 50, 100, 200, 300, 400, 500, 600, and 800 ppm). Net photosynthesis (A) values at various intercellular carbon dioxide concentrations (Ci) were used to generate carbon dioxide response (A/Ci) curves. Data from the A/Ci curves were used to estimate Vcmax and J values using the non-linear equation provided by Sharkey (2016). For light-adapted chlorophyll fluorescence measurements, a section of a fully mature leaf of each plant was enclosed in the LI-6400-40 chamber with 1500 µmol m^{-2} s^{-1} of PAR, 400 ppm of CO_2, and 40–60% humidity at 25 °C. We acclimatized the leaf for 5 min, after which we measured light-adapted fluorescence (LI-6400xt Instruction Manual, version 6, Li-Cor Inc., Lincoln, NE, USA) to determine the efficiency of photosystem II. SPAD leaf chlorophyll index was also measured on three fully expanded leaves per plant on either the second or third node for *Hydrangea* and *Cornus* by using a portable SPAD meter (SPAD-502; Minolta Corporation, Ltd., Osaka, Japan). The

variegated golden leaf color of *Hosta* produced unrealistic values of the SPAD index; hence, we did not measure the SPAD index for those plants.

We examined the leaves of each plant and scored them for visible pesticide injury based on a rating system on a scale of one to ten, with ten being a healthy leaf without damage, and one being a dead leaf. After scoring plants for visible symptoms, the leaves, stems, and roots were harvested, dried in an oven (45 °C), and weighed. All of the dry weights were combined to determine the total dry biomass (TBD). Samples of dried leaves, stem, and roots were sent to a commercial laboratory (Brookside Labs, Laboratories, Inc., New Bremen, OH, USA) to determine the residual levels of isoxaben, chlorpyrifos, and oxyfluorfen in the tissue. The QuEChERS technique was used to extract all tissue samples. Oxyfluorfen and chlorpyrifos were quantified using gas chromatography–mass spectrometry (GC-MS), and isoxaben was quantified using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Raina, 2011).

2.3. Statistical Analysis

All statistical analyses were carried out using SAS (version 9.4; SAS Institute, Inc., Cary, NC, USA). Regression analysis in SAS was carried out for the chlorophyll index and light-adapted fluorescence (Fv'/Fm') in response to pesticide treatments. For the total dry biomass (TDB), visual leaf injury, and residual pesticide concentration in the leaves, stems, and roots, we analyzed data using one way ANOVA for each species and pesticide. Vcmax and J estimates derived from A/Ci curves were also analyzed by using one way ANOVA for each taxon and each pesticide treatment.

Any data that did not meet the assumption of homogeneity of variance were transformed prior to statistical analysis.

3. Results

3.1. Leaf Visual Injury and Growth in Response to Pesticide Treatment

Leaf visual injury on plants was observed only for oxyfluorfen applications (Figure 1). Exposure to isoxaben or chlorpyrifos did not result in any visible damage to the taxa tested (data not shown). For oxyfluorfen, 0 and 0.005 mg/L did not produce any visual symptoms on any taxa. Increasing the oxyfluorfen dose to 0.01 mg/L produced leaf injury on *Hydrangea* and *Hosta*, but not on *Cornus*. Leaf injury was visible on *Cornus* plants only at the maximum dose (0.02 mg/L) of oxyfluorfen application. Visible leaf injury on *Hydrangea* and *Hosta* increased with an increasing dose of oxyfluorfen (Figure 1). Visible symptoms of oxyfluorfen exposure included leaf browning and smaller leaves (Figure 2). Oxyfluorfen application also reduced TDB for *Hydrangea*, but not for *Hosta* and *Cornus* (Table 1). For *Hydrangea*, increasing oxyfluorfen to 0.015 mg/L did not reduce the TDB, but further increase in dose reduced TDB. For *Hosta*, the decrease in the TBD was linear but was not statistically significant (Table 1). Exposure to isoxaben or chlorpyrifos in irrigation water did not affect the TDB of any of the taxa tested, except for *Hosta*, where the application of isoxaben first reduced TDB (till 0.7 mg/L) and then the biomass increased (1.4 mg/L).

3.2. Physiological Performance in Response to Pesticide Treatments

Irrigating *Hydrangea* and *Cornus* plants with simulated runoff containing oxyfluorfen reduced the SPAD chlorophyll index of leaves. For *Cornus*, the SPAD chlorophyll index decreased linearly with increasing oxyfluorfen concentration, while the SPAD index for *Hydrangea* decreased rapidly at oxyfluorfen concentrations above 0.01 mg/L (Figure 3). Isoxaben and chlorpyrifos did not affect the SPAD index in any of the three taxa (data not shown). Chlorpyrifos and oxyfluorfen did not affect the light-adapted fluorescence in any of the taxa (data not shown). Isoxaben reduced light-adapted fluorescence for *Hydrangea* and *Cornus*, but did not affect the Fv'/Fm' of *Hosta* (Figure 4). In both *Hydrangea* and *Cornus*, Fv'/Fm' decreased with increasing isoxaben concentration until 0.07 mg/L. Fv'/Fm' then remained constant, with further increases in isoxaben concentration (Figure 4). Irrigating with simulated runoff containing oxyfluorfen affected the photosynthetic rates of *Hosta* and *Hydrangea* (Figure 5). Exposure to oxyfluorfen reduced photosynthesis rates in *Hosta* when oxyfluorfen concentration in irrigation water was 0.01 mg/L or higher. For *Hydrangea*, exposure to oxyfluorfen decreased photosynthetic rates only when oxyfluorfen concentrations in irrigation were 0.015 mg/L or more. Exposure to oxyfluorfen in irrigation water did not affect photosynthesis in *Cornus*. Irrigation with water containing isoxaben slightly reduced photosynthesis for *Hosta* at concentrations of 0.07 mg/L or above (Figure 6). Isoxaben did not reduce photosynthesis in *Hydrangea* and *Cornus* (Figure 6). Chlorpyrifos did not affect the photosynthesis of any of the taxa tested (data not shown). Reduction in Vcmax and J

were only seen for oxyfluorfen application (Figure 7). Oxyfluorfen limited photosynthesis in *Hydrangea* by reducing Vcmax and J at concentrations of 0.015 m/L or above, and in *Hosta* by reducing Vcmax and J at a concentration of 0.01 mg/L or above (Figure 7).

3.3. Pesticide Absorption

Leaf pesticide concentration for the pesticides increased with increasing dose (Figure 8). However, taxa varied in their uptake and retention of each pesticide. For oxyfluorfen, *Hydrangea* had maximum absorption and retention in leaves, followed by *Hosta*, and then *Cornus* (Figure 8a). However, for isoxaben and chlorpyrifos, *Cornus* absorbed the highest amount, followed by *Hydrangea* and then by *Hosta* (Figure 8b,c). Isoxaben absorption and retention in leaves were consistently lower in all three taxa when compared to oxyfluorfen and chlorpyrifos. Stem and roots also absorbed and retained pesticides (Figure 9a–f). For all three pesticides, pesticide residues were always present in the stem (Figure 9a–c). The order of pesticide residue concentration in stem was chlorpyrifos > oxyfluorfen > isoxaben. *Hosta* plants do not have a true stem, therefore, we did not conduct a stem pesticide analysis in *Hosta*. Fine roots of *Hydrangea* absorbed and retained oxyfluorfen and isoxaben, but not chlorpyrifos (Figure 9d–f). For *Hydrangea*, absorption of oxyfluorfen was greater when compared to isoxaben. *Hosta* absorbed and retained all three pesticides, but unlike *Hydrangea*, its pesticide root retention order was chlorpyrifos > isoxaben > oxyfluorfen (Figure 9d–f). For *Cornus*, oxyfluorfen was not retained in fine roots, but fine roots absorbed and retained chlorpyrifos and oxyfluorfen (Figure 9d–f).

4. Discussion

4.1. Growth and Physiology

The potential for crop injury from residual pesticides can be a barrier for nursery operators to re-use runoff for irrigation. Additionally, unrealized reductions in crop growth from diluted, persistent pesticides can reduce profits by increasing production time or reducing plant quality. Leaves absorb pesticides that are applied to foliage, often producing leaf injury (Stevens and Baker, 1987). Visible injury is of concern to nursery producers, even if growth is not affected, because aesthetic appearance is important in marketing ornamental plants. Pesticide injury to leaves depends on the dose and type of pesticide used (Poudyal and Cregg, 2019). In this study, oxyfluorfen produced dose-dependent visible injury in all three taxa. Exposure to 0.02 mg/L of oxyfluorfen reduced visual leaf rating in *Hydrangea*, *Hosta*, and *Cornus* by 56.7%, 37.5%, and 18.4% when compared to the untreated control, respectively. Isoxaben and chlorpyrifos at the rates we used did not produce any visible injury, and hence irrigation with runoff containing these compounds can be considered relatively safe for use on these taxa. Oxyfluorfen works as a pre-emergent and post-emergence contact herbicide, therefore it is not surprising that it caused the greatest visible injury. When applied to leaves, oxyfluorfen inhibits chlorophyll formation in addition to causing lipid peroxidation and membrane degradation (Lee and Duke, 1994). In contrast, isoxaben is a pre-emergence herbicide that blocks germination (Heim et al., 1990), and chlorpyrifos is an insecticide. The sensitivity of plants to oxyfluorfen in this study is consistent

with observations by nursery growers who have reported crop damage following exposure to oxyfluorfen when runoff water was used as irrigation (personal communication). Oxyfluorfen application also produced leaf injury on rice (*Oryza sativa*) (Priya et al., 2017), yellowwood (*Cladrastis kentukea*) (Mathers,N/A), and sunflower (Jursik et al., 2011). Lactofen, a herbicide with a similar mode of action to oxyfluorfen, also produced leaf injury on soybean leaves (Wichert and Talbert, 1993). Oxyfluorfen (0.02 mg/L) reduced the TDB of *Hydrangea* by 21.5% and the TBD of *Hosta* by 43.1%. The leaf is where photosynthesis, a process to convert light, CO_2, and water to food, takes place, and photosynthesis governs plant growth (Kirschbaum, 2011). In our study, leaf injury from oxyfluorfen was observed primarily in *Hydrangea* and *Hosta*. For *Cornus*, leaf injury was only observed at the maximum dose (0.02 mg/L) of oxyfluorfen. This leaf injury in *Hydrangea* ultimately led to a reduction in TDB for *Hydrangea*. Isoxaben and chlorpyrifos did not injure leaves; hence, healthy growth was seen in plants receiving those pesticides.

Effects of exposure to pesticides in simulated runoff irrigation on photosynthetic parameters largely reflected sensitively as seen in visible injury to leaves. Isoxaben and chlorpyrifos did not affect the SPAD chlorophyll index, but oxyfluorfen reduced chlorophyll index for *Hydrangea* and *Cornus*. Oxyfluorfen is a protoporphyrinogen oxidase (PPO) inhibitor, and in the presence of light, this herbicide produces reactive oxygen species that break down chlorophyll and organelle membranes (Sherwani et al., 2015). Chlorophyll fluorescence can be an early indicator of pesticide damage and has been used to predict herbicide damage for various taxa (Silva

et al., 2014; Wang et al., 2018). In our study, however, oxyfluorfen or chlorpyrifos did not affect light-adapted chlorophyll fluorescence on any taxa. Although exposure to isoxaben in runoff did not affect the SPAD chlorophyll index, it did slightly reduce the light-adapted fluorescence for *Cornus* and *Hydrangea*. In a phytoremediation study by Fernandez et al. (1999), isoxaben also reduced chlorophyll fluorescence in canna, pickerel weed, and iris (Fernandez et al., 1999).

Photosynthesis and intercellular carbon dioxide response curves (A/Ci) can be used to determine the photosynthetic capacity of plants (Singh et al., 2013) and the shape of the A/Ci curve is generally determined by the capacity of rubisco for carboxylation (Vcmax) (at lower Ci rates < 200 ppm) and the rate of RuBP regeneration (J) (at higher Ci rates, >300 ppm) (Sharkey et al., 2007; Dinh et al., 2017). Visual observations of the A/Ci curve indicated oxyfluorfen concentrations of 0.015 and 0.02 mg/L reduced the carboxylation capacity of RUBISCO and the rate of electron transport for RuBP regeneration for *Hydrangea*. For *Hosta*, oxyfluorfen rates of 0.01 mg/L or higher reduced those parameters. These visual observations were also statistically confirmed by calculating Vcmax and J values. Oxyfluorfen reduced Vcmax and J values both for *Hydrangea* and *Hosta*, therefore reduction in rate of photosynthesis in both of those taxa was by the decrease in carboxylation capacity of RUBISCO enzyme (at lower Ci) and reduction in the rate of electron transport for RuBP regeneration (at higher Ci). Oxyfluorfen did not limit photosynthetic rates, Vcmax, and J for *Cornus* at any concentrations. Even though isoxaben is a pre-emergent herbicide, it slightly reduced photosynthesis in *Hosta* when the levels of isoxaben

were 0.7 mg/L or higher, but unlike oxyfluorfen, reduction in Vcmax and J was not observed. The lack of response in Vcmax and Jmax confirmed the reduction in photosynthesis to be minimal; therefore, it never translated to a decrease in growth. The decline in photosynthesis by oxyfluorfen corresponded well to the leaf injury. Photosynthetic response to pesticide exposure was more sensitive when compared to the TDB response to pesticide exposure.

4.2. Pesticide Absorption

Oxyfluorfen was absorbed and retained in leaves for all three taxa, and the absorption increased with increasing dose. Even though oxyfluorfen was retained on the leaves of all three taxa, leaf injury varied. *Hydrangea* had the maximum leaf injury, followed by *Hosta*, which is also supported by the fact that *Hydrangea* had the highest leaf retention of oxyfluorfen followed by *Hosta*. Taxa vary in their tolerance to oxyfluorfen, which is mainly governed by pesticide absorption, pesticide degradation inside leaves, and the affinity of the target sites (sites inside plants where herbicide binds to produce response) to herbicide (Chun et al., 2001). For *Cornus* to be tolerant, either most of the absorbed oxyfluorfen must have degraded inside the leaves or were stopped from reaching the target site. Isoxaben sensitivity is taxa-specific (Schneegurt et al., 1994), and a wide range of plants are tolerant to lower concentrations of post applied isoxaben (Wehtje et al., 2006). In the current study, isoxaben absorption and retention in leaves were dose-dependent and similar across taxa. Among the pesticides investigated, isoxaben was least absorbed and retained, which may be because the leaf absorption of isoxaben is very low, and isoxaben is

minimally translocated beyond the application point (Schneegurt et al., 1994; Wehtje et al., 2006). Isoxaben also did not produce visible symptoms or reduce growth. The mode of action for isoxaben is the inhibition of cell wall biosynthesis, which is dose-dependent (Heim et al., 1990). Our range of doses for isoxaben may not have been high enough to produce phytotoxicity. In a study by Heim et al. (1993), variation in the sensitivity of *Agrostis palustris* to isoxaben was associated with decreased sensitivity of isoxaben binding sites, which might have also occurred in our study. Fernandez et al. (1999), found that isoxaben reduced photosynthesis in three monocot species, while a slight reduction in photosynthesis for monocot-*Hosta* was also observed in our study. Isoxaben application did not reduce photosynthesis in *Hydrangea* and *Cornus* as isoxaben response is taxa-specific (Willoughby et al., 2003).

Similar to oxyfluorfen and isoxaben, absorption and retention of chlorpyrifos in leaves was also dose-dependent and increased with increasing dose. Chlorpyrifos may enter inside plant tissue through leaves or roots, and its absorption and retention vary within species (Lu et al., 2014). In a study by Fan et al. (2013), chlorpyrifos was absorbed and retained in the leaves of six different leafy vegetables with retention concentration varying within species (Fan et al., 2013). In our study, absorption and retention of chlorpyrifos did not affect growth and physiological performance on any of the taxa. In wheat, root application of chlorpyrifos led to the accumulation of chlorpyrifos in root and shoots, but growth was not affected (Copaja et al., 2014). Wheat and rapeseed also absorbed chlorpyrifos that was mixed in irrigation water, but growth was not affected

in either taxon (Wang et al., 2007). Chlorpyrifos does not have specific sites of action in plants but may produce phytotoxicity depending on dose, however, the dose of chlorpyrifos that we applied was not enough to produce any morphological or physiological symptoms in the three taxa that we tested.

In our study, the type and concentration of pesticides absorbed and retained in fine roots varied dramatically among the taxa. Isoxaben concentrations in stem and fine roots were lower when compared to chlorpyrifos or oxyfluorfen because isoxaben is less mobile in plants, and up to 99% of isoxaben applied may be adsorbed by pine bark (Schneegurt et al., 1994; Wehtje et al., 2006). Application of all three pesticides also resulted in the accumulation of those pesticides in the stem, which may either be through root absorption, translocation from the leaves, or both (Duke, 1990; Schneegurt et al., 1994; Chun et al., 2001).

5. Conclusions

Phytotoxicity due to pesticide exposure from runoff irrigation depends on the plant type, type of pesticide applied, and concentration of pesticide. In our study, 0.01 mg/L was the threshold level of oxyfluorfen to produce leaf visual injury in *Hydrangea* and *Hosta*, while 0.02 mg/L of oxyfluorfen was required to induce phytotoxicity in *Cornus*. Irrigation with simulated runoff containing isoxaben and chlorpyrifos were comparatively safe for all three taxa tested. Isoxaben caused a slight reduction in PSII efficiency, but neither isoxaben or chlorpyrifos affected dry weight biomass, photosynthetic biochemistry, or caused visible leaf injury as oxyfluorfen did. This

response likely reflects the fact that oxyfluorfen is an herbicide that has post-emergent activity and therefore has the potential to affect sensitive plants following prolonged low-dose exposure. The other pesticides examined in this study are an insecticide (chlorpyrifos) and an herbicide without post-emergent activity (isoxaben), which may be less likely to impact plant growth and physiological function. Among the three taxa, *Hydrangea* was most sensitive, followed by *Hosta*, and then by *Cornus*. Differences among taxa in their sensitivity to oxyfluorfen may also be due in part to differences in plant uptake and translocation. The taxa that were most affected by oxyfluorfen exposure, *Hydrangea* and *Hosta*, also had the highest leaf residual concentrations of that compound. Growth impacts of pesticide exposure in irrigation water are also linked to physiological function. Pesticides had more significant effect on photosynthesis compared to growth. The results of this study establish the potential of using runoff water containing isoxaben and chlorpyrifos. However, consideration should be made on the concentration of pesticides in runoff and plant taxa irrigated. As with all nursery research, a limitation of the current study is that we only considered three taxa, whereas most commercial nurseries produce dozens, if not hundreds, of different types of plants. We specifically selected taxa that had shown sensitivity to pesticides in similar studies, but it is possible that some taxa may have lower thresholds for pesticide impacts. Likewise, in addition to the three pesticides studied, other compounds including mefenoxam, oryzalin, glyphosate, acephate, and bifenthrin may be found in nursery retention ponds (Poudyal and Cregg, 2019). We suggest that researchers conduct similar studies with other commonly used pesticides in a nursery and also determine the pesticide sensitivity of the plants

that are different from ours. Their research in combination with ours will provide a stronger base for the adoption of irrigation practice using runoff water.

APPENDIX

APPENDIX

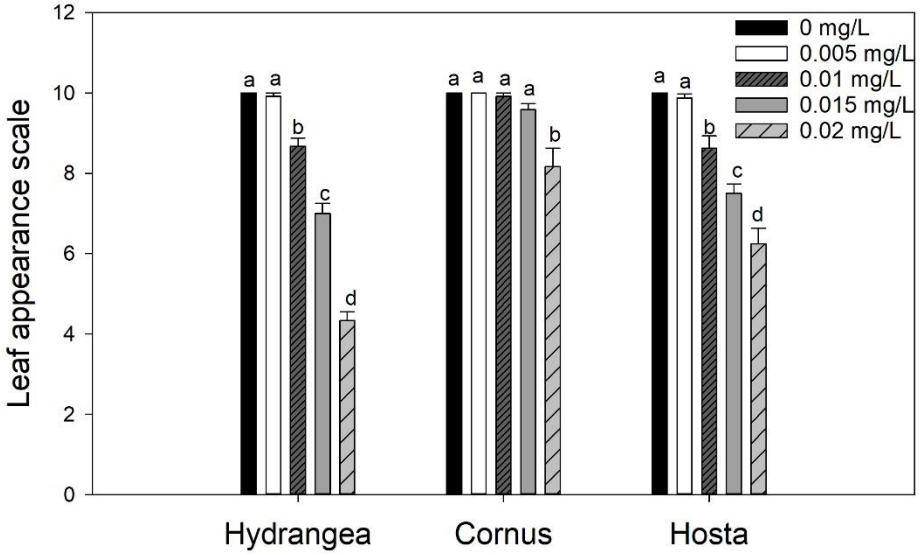

Figure III - 1. Mean leaf visual rating of *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold Standard' plants irrigated with simulated runoff containing five concentrations of oxyfluorfen for three months. Visual rating was based on a scale of 1 to 10 (10 = no injury to 1 = dead plant). Means within a taxon followed by the same letter are not different at $p < 0.05$. Mean separation was by the Fisher least significance difference (LSD) test.

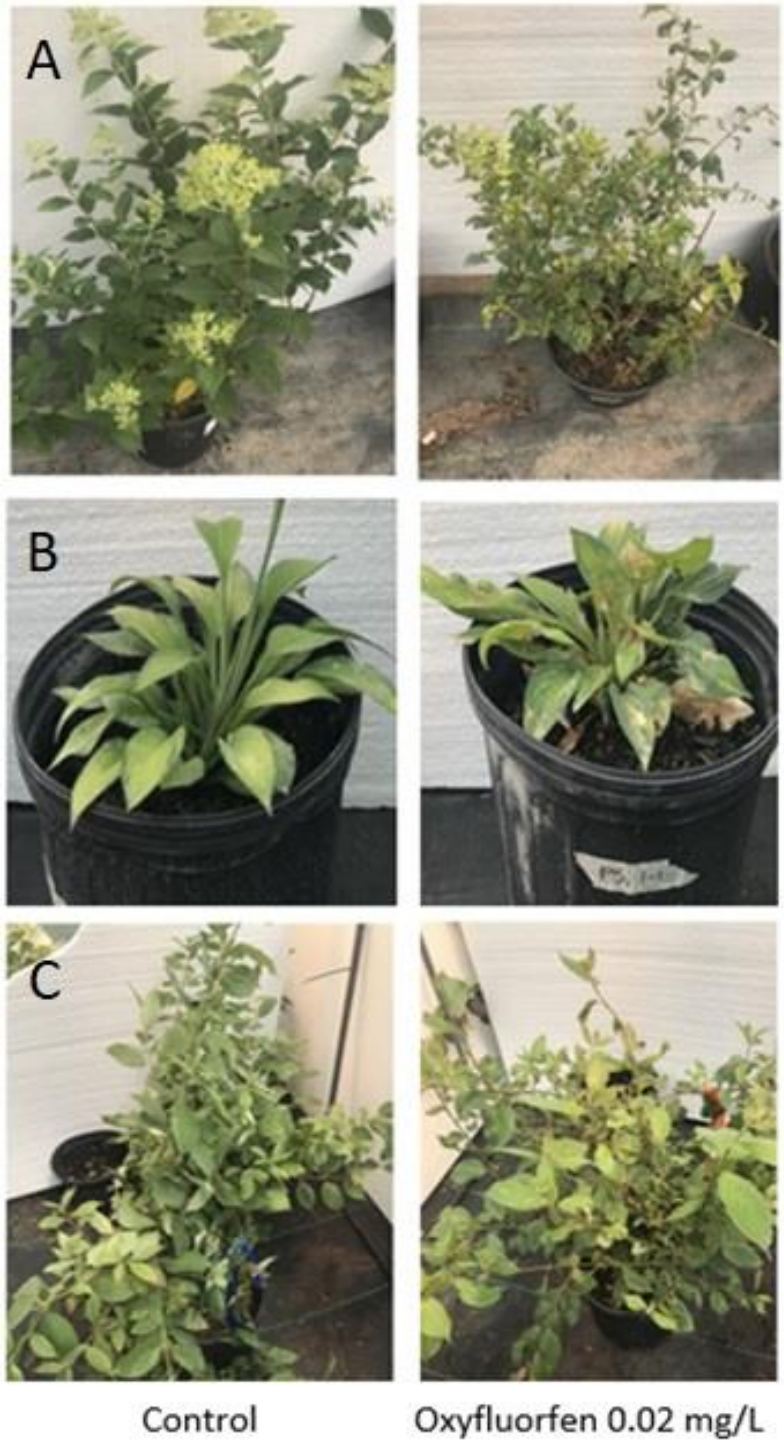

Figure III - 2. Images of Hydrangea paniculata ‘Limelight’ (A), Hosta ‘Gold Standard’ (B) and Cornus obliqua ‘Powell gardens’ (C) exposed to 0 (control) or 0.02 mg/L of oxyfluorfen application irrigated for three months.

Table III - 1. Mean total dry biomass (g) for *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold standard' plants irrigated for three months with simulated runoff containing oxyfluorfen, isoxaben, or chlorpyrifos. Means within a column followed by the same letter for a given taxon are not different at $p < 0.05$. Post-hoc mean separation was done using the Fisher least significance difference (LSD) test.

	Oxyfluorfen		
Concentration (mg/L)	*Hydrangea*	*Cornus*	*Hosta*
0	135.71ab	189.65a	31.58a
0.005	152.97a	180.68a	30.23a
0.01	153.11a	188.44a	23.63a
0.015	130.65ab	210.92a	22.72a
0.02	106.49b	207.20a	17.93a

Concentration (mg/L)	Chlorpyrifos		
	Hydrangea	*Cornus*	*Hosta*
0	135.71a	189.65a	31.58a
0.05	139.71a	166.40a	29.76a
0.1	138.25a	213.63a	41.32a
0.2	138.62a	197.22a	32.38a
0.4	143.45a	194.87a	23.71a

	Isoxaben		
Concentration (mg/L)	*Hydrangea*	*Cornus*	*Hosta*
0	135.71a	189.65a	31.58a
0.15	140.63a	225.85a	19.11bc
0.35	155.07a	198.92a	16.54c
0.7	161.66a	211.75a	13.05c
1.4	141.34a	186.94a	27.20ab

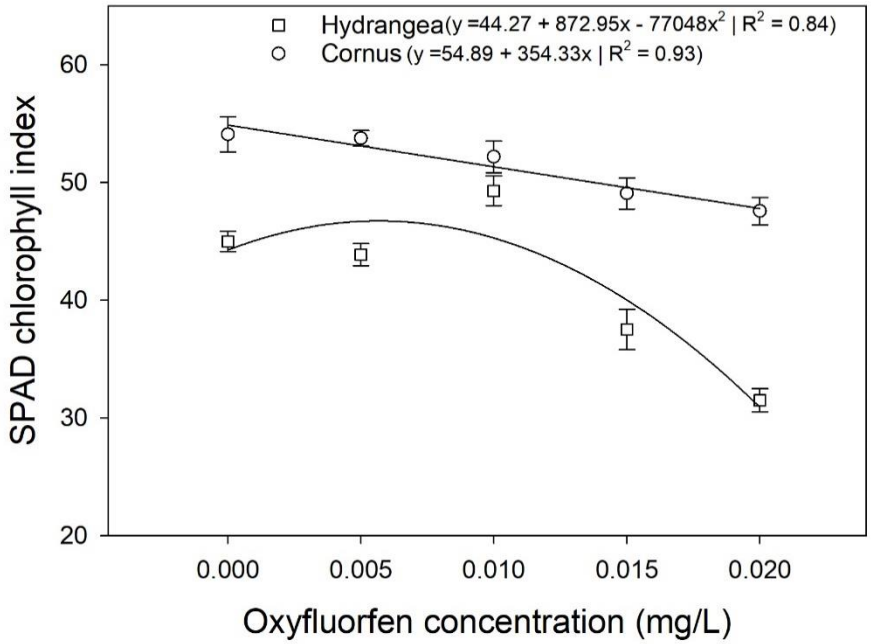

Figure III - 3. Mean chlorophyll index (CI) of *Hydrangea paniculata* 'Limelight' and *Cornus obliqua* 'Powell Gardens' in response to simulated runoff containing five different concentrations of oxyfluorfen applied for three months. CI for *Hydrangea* followed quadratic regression while the CI of *Cornus* decreased linearly.

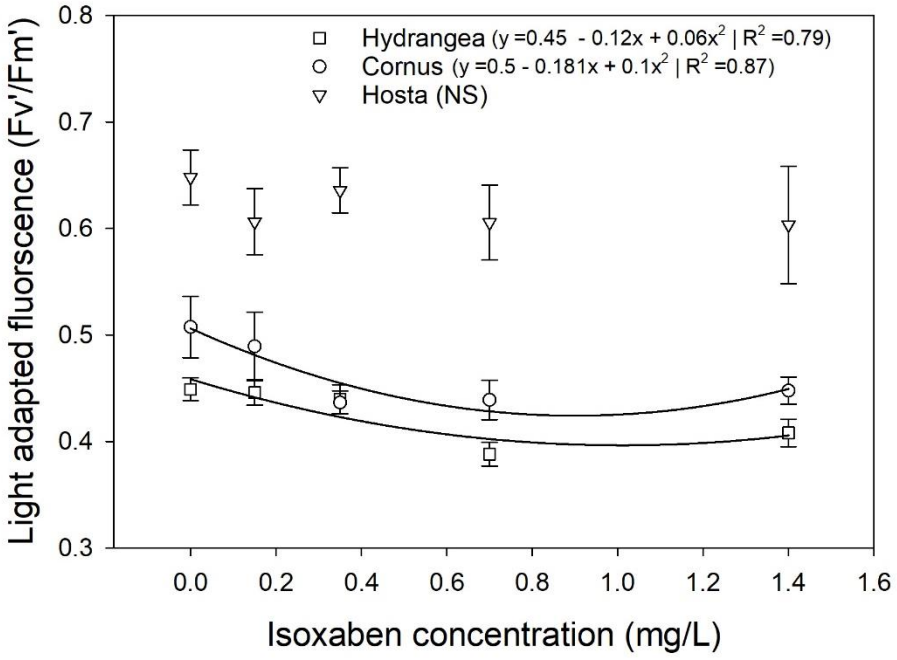

Figure III - 4. Mean light-adapted fluorescence (Fv'/Fm') of *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold Standard' plants in response to simulated runoff containing five different concentrations of isoxaben applied for three months. Fv'/Fm' for *Hydrangea* and *Cornus* both followed quadratic regression, while regression of *Hosta* was not significant at $p < 0.05$.

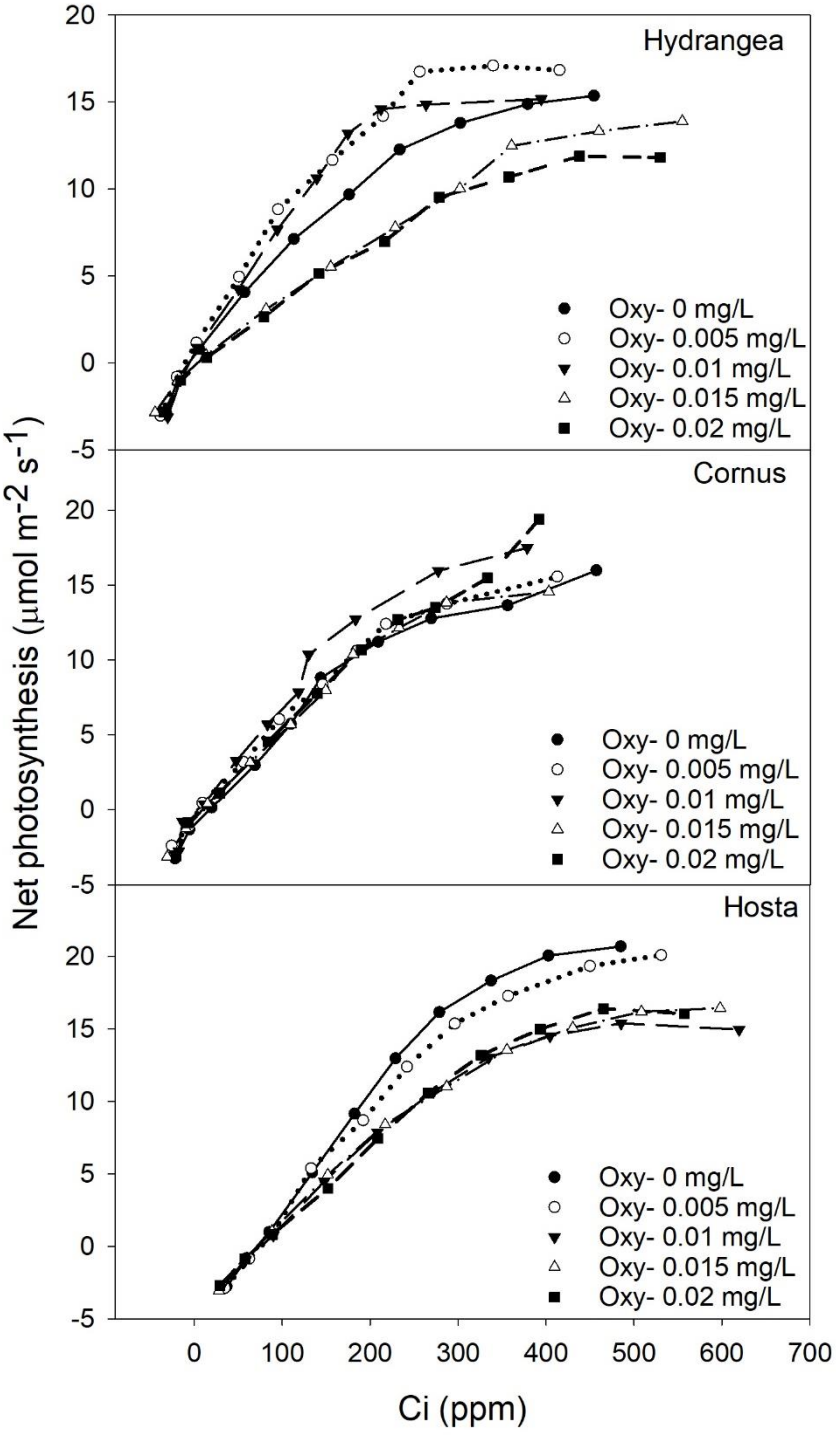

Figure III - 5. Carbon dioxide response (A/Ci) curve of *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold Standard' plants exposed to five different concentrations of oxyfluorfen (Oxy) for three months.

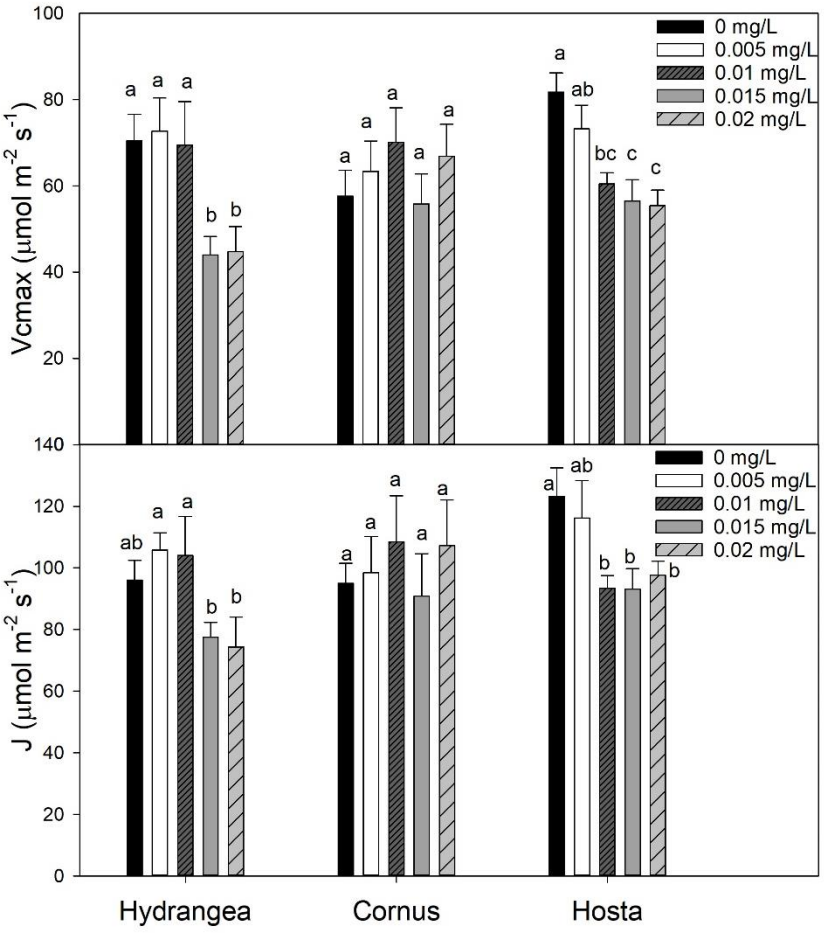

Figure III - 6. Mean Vcmax (maximum rate of RUBISCO for carboxylation) and J (rate of electron transport for RuBP regeneration) of *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold Standard' plants irrigated with simulated runoff containing five concentrations of oxyfluorfen for three months. Means within a taxon followed by the same letter are not different at $p < 0.05$. Mean separation was by the Fisher least significance difference (LSD) test.

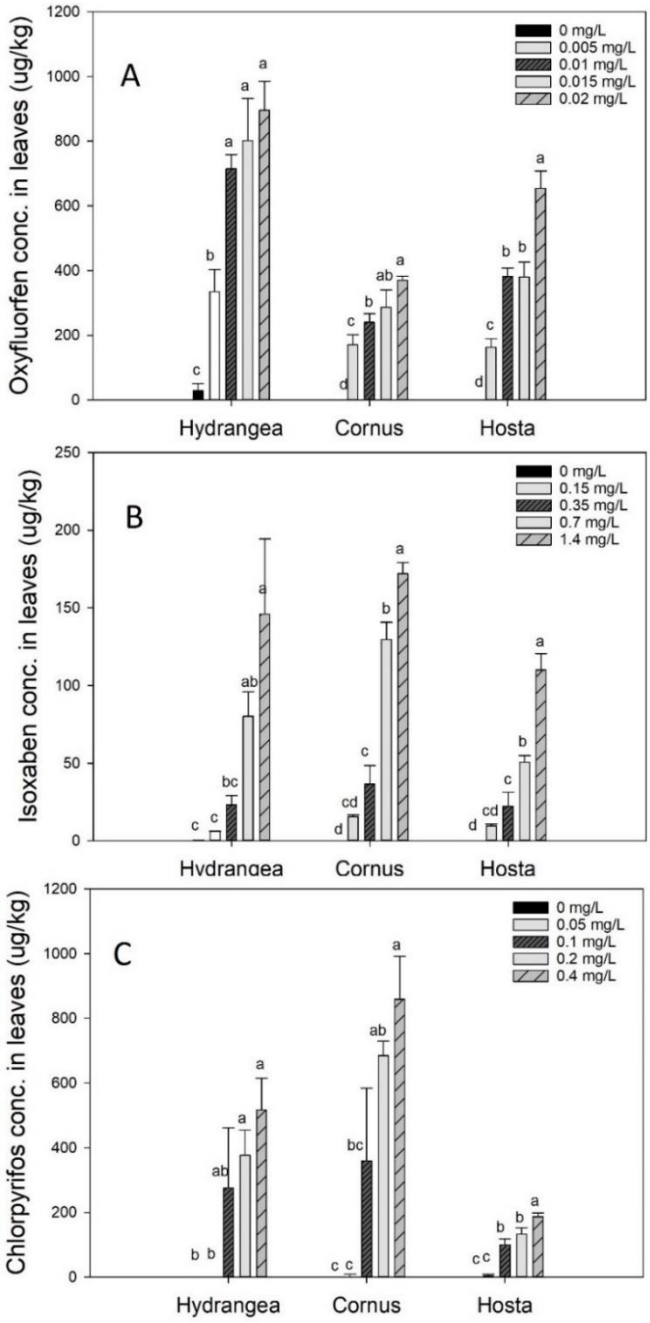

Figure III - 7. Concentration of oxyfluorfen (**A**), isoxaben (**B**) ,and chlorpyrifos (**C**) in leaves for *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold Standard' plants following irrigation with simulated runoff containing five different concentrations of oxyfluorfen, isoxaben, and chlorpyrifos applied for three months. Means within a taxon followed by the same letter are not different at $p < 0.05$. Post-hoc mean separation was done using the Fisher least significance difference (LSD) test.

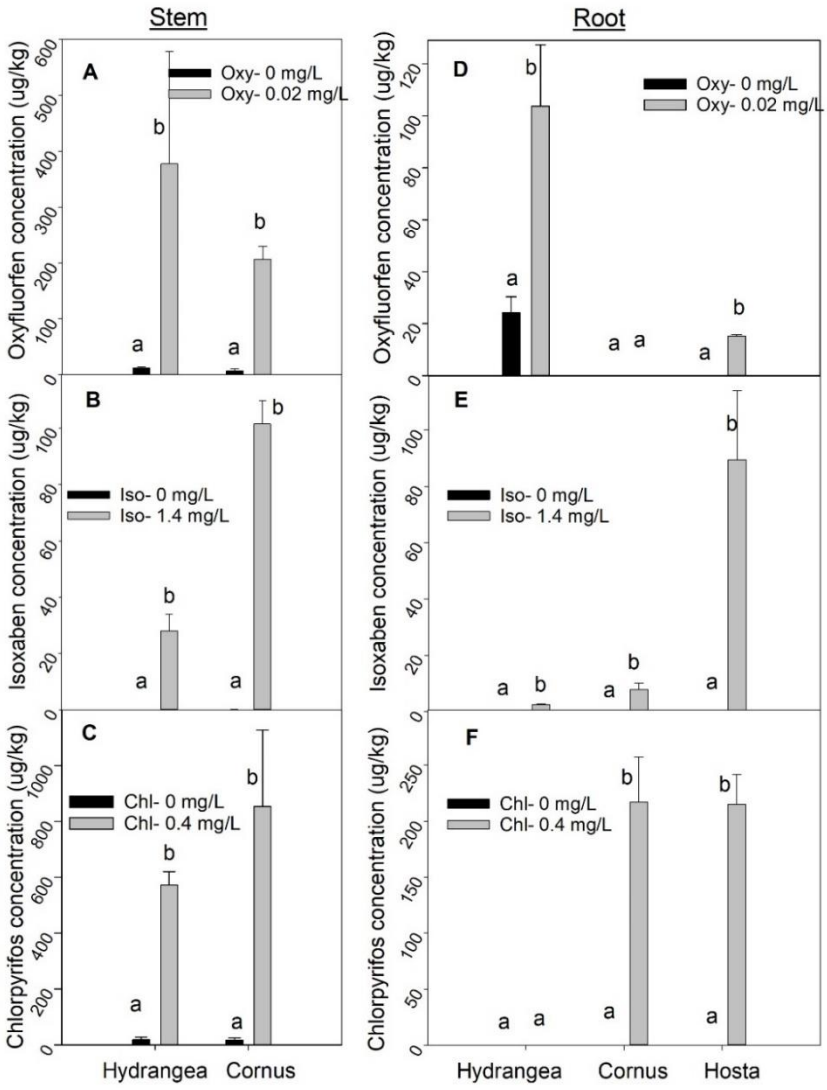

Figure III - 8. Concentration of oxyfluorfen (**A**), isoxaben (**B**), and chlorpyrifos (**C**) in the stem for *Hydrangea paniculata* 'Limelight' and *Cornus obliqua* 'Powell gardens' and concentration of oxyfluorfen (**D**), isoxaben (**E**), and chlorpyrifos (**F**) in root for *Hydrangea paniculata* 'Limelight', *Cornus obliqua* 'Powell Gardens', and *Hosta* 'Gold Standard' plants following irrigation with simulated runoff containing five concentrations of oxyfluorfen (Oxy), isoxaben (Iso), and chlorpyrifos (Chl), applied for three months. Means within a taxon followed by the same letter are not different at $p < 0.05$. Post-hoc mean separation was done using the Fisher least significance difference (LSD) test. Bar graphs for treatment are missing when the residual pesticide concentration is very low (zero or close to zero).

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SECTION IV

SENSITIVITY OF HYDRANGEA TO RESIDUAL HERBICIDES IN RECYCLED IRRIGATION VARIES WITH PLANT GROWTH STAGE

Sensitivity of Hydrangea to residual herbicides in recycled irrigation varies with plant growth stage

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Abstract

Recycling irrigation runoff is a viable option to achieve sustainability in horticultural production systems, but residual herbicides present in recycled water may be phytotoxic. The sensitivity of plants to residual herbicides may vary depending on the growth stage of the plant. Therefore, if sensitive growth stages are avoided, the risk associated with using recycled water may be reduced. Here, we quantified the effect of residual oryzalin and oxyfluorfen exposure at various growth stages of Hydrangea paniculata. Exposure to both herbicides reduced plant growth, leaf visual rating, SPAD index, net photosynthesis and light-adapted fluorescence of H. paniculata. Herbicide injury was higher for plants exposed to herbicides at early growth stages; however, the recovery rate of those plants was also rapid. For oxyfluorfen, leaf damage was less noticeable as plants continued to produce healthy new growth immediately after the end of exposure but for oryzalin, even newly formed leaves developed herbicide injury, therefore leaf damage continued to progress before recovering. Physiological measurements such as SPAD index, net photosynthesis and light-adapted fluorescence responded more quickly compared to growth index and leaf visual rating hence provided an early indicator of plant recovery. It is best to avoid early growth stages when irrigating with recycled water that may contain herbicides. However, damage caused by residual herbicide exposure at all growth stages was transient, and plants recovered over time. When assessing herbicide damage and recovery, physiological measurements such as net

photosynthesis and light-adapted fluorescence can provide rapid insights on instant plant performance.

Keywords: Oryzalin; Oxyfluorfen; Nursery; Ornamental crop; Phytotoxicity

1. Introduction

Production of container-grown ornamental nursery plants is an intensive horticultural system that requires frequent inputs of water and agrochemicals to produce visually appealing plants. Irrigation in nurseries often generates substantial amounts of runoff and up to 70-80% of applied water may be lost from nursery production areas.(Beeson and Knox, 1991; Fain et al., 2000; Poudyal and Cregg, 2019). Runoff generated from nurseries often contains various agrochemicals, which, if released without remediation, may degrade neighboring ecosystems. Public awareness of non-point source pollution is growing, and so are the regulations to reduce irrigation return flow. Several states including California, Florida, Texas, Oregon, and Maryland restrict water discharge from nurseries, and other states will likely follow.(Oki and White, 2012; Fulcher et al., 2016). As water security, accountability and costs associated with withdrawals from primary water sources are rising (Rodell et al., 2018; de Amorim et al., 2018), recycling return flow is becoming environmentally sustainable and economically viable (Fulcher et al., 2016; Ferraro et al., 2017; Pitton et al., 2018). Therefore, nursery growers in states with and without mandatory return flow capture are starting to recycle water for irrigating ornamental crops.

Recycling nursery return flow for irrigation conserves water and can improve water security but it also holds some degree of risk to growers. Residual pesticides in recycled water may be phytotoxic to sensitive crops (Poudyal et al., 2019; Poudyal and Cregg, 2019), and some growers report evidence of phytotoxicity associated with pesticides (personal communication).

Chronic, low-dose exposure to pesticides in irrigation water can result in reduced plant growth, chlorosis, leaf distortion and other visible plant injury. For example, pendimethalin (2.24 kg a.i./ha) reduced plant width in heather (*Calluna vulgaris* L.) and isoxaben 0.05 kg a.i./ha reduced plant height in wintercreeper euonymus (*Euonymus fortunei* Turcz.), when applied as overhead spray (Regan and Ticknor, 1987). Glyphosate residue in the rhizosphere reduced growth and biomass production in sunflower (*Helianthus annuus* L.)(Tesfamariam et al., 2009) and the application of imazapyr and triclopyr for weed management in power transmission lines reduced germination rate and vegetative growth of non-target plants; yarrow (*Achillea millefolium* L.) and fireweed (*Chamerion angustifolium* L.) (Isbister et al., 2017).

The concentration of pesticides in recycled water is orders of magnitude lower compared to standard application rates, but still may cause sub-lethal effects on plants. Sensitivity of plants and their capacity to overcome injury may depend on the growth stages of plants (Follak and Hurle, 2004). Most leaves in young plants or actively growing shoots are new and have thinner cuticles compared to mature plants and shoots (Jursik et al., 2013; Rouse and Dittmar, 2013), hence young plants and new shoots are more prone to phytotoxicity compared to matured plants and shoots, but may not always be the case (Richardson, 1972). Phytotoxic symptoms produced by short term exposure to pesticides are either reversible or irreversible (Follak and Hurle, 2004), and the latter is of most concern to growers. Peach seedlings sprayed with simazine at 3 mg/L and terbacil at 3 mg/L showed excellent recovery from the damage but the seedlings sprayed with oryzalin at 6

mg/L did not recover (Lourens et al., 1989). Trimec Classic (2,4-D + MCPA + dicamba) and glyphosate at 1.6 kg a.i/ha were applied as overhead spray in rose plants and the plants were evaluated for pesticide related injury. Injury by Trimec recovered after 11 weeks of exposure but the injury caused by glyphosate did not recover. Peach did not recover from the phytotoxicity cause by oryzalin (6 mg/L) throughout the study period (Gonzalez and Karlik, 1999).

Herbicides commonly used in container nursery production, including oryzalin and oxyfluorfen, are often found in nursery return flow (Keese et al., 1994; Riley et al., 1994; Goodwin and Beach, 2001). Oryzalin is a pre-emergent herbicide belonging to the dinitroaniline family; it binds to free tubulin and restricts the formation of microtubules, arresting cells in the dividing stage, but when the herbicide is washed off the new microtubules reappear. After exposure to oryzalin, younger cells show quick recovery and reassembly of microtubules while older cells take longer to recover (Wasteneys and Williamson, 1989). Oxyfluorfen is a protoporphyrinogen oxidase (PPO) inhibitor and is applied as both pre-and post-emergent herbicide. Photo-oxidative damage caused by oxyfluorfen can reduce net photosynthesis (*A*) and chlorophyll fluorescence (Sharma et al., 1989). Oxyfluorfen also causes disturbances in mitotic cell division, producing clastogenic effects and C-mitotic effects (Dragoeva et al., 2012). Plants may recover from phytotoxic damage caused by oxyfluorfen depending upon the length of exposure and time available for recovery. Complete recovery from phytotoxicity in rice was seen just a month after oxyfluorfen exposure (Priya et al., 2017). Plant injury associated with oxyfluorfen exposure is

often more acute when plants are exposed to oxyfluorfen at early growth stages compared to late growth stages (Akey and Machado, 1985; Nosratti et al., 2017).

In order to manage risks associated with recycled water for irrigation, we need to develop an improved understanding of the basis of plant injury from chronic low-dose pesticide exposure. Recycling return flow water for irrigation is a viable option and, if the sensitive growth stages are avoided, the risk associated with irrigation from recycled water can be minimized. Quantifying chlorophyll fluorescence and *A* of plants exposed to the herbicide can reveal physiological herbicide injury (Moreland et al., 1972; Sharma et al., 1989; Krugh and Miles, 1996; Baker, 2004; PAN et al., 2009) and can be used to monitor herbicidal stress in plants. In addition to physiological performance; growth, visual appearance, and flower quality are also essential attributes of ornamental plants as customers are more likely to buy visually appealing plants. Therefore morphological assessments, in addition to physiological performance, can provide a complete picture of herbicide injury in plants. This study was focused on (1) quantifying the physiological and morphological effects of residual oryzalin and oxyfluorfen in simulated recycled water at various growth stages of *Hydrangea paniculata* Siebold. (Limelight), (2) identifying variation in sensitivity among growth stages of plants to residual herbicide exposure, and (3) determining time required to recover from herbicide damage. We used *Hydrangea paniculata* as it is one of the most popular shrub in the U.S. (Odom, 2016; “Shrubs: In-demand,” 2018) and are sensitive to residual herbicide in irrigation water (Poudyal et al., 2019).

2. Materials and methods

2.1. Plant material and treatments

This study was conducted in a greenhouse at the Michigan State University Horticulture Teaching and Research Center (HTRC) located in Holt, Michigan, USA (42.67° N, 84.48° W). *Hydrangea paniculata* Siebold. 'Limelight' plants grown in pine bark and peat moss substrate (80: 20; Volume: Volume) were used for our study. Starter plants from 10 cm plugs (liners) of *H. paniculata* were planted on May 24, 2018, and grown outdoors in 11.3 L plastic containers at the HTRC and received 19 mm of daily overhead irrigation and medium recommended dose (60 g per container) of controlled-release fertilizer (18–5–8; N-P_2O_5-K_2O with micronutrients, 5–6 months, ICL Specialty fertilizers, Summerville, SC, USA) applied as a top-dressing. Plants were brought into an unheated plastic hoop house on October 26, 2018, and leaves were allowed to senesce and the plants to go dormant. All plants were pruned consistently, leaving only three shoots of 10 cm length per plant.

All the plants were brought into the greenhouse on January 15, 2019, and were fertilized with 60 g of the same fertilizer as mentioned above and irrigated via a drip irrigation system. The temperature in the greenhouse was set to 23°C and plants received natural light. Buds began to sprout on plants on January 27, 2019, and by Feb 6, 2019, all the plants had visible leaves on at least six different nodes. As all the plants had initiated growth by Feb 6, 2019, this day was referenced as 'initiation of growth' for the study.

Plants were assigned at random to two treatment groups; one set receiving simulated recycled irrigation containing 0.02 mg/L of oxyfluorfen (Goal 2XL; Dow AgroSciences LLC, Indianapolis, IN) and the other set receiving simulated recycled irrigation containing 8 mg/L of oryzalin (Surflan AS; United Phosphorus Inc., King of Prussia, PA). We prepared the desired concentration of oxyfluorfen and oryzalin solution by dissolving the appropriate amount of each herbicide in 50 liters of water. Two 100 L black plastic tanks were used to prepare and store herbicide solution. A submersible sump pump was used to agitate the herbicide solution and to manually apply herbicide solution as overhead irrigation on all the leaves of the plant and on the substrate. Herbicide solution was applied daily with an irrigation wand (Yardworks® Front Trigger Red 7-Pattern Nozzle, Model Number: 56715) and lasted for a minute. Herbicide solutions were freshly prepared two times a week. We selected 0.02 mg/L of oxyfluorfen and 8 mg/L of oryzalin as herbicide treatments as these are the maximum concentrations reported in nursery irrigation return flow or retention reservoirs (Keese et al., 1994; Riley et al., 1994; Briggs et al., 2003).

Each treatment group was further divided into five sub-groups, with five individual plants (replication) per sub-group. One sub-group of plants served as an untreated control; the remaining four groups received herbicide exposure at four different growth stages, i.e., five days after initiation of growth (GS+5; maximum of two nodes per branch), 15 days after initiation of growth (GS+15; maximum of five nodes per branch), 25 days after initiation of growth (GS+25; maximum

of seven nodes per branch) and 35 days after initiation of growth (GS+35; maximum of nine nodes per branch). A flow chart for herbicide exposure is described in Table 1. Plants were temporarily isolated with foam panels during the spraying process to avoid cross-contamination and then put back in place. After ten days of continuous exposure, plants were returned to the drip irrigation system and observed for damage and recovery over the course of the next 20 days and also at the end of the study at 65 days. Each time the plants were evaluated for treatment responses, simultaneous observations of plants from the control group were also carried out.

In addition to the plants mentioned above, three sets of *H. paniculata* with three plants per set were grown separately until bloom under drip irrigation following a similar management strategy as previous mentioned plants. When flower panicles were approaching complete bloom, flowers on the first set were sprayed with oxyfluorfen, flowers on the second set were sprayed with oryzalin using the same application rates, durations, and methods as for the whole-plant exposure experiments above. A third set of three plants were allowed to bloom and acted as a control.

2.2. Assessment of physiological and morphological effect of herbicide

Herbicide injury was assessed for each treatment group at the end of each ten-day herbicide exposure period. Injury was also assessed on 10 and 20 days after cessation of herbicide exposure to determine plant recovery. One final assessment was conducted at the end of the study i.e. 65 days after first leaf emergence. On each assessment of phytotoxicity, control plants were assessed simultaneously to compare with the herbicide exposure group.

At each assessment, leaves were examined for the visible damage (e.g., discoloration, stunting, and curling) and scored on the scale of zero (all dead leaves) to ten (no leaf damage). Growth index (GI; an average of plant height and two perpendicular widths) was measured on each plant.

A portable photosynthesis system (LI-6400 XT, Li-Cor, Inc., Lincoln, NE) mounted with a leaf chamber fluorometer (LI-6400-40, Li-Cor, Inc., Lincoln, NE) was used to measure *A* and light-adapted fluorescence (*Fv'/Fm'*). A section of a fully mature leaf on either the 3rd or 4th node from the shoot of each plant was used for physiological measurements. Photosynthetically active radiation (PAR) in the chamber was set to 1500 µmol m^{-2} s^{-1}, block temperature was set to 25°C, and 400 ppm of CO_2 was supplied, and relative humidity in the chamber varied between 40–60%. Each leaf was then acclimatized for five minutes. We first measured *A* and then *Fv'/Fm'*. We also measured the SPAD index (leaf chlorophyll index) on three leaves per plant on either the 3rd or 4th node from the top using a portable SPAD meter (SPAD-502; Minolta corporation, Ltd., Osaka, Japan). At the end of the study, plants were harvested and dried in an oven (45°C), to determine total above-ground biomass (TDB; the weight of leaves and stem after drying in an oven at 45°C for three days).

Flowers were assessed for herbicide phytotoxicity on nine additional plants. After ten days of herbicide exposure, panicles were left to completely bloom (for 12 days) and then were assessed

for their GI (average of height and two perpendicular widths), total flower mass (TFM; the weight flower after drying in an oven at 45°C for 3 days) and visual injury (on the scale of zero to ten).

2.3. Statistical analysis

The experiment was conducted as a completely randomized design with five replications per treatment. All the statistical analyses were carried out using SAS (version 9.4; SAS Institute, Inc., Cary, NC). Before analysis, all the data except visual injury were converted to percentage based on means of the control. The mean value of control was assumed to be 100 percent and the variation in control was also calculated based on mean value of 100 percent. For each herbicide, we analyzed visual leaf injury, and percentage change in GI, A, *Fv'/Fm'*, and SPAD index using one way ANOVA. Final data after 65 days of the initiation of growth for TDB, visual leaf injury, GI, A, *Fv'/Fm'*, and SPAD index were analyzed, using one way ANOVA. Preliminary analyses indicated significant differences for some evaluation parameters between herbicides and growth stage × herbicide interactions; therefore, data were analyzed separately for each herbicide. Fisher's least significant difference post-hoc mean separation was carried out for ANOVA with p-value of 0.05 or less to determine a difference within treatments, for each herbicide. Any data that did not meet the assumption of homogeneity of variance were transformed before statistical analysis.

3. Results

3.1. Morphological responses to herbicide exposure

Exposure to simulated recycled irrigation containing oryzalin reduced GI of *H. paniculata* compared to plants that were not exposed (Fig. 1). The largest reduction in GI (20%) was observed immediately after the end of oryzalin exposure, when plants were exposed at the earliest growth stage i.e., GS+5. However, plants exposed to oryzalin at GS+5 recovered quickly compared to other growth stages. Oryzalin exposure at GS+15, GS+25, and GS+35 resulted between 9 to 12% reduction in GI immediately after the end of the exposure. Even with lower reduction compared to GS+5, GI of plants exposed at GS+5, GS+15, and GS+25, was still lower compared to control plants, 20 days from the end of oryzalin exposure. Reduction in GI caused by oxyfluorfen exposure was similar to that of oryzalin. Reduction in GI, immediately after the end of oxyfluorfen exposure, was highest (32%) when plants were exposed to oxyfluorfen at GS+5 and least (7.5%) when plants were exposed to oxyfluorfen at near maturity i.e., GS+35. GI of plants receiving oxyfluorfen exposure at GS+25 recovered completely in 20 days after the end of exposure and GI in plants receiving oxyfluorfen exposure at GS+35 recovered completely just in ten days after the end of oxyfluorfen exposure. Plants receiving oxyfluorfen exposure at GS+5 and GS+15 did not recover completely even after 20 days from the end of oxyfluorfen exposure (Fig. 1).

The location of leaf injury was similar for both herbicides and occurred on younger leaves towards the tip of the stem. In contrast, the type of damage caused by each herbicide was different.

Oryzalin exposure distorted leaf shape and produced random yellow patches in leaves while oxyfluorfen exposure reduced leaf size and caused complete or interveinal necrosis (Fig 2). Immediately after the end of oryzalin exposure, plants exposed at GS+5 had the lowest leaf visual rating (7.4), while plants exposed at all other growth stages had lower but similar (8.2 to 8.8) leaf damage (Fig. 3). Plants did not recover from leaf injury immediately after end of oryzalin exposure. Instead, leaf visual rating declined from one day after the end of exposure to 10 days after the end of exposure. Leaf injury across all growth stages started recovering (by growth of healthy new leaves) rapidly and was only 10% lower compared to control on the 20th day after the end of oryzalin exposure (Fig. 3). Leaf injury for oxyfluorfen exposure followed a similar pattern as GI. Immediately after the end of oxyfluorfen exposure, the lowest leaf visual rating (4.8) was observed on plants exposed to oxyfluorfen at GS+5, whereas leaf visual rating was highest (8) on plants exposed to oxyfluorfen at GS+25. Unlike oryzalin, plants exposed to oxyfluorfen at all growth stages started recovering (by growth of healthy new leaves) immediately after the end of oxyfluorfen exposure. After 20 days from the end of oxyfluorfen exposure, plants exposed at GS+5 had lowest leaf injury, while plants exposed at GS+35 had maximum leaf injury. However, leaf visual rating for plants receiving oxyfluorfen exposure at all growth stages was still 6 to 13% lower compared to control even on the 20th day after the end of oxyfluorfen exposure (Fig. 3).

3.2. Physiological responses to herbicide exposure

Exposure to both herbicides reduced SPAD chlorophyll index. SPAD index was reduced on plants across all growth stages on the 1st and the 10th day after the end of herbicide exposure. However, on the 20th day after the end of the herbicide exposure SPAD index of exposed plants was similar to control plants regardless of when plants were exposed to herbicide (Fig. 4).

Exposure to each herbicide at all growth states reduced *A* at one or ten days after exposure, or both (Fig. 5). However, *A* recovered to the same level as non-exposed plants for all plants regardless of exposure dates or herbicide by day 10 or 20 (Fig. 5). Net photosynthesis did not decrease immediately after the end of oryzalin exposure on GS+5 and GS+25 plants. However, *A* was consistently lower across all growth stages on the 10th day from the end of oryzalin exposure. The largest reduction in *A* (36%) occurred when plants were exposed at GS+15, immediately after the end of oryzalin exposure. However, plants receiving oryzalin exposure across all growth stages had similar *A* compared to control, 20 days after the end of oryzalin exposure. For oxyfluorfen exposure, reduction in *A* was observed for all growth stages, immediately after the end of oxyfluorfen exposure. However unlike oryzalin, plants across all the growth stages slowly and progressively recovered in next 10 days, at which time the *A* of plants exposed to oxyfluorfen and control was similar.

Oryzalin exposure at GS+15 and GS+35 immediately reduced *Fv'/Fm'* but *Fv'/Fm'* for GS+5 and GS+25 was not reduced immediately. 10 days after the end of oryzalin exposure

Fv'/Fm' was still lower on GS+35 and was further lowered for GS+5 but for GS+15 *Fv'/Fm'* was fully recovered. Reduction in *Fv'/Fm'* was never observed on GS+25. However 20 day after the end of oryzalin exposure *Fv'/Fm'* for all growth stages was similar to that of control (Fig. 6). Oxyfluorfen exposure at all growth stages, except GS+25, immediately reduced *Fv'/Fm'* (Fig. 6). However 10 days after the end of oxyfluorfen exposure *Fv'/Fm'* completely recovered for three out of four growth stages, except GS+25, which completely recovered by 20 days of the end of oxyfluorfen exposure (Fig. 6). Overall *Fv'/Fm'* recovery was slightly faster for oxyfluorfen compared to oryzalin.

3.3. Final evaluation

Plants receiving oryzalin exposure at different growth stages had similar TDB, GI, SPAD index, *A,* and *Fv'/Fm'* at 65 days after the emergence of first leaf. They only differed in the leaf visual rating. Leaves injury did not recover completely on plants receiving oryzalin exposure at GS+15, GS+25 and GS+35. Sixty-five days after the emergence of the first leaf, leaf visual rating was lowest for plants in treatment group GS+35 and GS+25 (Table 2).

Sixty-five days after the emergence of the first leaf, plants receiving oxyfluorfen exposure at GS+5 and GS+15 had 31% and 15% lower TDB compared to control. Plants receiving oxyfluorfen at all other growth stages had TDB similar to that of control. In contrast, the leaf visual rating was lower for plants receiving oxyfluorfen exposure at later growth stages. Leaf visual rating for GS+35, GS+25 and GS+15 was reduced by 17%, 8% and 4%, respectively. GI, SPAD index,

A and *Fv'/Fm'* at the end of the vegetative stage completely recovered in all the plants exposed to oxyfluorfen (Table 2).

3.4. Evaluation of flowers

GI, TFM, and visual rating of flowers of *H. paniculata* were not affected ($p>0.05$) by exposure to either oxyfluorfen or oryzalin. GI for control, oryzalin and oxyfluorfen were 8.86 ± 0.2 g, 7.19 ± 0.38 g and 7.41 ± 0.18 g respectively. TFM for control, oryzalin and oxyfluorfen were 17.49 ± 0.56 cm, 17.21 ± 0.56 cm and 17.41 ± 43 cm respectively and visual rating for flowers receiving any of three treatments were 10 out of 10.

4. Discussion

4.1. Morphological response depends on the growth stage of plant

Studies evaluating the effect of herbicides at specific leaf stages of weed and crops are common (Roe and Buchman, 1963; Klingaman et al., 1992). However, researchers acknowledge that studies concerning herbicide sensitivity at varying stages of plant are comparatively rare (Shim et al., 2003). Some herbicides may injure younger leaves, while others may produce damage on older leaves (Kuk et al., 2006; Yoon et al., 2011). In our study, we increased the duration of herbicide exposure but reduced the concentration of herbicide compared to general herbicide application practice in order to simulate irrigation with recycled water. Both oxyfluorfen at 0.02 mg/L and oryzalin at 8 mg/L produced phytotoxicity in *H. paniculata* and injury was primarily

observed in younger and growing leaves for all four growth stages that we tested. However, the maximum morphological damage occurred for GS+5 plants; hence it was the most sensitive growth stage for both herbicides. Younger leaves adsorb, retain and translocate higher concentration of herbicides because of thinner cuticle and wax layer compared to mature leaves (Akey and Machado, 1985; Zhu et al., 2018) and higher number of exposed leaves due to lesser canopy density in younger leaves increase pesticide interception (Sellers et al., 2003). Antioxidant capacity of leaves is known to increase herbicide resistance and is relatively low in younger leaves (Moustaka et al., 2015; Nobossé et al., 2018). All leaves in GS+5 plants were young, rapidly growing, and had open canopy at the time of herbicide exposure; therefore, they sustained maximum herbicide damage. Plants that received herbicide at GS+15, GS+25 and GS+35, had some mature leaves that were increasingly tolerant to residual concentration of herbicide resulting in lower herbicide injury. Dithiopyr, a similar herbicide as oryzalin, produced a greater reduction in growth when applied at early growth stages (McCullough et al., 2014) and oxyfluorfen injury was also found to be higher on plants exposed to oxyfluorfen at early growth stages compared to late growth stages (Akey and Machado, 1985; Nosratti et al., 2017). In our study, after the end of herbicide exposure, leaf visual rating on plants exposed to oxyfluorfen started to recover immediately. This is consistent with oxyfluorfen mode of action as it is minimally translocated from the application site and mostly works as a contact herbicide (Chun et al., 2001). In contrast, for plants exposed to oryzalin, herbicide damage increased from one to ten days after the end of exposure as oryzalin is readily absorbed and sometimes translocated from newly growing leaves

(Appleby and Valverde, 1989; Sterling, 1994). Therefore, even after the end of oryzalin exposure, oryzalin absorbed and retained in leaves was affecting newly forming and enlarging leaves. Another reason for the difference in recovery may be due to the mode of action of these herbicides. Oxyfluorfen produces reactive molecules that disrupt cell membranes and cause cell death; this reaction is immediate in the presence of light (Kunert et al., 1985; Anatra-Cordone et al., 2005). while oryzalin restricts the formation of microtubules that do not produce immediate visual injury or other effects (Hugdahl and Morejohn, 1993). Oxyfluorfen has minimal impact on cell division and growth while the mode of action for oryzalin is predominantly related to cell division and growth; therefore, the effect of oryzalin is delayed and persists longer.

The effect of sub-lethal dose of herbicide may vary depending on the growth stage of plants (Boutin et al., 2014). In our study maximum visual damage was observed when plants were exposed to herbicide at early growth stage and similar to our finding, soybean plants also had a maximum visual injury at early growth states when exposed to a sub-lethal dose of 2,4-D (Scholtes et al., 2019). Recovery in GI and leaf visual rating was rapid in plants exposed to herbicides at GS+5, because cell multiplication and growth are rapid at early vegetative stages compared to other stages of plant growth (Van De Sande-Bakhuyzen and Alsberg, 1927; Goudriaan and Van Laar, 1994).

4.2. Physiological measurements provide a rapid indicator of herbicide damage and recovery

In our study, physiological measurements (SPAD index, *A* and *Fv'/Fm'*) responded to herbicide exposure. Oxyfluorfen directly reduces chlorophyll formation but oryzalin does not have a direct impact on chlorophyll, and this was evident in our study through SPAD index. The reduction in the SPAD index was higher for oxyfluorfen compared to oryzalin, during the early growth stage i.e., GS+5 (statistical comparison not shown).

Physiological measurements such as *A* and *Fv'/Fm'* may be used as early indicators of herbicide damage (Yanniccari et al., 2012; Wang et al., 2018). In our study, both *A* and fluorescence parameters had a faster and greater response to herbicide compared to visual injury and growth, at later growth stages from GS+15 to GS+35 and GI and leaf visual rating had greater response at early growth stage i.e., GS+5. For oryzalin, leaf visual rating was lowest ten days after the end of the exposure, but *A* and *Fv'/Fm'* had already started to recover. The increase in *A* and *Fv'/Fm'* was followed by visual leaf recovery evident on the 20th day after the end of oryzalin exposure. Thus leaf damage by herbicides such as oryzalin that do not produce immediate visible damage can be identified quickly by using physiological tools and those tools can be used as early indicators for herbicide damage, preferably at later growth stages when visible damage take some time to appear . In contrast to our result, exposure to oryzalin did not reduce *A* for dwarf gardenia (*Gardenia jasminoides* 'Radicans' Thunb.) and fountain grass (*Pennisetum rupelli* Steud.)

probably because the concentration used was eight times lower compared to ours (Bhandary et al., 1997). As discussed earlier, recovery from injury associated with oxyfluorfen exposure was faster than recovery from oryzalin and was obvious during physiological evaluations. For oxyfluorfen, both *A* and *Fv'/Fm'* completely recovered from herbicide damage as early as ten days after the end of exposure and was followed by morphological recovery. Thus physiological tools can also be efficiently applied to detect herbicide recovery in addition to herbicide damage. Physiological recovery of plants from oryzalin exposure was slower compared to oxyfluorfen. Physiological parameters such as *A* and *Fv'/Fm'* were same or lower 10 day after the end of oryzalin exposure compared to a day after the end of oryzalin exposure, expect on GS+15 for *Fv'/Fm'*. Overall, plants exposed to oryzalin took somewhere from 10 to 20 days for *A* and *Fv'/Fm'* to completely recover. However, this was still quicker than recovery of GI and visible symptoms.

4.3. Flowers were not damaged by residual oryzalin and oxyfluorfen

Both oxyfluorfen and oryzalin exposure did not produce any effect on flowers in *H. paniculata*. Oxyfluorfen mode of action requires the presence of chlorophyll within the chloroplast, in flowers (petals), there are chromoplasts instead of chloroplast which is the main reason behind the resistance of flowers to oxyfluorfen (Thomson and Whatley, 1980; Lysenko and Varduny, 2013). Stomatal density in flowers is lower compared to leaves (Zhang et al., 2018) and lower stomatal density also reduces herbicide penetration and damage. Thus exposing flowers to these herbicides did not produce injury. Oryzalin application impacts flower morphology at a

cellular level by swelling the tip of conical cells, changing the epidermal cell angle and producing shorter cells (Ren et al., 2017) but was not observed at the morphological scale in our study.

4.4. Leaf visual injury takes the longest to recover

Oryzalin did not reduce TDB at the end of vegetative growth stage which possibly is because oryzalin had lower leaf damage and less reduction in the SPAD index compared to oxyfluorfen. At the end of the vegetative growth stage (65 days after leaf initiation), GI for oxyfluorfen exposure was the same across all growth stages but TDB was lower for GS+5 and GS+15 (Table 2). Therefore oxyfluorfen exposure at early growth stages (GS+5 and GS+15) will increasing radial growth but reduce plant density. Reduction in TDB caused by oxyfluorfen application at early growth stages did not recover even after 50 days of oxyfluorfen exposure but exposure at later growth did not reduce final TDB. Similarly, in other studies, oxyfluorfen application in strawberry to control broadleaf weeds produced transient foliar injury that usually did not translate to yield loss (Daugovish et al., 2008) and oryzalin application at 1 mg/L did not reduce root and shoot weight in dwarf gardenia (Bhandary et al., 1997).

Visual leaf injury for plants exposed to both herbicides at GS+15, GS+25 and GS+35 were still present at the end of the vegetative growth stage, and the leaf injury from oxyfluorfen exposure was higher (4-13%) compared to oryzalin (5-10%) exposure. Therefore leaf injury needs the longest time to recover compared to other morphological and physiological parameters. In other studies, oryzalin applications at various rates on sweet potato produced sustained leaf distortion

(<10 %) and plant stunting (<12 %)(Chaudhari et al., 2018) and oxyfluorfen produced lasting leaf injury in cabbage (*Brassica oleracea* L.), tomato (*Lycopersicon esculentum* Mill.), cucumber (*Cucumis sativus* L.), and lettuce (*Lactuca sativa* L.) (Grabowski and Hopen, 1985). However leaf injury produced by herbicide at early growth stages may completely recover. Visual leaf injury caused by oryzalin application, immediately after transplanting, in sweet potato reversed and did not translate to a reduction in yield (Chaudhari et al., 2018) and foliar injury in broccoli produced by post-emergence application of oxyfluorfen completely recovered in late-maturing varieties, while early maturing varieties had sustained foliar injury and yield loss (Farnham and Harrison, 1995). Similarly, in our study visual leaf injury for GS+5 completely recovered while visual leaf injury cause by herbicide exposure at later growth stage did not recover.

5. Conclusion

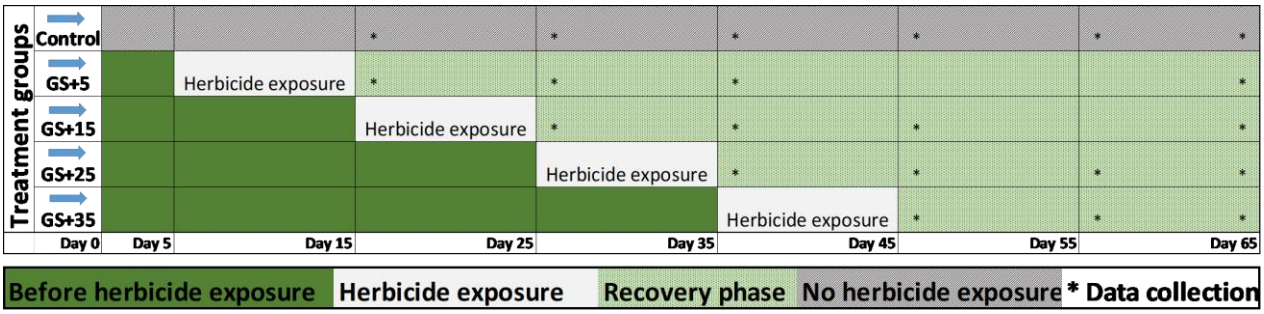
Residual herbicides such as oxyfluorfen or oryzalin present in recycled water may produce sub-lethal effects on woody ornamentals when used for irrigation. Young and growing leaves are more susceptible to herbicidal injuries compared to mature leaves. Early growth stages of plants have a higher ratio of young to mature leaves and therefore are more prone to herbicide damage. Leaf injury from some herbicides will immediately begin to recover while leaf injury from others will continue to increase before starting to recover. Physiological measurements of herbicide damage can be assessed earlier compared to morphological measurements, particularly for herbicides that do not produce damage immediately after exposure, and can reflect immediate plant

performance. Physiological measurements are more sensitive to herbicide injury at later growth stages while morphological measurement may be sensitive at early growth stages. Hence, those tools can be used as an early indicator of damage and recovery. Damage caused by herbicides such as oxyfluorfen that directly destroy photosynthesis apparatus is more severe and may permanently reduce TDB if plants are exposed at early growth stages. Flowers were not affected by 0.02 mg/L of oxyfluorfen and 8 mg/L or oryzalin exposure because of the differences in cell structure compared to leaves. The limitation of our study is the use of only one plant taxon and two herbicides; results may be different if different taxa or herbicides with a different mode of action are used.

APPENDIX

APPENDIX

Table IV - 1. Flow chart for herbicide exposure.



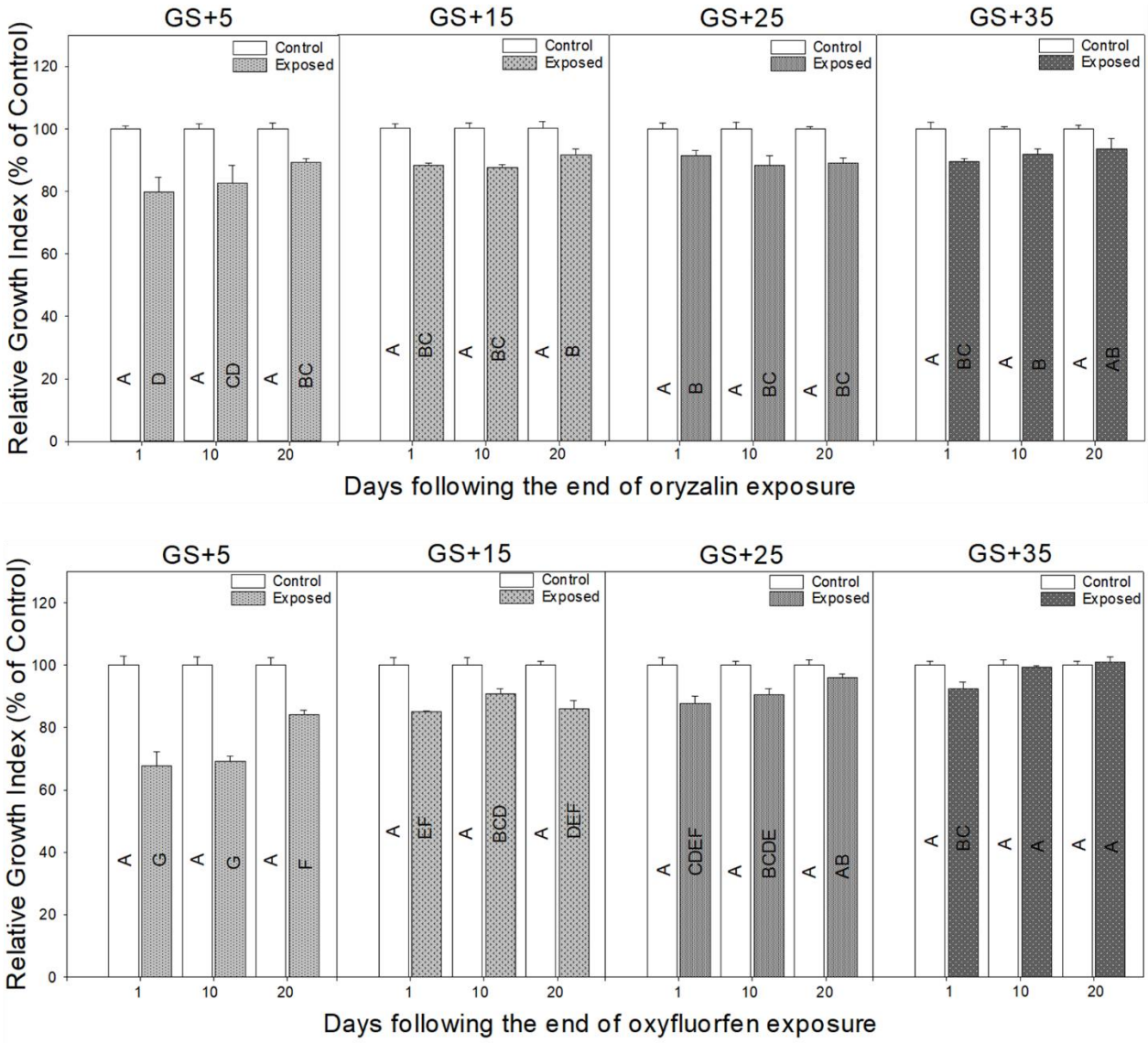


Figure IV - 1. Relative growth index of *H. paniculata* 'Limelight' in response oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS+5 received herbicide exposure five days after initiation of growth, GS+15 received herbicide exposure 15 days after initiation of growth, GS+25 received herbicide exposure 25 days after initiation of growth and GS+35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each herbicide were carried out using Least Significant Difference (LSD) post-hoc test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at p=0.05.

Figure IV - 2. Representative herbicide damage immediately after the end of oxyfluorfen exposure (A) and ten days after the end of oryzalin exposure (B). Plants were exposed to oxyfluorfen or oryzalin at growth stage (GS), GS+15 for ten days. Both plants received a score of seven out of ten for leaf visual rating.

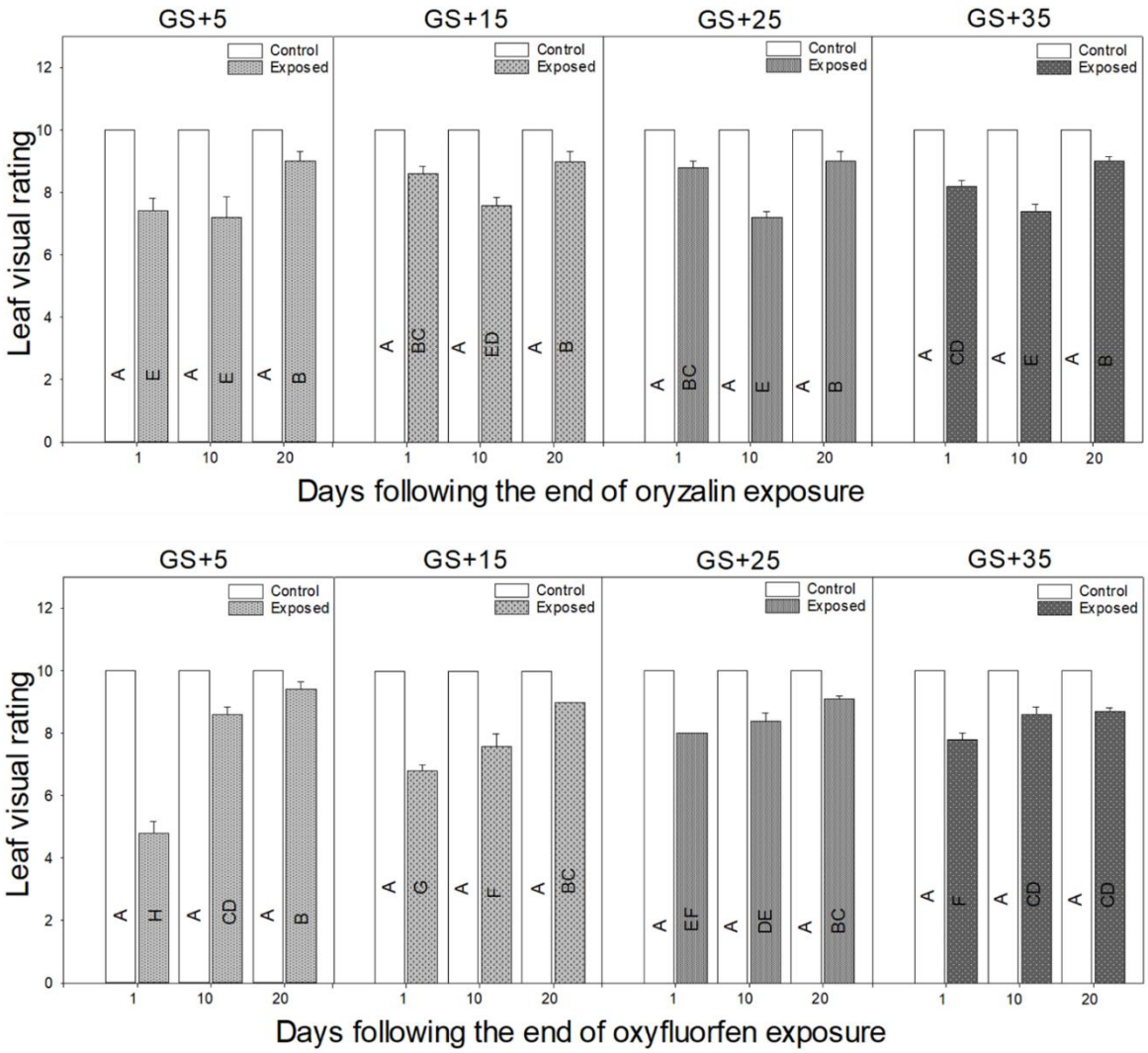


Figure IV - 3. Leaf visual rating of *H. paniculata* 'Limelight' in response oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS+5 received herbicide exposure five days after initiation of growth, GS+15 received herbicide exposure 15 days after initiation of growth, GS+25 received herbicide exposure 25 days after initiation of growth and GS+35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each herbicide were carried out using Least Significant Difference (LSD) post-hoc test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at p=0.05.

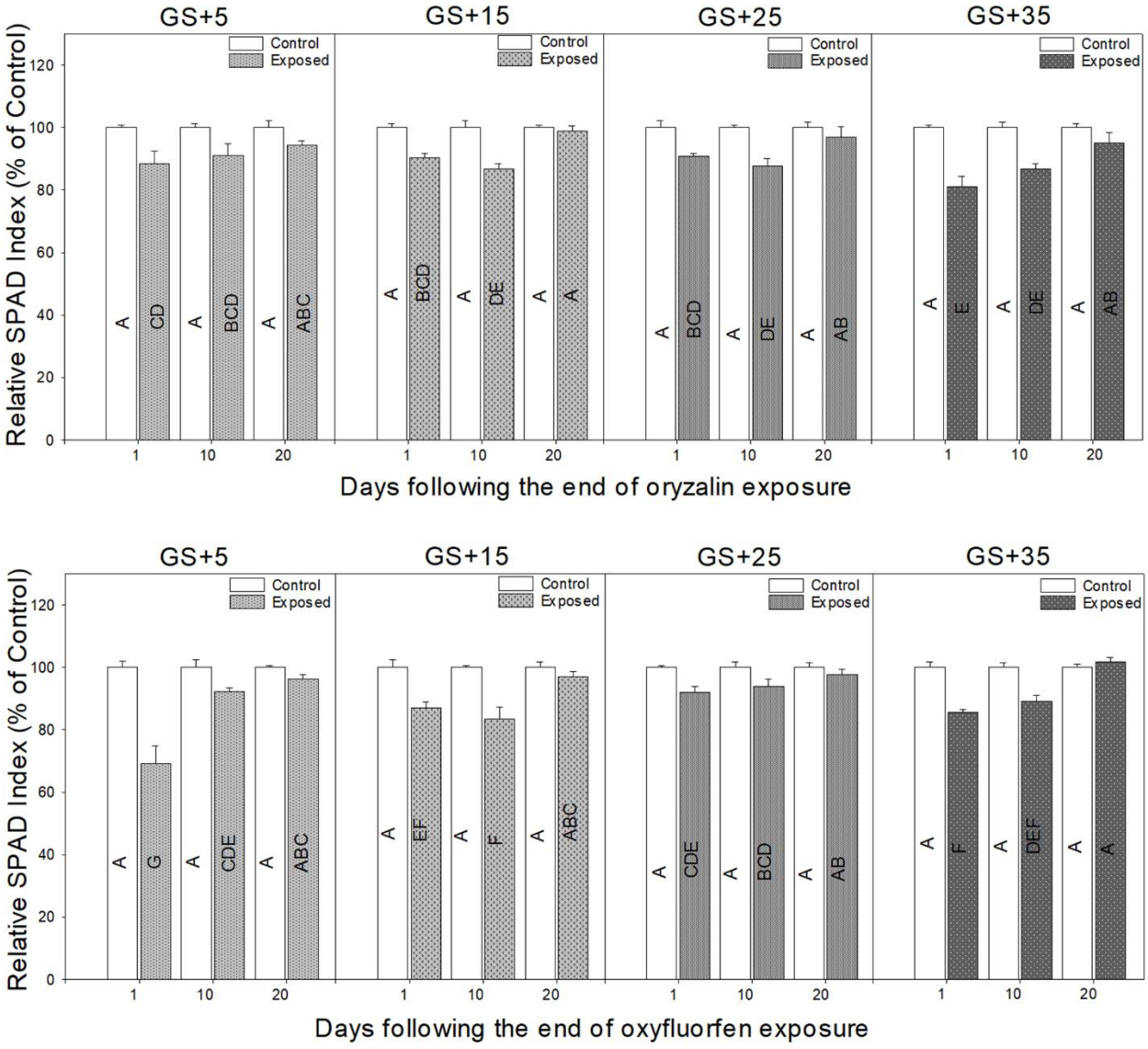


Figure IV - 4. Relative SPAD index of *H. paniculata* 'Limelight' in response oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS+5 received herbicide exposure five days after initiation of growth, GS+15 received herbicide exposure 15 days after initiation of growth, GS+25 received herbicide exposure 25 days after initiation of growth and GS+35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each herbicide were carried out using Least Significant Difference (LSD) post-hoc test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at p=0.05.

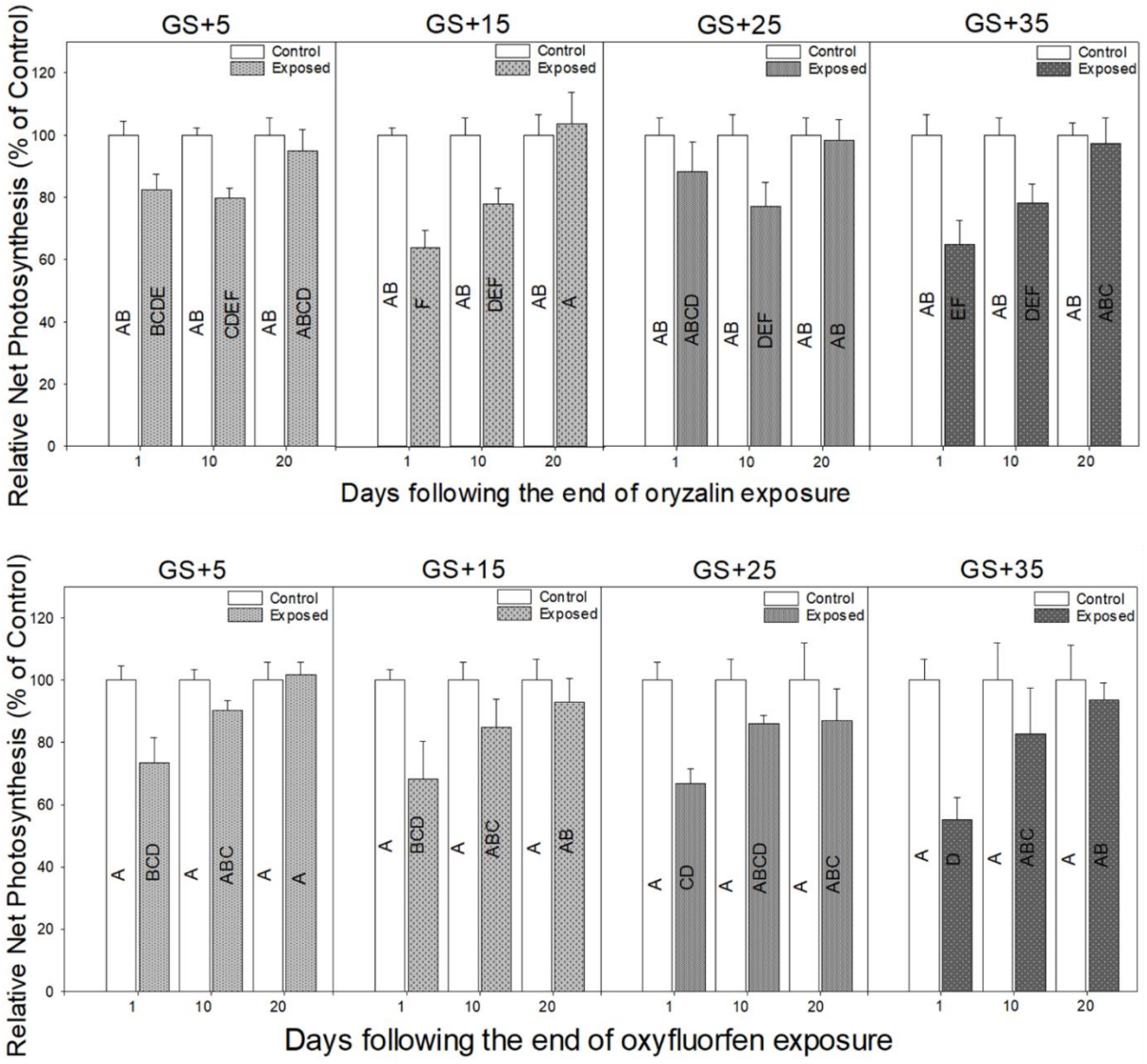


Figure IV - 5. Relative net photosynthesis of *H. paniculata* 'Limelight' in response oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS+5 received herbicide exposure five days after initiation of growth, GS+15 received herbicide exposure 15 days after initiation of growth, GS+25 received herbicide exposure 25 days after initiation of growth and GS+35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each herbicide were carried out using Least Significant Difference (LSD) post-hoc test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at p=0.05.

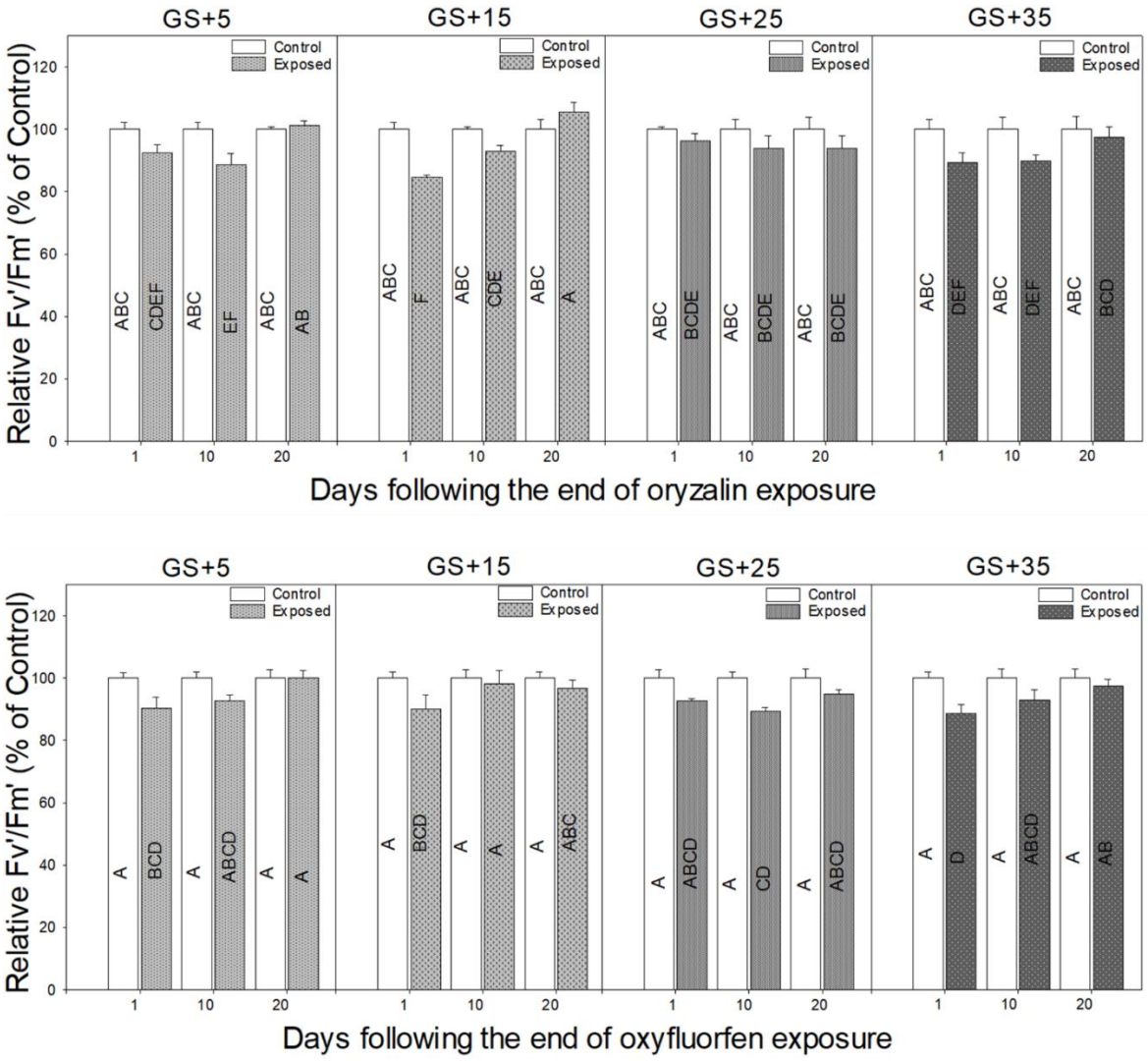


Figure IV - 6. Percent reduction in light-adapted fluorescence of *H. paniculata* 'Limelight' in response oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS+5 received herbicide exposure five days after initiation of growth, GS+15 received herbicide exposure 15 days after initiation of growth, GS+25 received herbicide exposure 25 days after initiation of growth and GS+35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each herbicide were carried out using Least Significant Difference (LSD) post-hoc test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at p=0.05.

Table IV - 2. Total dry above-ground biomass (TDB), leaf visual rating (VR), SPAD index (SPAD), growth index (GI), photosynthesis (A) and light-adapted chlorophyll fluorescence (Fv'/Fm') of *H. paniculata* 'Limelight' at 65 days after initiation of leaf growth. Plants were exposed to either oryzalin (8 mg/L) or oxyfluorfen (0.02 mg/L) at various growth stages (GS) for ten days. GS+5 received herbicide exposure five days after initiation of growth, g GS+15 received herbicide exposure 15 days after initiation of growth, GS+25 received herbicide exposure 25 days after initiation of growth and GS+35 received herbicide exposure 35 days after initiation of growth. Mean separations for each herbicide were carried out using Least Significant Difference (LSD) post-hoc test. Means within each herbicide that are followed by the same letters are not significantly different at given p-values.

Oryzalin exposure

GS	TDB (g)	VR	SPAD	GI (cm)	A	Fv'/Fm'
Control	143.85	10.00a	36.96	96.47	15.97	0.60
GS + 5	124.61	9.70ab	37.22	92.60	16.18	0.59
GS + 15	126.85	9.50b	35.30	91.40	14.79	0.60
GS + 25	119.85	9.30bc	36.64	89.00	15.21	0.60
GS + 35	127.65	9.00c	35.12	90.27	15.54	0.58
p-value	*NS*	*<0.0005*	*NS*	*NS*	*NS*	*NS*

Oxyfluorfen exposure

GS	TDB (g)	VR	SPAD	GI (cm)	A	Fv'/Fm'
Control	128.72a	10.00a	37.12	91.80	16.52	0.56
GS + 5	88.65c	10.00a	36.78	88.20	15.99	0.53
GS + 15	104.46bc	9.60b	35.16	91.07	15.20	0.55
GS + 25	108.60abc	9.20c	36.90	89.20	15.74	0.52
GS + 35	128.37ab	8.70d	37.72	92.73	15.46	0.54
p-value	*<0.05*	*<0.0005*	*NS*	*NS*	*NS*	*NS*

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SECTION V

EFFECT OF RESIDUAL PESTICIDES IN RECYCLED NURSERY RUNOFF ON GROWTH AND PHYSIOLOGY OF ORNAMENTAL SHRUBS

Effect of residual pesticides in recycled nursery runoff on growth and physiology of ornamental shrubs

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Abstract

Nursery runoff may contain pesticide residues which, if released off-site, could impair surrounding ecosystems. As a solution, nursery growers can retain runoff water on-site and recycle retained water to irrigate plants. However, concerns related to potential phytotoxicity caused by residual pesticides in recycled water discourage growers from recycling water. To evaluate plant quality irrigated with recycled water, we conducted a three-year field study simulating a commercial nursery growing practice. Irrigation treatments were applied to six ornamental taxa grown in a nursery production bed. Irrigation treatments were raw groundwater from the onsite well (control), water recycled from a separate nursery bed containing plants that were treated with nine pesticides regularly over the growing seasons, and recycled water from the nursery bed that had been remediated using heat-expanded shale aggregates and woodchip bioreactors. Plants receiving recycled water (runoff water with and without remediation) did not produce pesticide-related visual injury. However, result for growth index, chlorophyll SPAD index, dark-adapted fluorescence, and shoot biomass were irregular among raw groundwater and recycled water; for most instances, pesticides in recycled water did not reduce any of those parameters. Net photosynthesis and light-adapted fluorescence were similar for raw groundwater and recycled water. Results from this study demonstrate the possibility of using recycled for irrigation of woody ornamental shrubs.

Keywords: Nursery sustainability; Specialty crops, Irrigation return flow, Remediated water, Bioreactors

1. Introduction

Unsustainable withdrawal and luxuriant use of raw groundwater are common water-related problems around the globe, including the U.S., where irrigation accounts for 38% of freshwater withdrawal (Dieter et al., 2015; Boretti and Rosa, 2019). Water availability is decreasing and so is the total water use in U.S. but the demand for freshwater and water use in many sectors of agriculture is increasing (United States Geological Survey, 2015; Rodell et al., 2018). Concerns regarding water scarcity are particularly acute for container production of nursery crops, which is an intensive system that requires relatively high inputs of water. Reduced water availability has created restrictive regulations and is forcing container-crop producers to look for alternatives to using raw groundwater (Beeson et al., 2004; Fulcher et al., 2016). Nurseries growers also rely heavily on agrochemicals. These agrochemicals may collect in runoff water and can be transported from the production area, potentially contaminating surrounding ecosystems. Green industry is admired for its ecological contribution and nursery being part of green industry, nursery producers are interested towards implementing strategies that can alleviate environmental consequences of their production systems (Mangiafico et al., 2008; Wilson and Broembsen, 2015; Fulcher et al., 2016; Majsztrik et al., 2017). To address those problems, it is becoming more common for nursery growers to capturing and recycling nursery runoff (Fain et al., 2000; Meador et al., 2012; Mack et al., 2017). Although recycling runoff water may be a sustainable solution, agrochemicals and pathogens present in recycled water could impact plant quality and health, creating risk for growers

(Poudyal and Cregg, 2019). Disease infestation is the primary concern of nursery growers when using recycled water as recycled water may disseminate plant pathogens (Pottorff and Panter, 1997; Hong et al., 2003). However, technologies such as UV radiation, slow sand filtration, crushed brick filtration, chlorination, and ozone treatments can help to limit the spread of plant diseases in nurseries (Stewart-Wade, 2011; Nyberg et al., 2014; Younis et al., 2019). In addition to plant diseases, growers are also concerned about crop damage from pesticides in recycled water. Pesticides such as acephate, isoxaben, bifenthrin, prodiamine, glyphosate, triflumizole, mefenoxam, thiophanate-methyl, and chlorpyrifos are commonly applied in nurseries (United States Department of Agriculture and National Agricultural Statistics Service, 2011; Poudyal and Cregg, 2019) and have been found in nursery runoff (Poudyal and Cregg, 2019). Herbicides are of particular concern because they directly impact plant growth and physiology; isoxaben destroys cellular membrane and structures, prodiamine hinders cell division and multiplication and glyphosate inhibit the production of essential plant enzymes (Amrhein et al., 1980; Heim et al., 1990; Brosnan et al., 2014). In addition, insecticides such as acephate, chlorpyrifos and bifenthrin cause indirect phytotoxicity by disrupting the physiological process and by producing reactive oxygen species (Spiers et al., 2006; Parween et al., 2016). The effect of fungicides on plant growth is not fully explored but instances of both positive and negative responses of plants toward fungicides are evident (Tjosvold et al., 2005; Dias, 2012; Petit et al., 2012). The occurrence of phytotoxicity and its severity also depends on the concentration of pesticides and frequency of exposure, a pesticide that is safe at lower concentrations may produce phytotoxic symptoms if the

concentration is increased (Veeraswamy et al., 1993; Bhandary et al., 1997a). The concentration of pesticides in recycled water are often substantially lower compared to application rates because of dilution caused by the volume of water in the receiving reservoir, subsequent irrigation, pesticide adsorption, microbial degradation, hydrolysis, photodegradation and volatilization (Wilson et al., 1996; Lu et al., 2006; Poudyal and Cregg, 2019). Therefore reduced concentrations of pesticide in the recycled water compared to the general application rates pave its possibility for reuse. Nonetheless, irrigation with recycled water can potentially result in chronic, low-dose exposure of plants to residual pesticides and may reduce plant quality.

Technologies such as vegetative waterways, constructed wetlands, sediment traps and sand filtration have effectively been used to remove pesticides from runoff water (Briggs et al., 1998; Stearman et al., 2003; Kabashima et al., 2004; Warsaw et al., 2012; Hedegaard and Albrechtsen, 2014). In addition to those technologies, woodchip bioreactors are also gaining popularity for its potential to reduce contaminants, including pesticides, in recycled water. Woodchips adsorb pesticides and also host a wide range of microbial organisms capable of degrading pesticides (Morillo et al., 2017; Abatenh et al., 2017; Abdi et al., 2020); furthermore, they are inexpensive and easily available. Thus wood chip bioreactors could be used for the remediation of pesticides in recycled water. There are numerous scientific studies related to the remediation of pesticide in a laboratory or small scale (Morillo et al., 2017), but growers need rapid, production-scale remediation systems to handle their water treatment requirements. In our study, we built a 2-stage

bioreactor system and evaluated their potential to lower pesticide concentration in runoff water. Documenting the effect of residual pesticides on growth and quality of ornamental shrubs is crucial for nursery growers to implement recycling of nursery runoff. Therefore, we conducted a trial to determine the response of nursery plants to irrigation with recycled runoff (RR) water that was collected from an experimental nursery of container-grown plants. The nursery plot was managed based on standard commercial nursery practices for the region, including multiple pesticide applications each season. In addition to evaluating plants irrigated with RR, we also evaluated plants irrigated with remediated recycled runoff (RRR) water and raw groundwater (RGW). Remediated recycle runoff water was RR water that had been remediated through 2-stage bioreactor and RGW was the water from local well (control). The objective of the study was to assess the growth, physiology and quality of ornamental shrubs irrigated with the different water sources. To achieve our objective, we conducted a three-year field research using conventional management practices. For an ornamental nursery grower, plant growth and quality are of utmost importance as consumer buying preferences are based on plant quality (Khachatryan and Choi, 2017). Therefore, when evaluating the suitability of recycled water (refer to both RR water and RRR water) for ornamental plants, it is vital to assess plant quality in addition to plant growth and performance. Plant visual assessment, chlorophyll SPAD index, growth index and plant biomass can be used to assess plant quality (Grieve and Poss, 2010; Furtini Neto et al., 2015). In addition, physiological measurements such as photosynthesis, light-adapted fluorescence and dark-adapted

fluorescence can reflect plant health and identify pesticide stress (Petit et al., 2012; Silva et al., 2014; Wang et al., 2018).

2. Materials and Methods

2.1 Field layout and water treatments

Field studies were conducted at Michigan State University Horticulture Teaching and Research Center (42°40’ 023” N and 84° 29’ 04” W) during the summers of 2017 - 2019. In each year we compared the responses of container-grown nursery plants to irrigation from three irrigations sources; RR, RRR and RGW. A layout of the experimental design is provided in Fig. 1.

2.1.1. Irrigation water sources

A plant evaluation bed (12.5 m x 25 m) and a runoff bed (runoff bed; 12.5 m x 25 m) were built 50 m apart. They were slightly sloped to facilitate runoff drainage and capture. The runoff bed was first topped with black impermeable pond liner (1.15 mm thick) and then with landscape fabric/weed barrier. At the lower end of the runoff bed, a runoff collection reservoir (P1) capable of holding 4000 L of water was dug to capture runoff. A pond liner was also installed on P1 to restrict water infiltration. Adjacent to runoff bed six 2-stage bioreactors were built to remediate a portion of runoff water from P1. A single bioreactor consisted of an open-top box (1.2 m x 2.4 m x 1.2 m), lined internally with a pond liner and half-filled with hardwood woodchips (average

woodchip volume of 18.2 cm^3). Two 1.2 m long polyvinyl chloride tubes (10 cm diameter) filled with heat-expanded shale aggregate (Haydite grade B; majority particle size 0.95 cm, Digeronimo Aggregates LLC, Ohio) were placed on the top of the box. Adjacent to the bioreactors, a remediated runoff collection reservoir (P2) able to hold 4000 L of water was also constructed and lined internally with a pond liner.

2.1.2. Runoff generation zone

988 woody ornamentals plants of various shrub taxa grown in an 11.3 L black plastic containers filled with pine bark and peat moss substrate (80:20; Volume: Volume) were transferred to the runoff bed (pot to pot spacing: 0.53m) on 12 May 2017, 24 May 2018, and 25 May 2019, and fertilized with 50 g of slow-release fertilizer (Osmocote blend; 18:2.2:6.6 N:P:K with micronutrients, 8-9 months, Product code # A90177, ICL Specialty fertilizers, Summerville, SC, USA). The plant taxa grown in the runoff bed varied among years but included common nursery shrubs such as *Deutzia gracilis* Siebold & Zucc. 'slender deutzia' (Yuki Snowflake®), *Hydrangea paniculata* Siebold 'panicle hydrangea' (Fire light and lime light), *Hydrangea arborescens* L. 'smooth hydrangea' (Invincibelle Spirit II), *Hydrangea macrophylla* Thunb. 'bigleaf hydrangea' (Let's dance blue jangles), *Weigela florida* (Bunge) A. DC. 'oldfashioned weigela' (Wine & Roses®), *Spiraea japonica* L. 'Japanese meadowsweet' (Double play pink), *Berberis thunbergii* DC. 'Japanese barberry' (Rose glow), *Continus Coggygria* Scop. 'smoke bush' (Wine Craft Black), *Potentilla fruiticosa* L. 'shrubby cinquefoil' (Happy face), *Rosa Sp* L. 'landscape rose'

(Oso easy double re) and *Rosa x Hansa* L. 'landscape rose'. We managed the runoff bed to closely replicate commercial nursery practices. Plants in the runoff bed were watered using overhead sprinkler irrigation delivering 19 mm of irrigation daily. Pesticides were applied at recommended label rates to the runoff bed using a 1.2 m overhead spray boom with 4 flat-fan nozzles during the growing season in each year (Table 1), except for glyphosate which was sprayed using backpack sprayer to avoiding direct contact to plants in the container. A gas-powered pump delivered 25 L of pesticides and 5 L of herbicide for each application event (Table 1). Pesticide application was scheduled on a day with no rain forecast. Herbicide (isoxaben, prodiamine or glyphosate) was applied early in the morning (8 am) and then the bed was irrigated. After irrigation, we waited for the runoff to cease and then the insecticides and/or fungicides were applied in the bed as a tank mixture. The day after pesticide application and thereafter, regular irrigation was resumed. Irrigation water leaving the runoff bed was collected in P1. A fraction of water from P1 was pumped to the bioreactors, where it first passed through the heat-expanded shale aggregates in the PVC pipes which then flowed into the woodchips in the bioreactor. Water from the bioreactors was then collected in P2. Raw groundwater was the water obtained from the farm well, RR water was the water collected from runoff bed in P1, without any RRR water was the water from P1 that went through the bioreactors and was collected on P2. In this study, recycled water refers to water both from P1 (RR water) and P2 (RRR water).

2.1.3. Plant evaluation zone

The plant evaluation bed was divided into 12 irrigation zones, each 2.4 m x 4.8 m. The plant evaluation bed had four rows serving as blocks and three zones within each block. Each irrigation zone received raw groundwater (RGW) or RR water or RRR water. Each zone in the plant evaluation bed had six different plant taxa and eight replication per taxon. Starter plants from 10 cm plugs (liners) of *Hydrangea macrophylla* Thunb. 'bigleaf hydrangea' (Let's dance blue jangles), *Hydrangea paniculata* Siebold 'panicled hydrangea' (Limelight), *Thuja occidentalis* L. 'arborvitae' (American Pillar), *Juniperus horizontalis* Hornibr. 'creeping juniper' (Blue rug), *Hydrangea arborescens* L. 'smooth hydrangea' (Invincibelle Spirit II®) and *Rosa sp*. L. 'landscape rose' (Oso Easy Double Red®) were obtained from commercial nursery and were transplanted in an 11.3 L black plastic container filled with pine bark and peat moss media (80:20; Volume: Volume) and moved to the plant evaluation bed and spaced 0.53 m apart (pot to pot). In 2017, plants that were transplanted as liners on August 27, 2016, were used. Those plants were brought to the overwintering hoop after approximately two month of growth outside, on October 20, 2016, and plants underwent dormancy. Those plants were moved to the plant evaluation bed on 12 May 2017. Plants used in the 2018 and 2019 studies were transplanted from liners on 24 May 2018 and 25 May 2019, respectively. Every year after moving plants to the plant evaluation bed, plants were fertilized with 50 g of slow-release fertilizer (Osmocote blend; 18:5:8 $N:P_2O_5:K_2O$ with micronutrients, 8-9 months, Product code # A90177, ICL Specialty fertilizers,

Summerville, SC, USA) per plant. Fertilizer was applied on 17 May 2017, 8 June 2018, and 22 June 2019. Pest infestation did not occur in the plant evaluation bed hence we did not apply pesticide on plant evaluation bed.

2.2 Plant evaluation

Plant evaluation bed received irrigation treatments from 1, August 2017, to 10 September 2017; from 05 July 2018, to 25 August 2018; and from 16 July 2019, to 25 September 2019. Precipitation and reference evapotranspiration for the duration of the study is provided in Fig. 2. Irrigation treatments were applied as overhead irrigation each morning using an automated irrigation timer (5 am for RR water, 5:30 am for RRR water and from 7:30 am for RGW), as those times were most likely to have the lowest wind speeds each day.

All plants on the plant evaluation bed were evaluated for pesticide-related visual injuries (PVI) three to seven days following each pesticide application to plants on the runoff bed. Potential PVI's included discoloration, necrotic spots, stunting, and curling, on a scale of 0 to 10 (0 being a completely dead plant and 10 being a healthy plant). After the completion of the treatment period for each year, plants in all three treatment groups were compared based on growth index (GI), PVI, chlorophyll SPAD index, dark-adapted fluorescence (*Fv/Fm*), light-adapted fluorescence (*Fv'/Fm'*), photosynthesis (*A*) and dry biomass. All measurements were conducted immediately after the end of irrigation treatment on plant evaluation bed. Growth Index, PVI and *Fv/Fm* were

measured on all eight replications per taxa in all 12 zones but shoot and root biomass, *A*, *Fv'/Fm'* and chlorophyll SPAD were measured only on a subsample of four plants per taxa in each zone.

GI was calculated as the average of plant height and two perpendicular widths for all six species. Chlorophyll SPAD index was measured using a portable SPAD meter (SPAD-502; Minolta corporation, Ltd., Osaka, Japan) as an average of three leaves per plant. It was only measured for four out of six species excluding, *T. occidentalis*, and *J. horizontalis*, because of their overlapping scale-like leaf structure. *Fv/Fm* was measured using a portable fluorometer (OS30p+; Opti-Sciences, Inc., Hudson, NH, US) on fully matured leaves at the 3rd or 4th node from the top after acclimatizing those leaves with a dark-adaption kit (Opti-Sciences, Inc., Hudson, NH, US) for 30 minutes, *J. horizontalis* was excluded from the measurement in 2017, but in 2018 and 2019, all six species were measured. In 2018 and 2019, *Fv'/Fm'* and *A* were measured on a fully mature leaf on either the 3rd or 4th node from the apex. We measured *Fv'/Fm'* and *A* on four out of six species similar to the chlorophyll SPAD index, excluding *T. occidentalis* and *J. horizontalis*. A portable photosynthesis system (LI-6400 XT, Li-Cor, Inc., Lincoln, NE) mounted with a leaf chamber fluorometer (LI-6400-40, Li-Cor, Inc., Lincoln, NE) was used for the measurements. Photosynthetically active radiation (PAR) in the chamber was set to 1500 µmol m-2 s-1, block temperature was set to 25°C and 400 ppm of CO2 was supplied. The relative humidity in the chamber varied between 40–60%. Each leaf was acclimatized for five minutes before measuring *A* followed by measuring *Fv'/Fm'* on the same leaf. In 2018 and 2019, after all the non-destructive

measurements, plants were harvested and dried in an oven at 50°C and weighed to determine dry biomass. Both shoot and root biomass were measured in 2018, but in 2019 we only measured shoot biomass.

2.3 Pesticide sampling

Pesticide samples were collected in 2018 and 2019. Pesticide samples for both RR water and RRR water were collected one day prior and one day after pesticide applications to the runoff bed. Raw groundwater was sampled for pesticides a total of six times, three times in 2018 and three times in 2019. Main water lines running from P1 and P2 to plant evaluation bed were tapped near plant evaluation bed to collect respective water samples. RGW samples were also collected from main water lines suppling RGW to plant evaluation bed (Fig. 1). All water samples were collected in 50 ml amber vials and were immediately frozen. The frozen samples were then sent to a ISO 17025 accredited commercial laboratory (Brookside Laboratories, Inc., New Bremen, OH, USA) to determine pesticide concentrations in RR water, RRR water and RGW. For all compounds other than glyphosate, the analysis was performed on an LC-MS/MS using direct aqueous injection. Glyphosate was analyzed through direct aqueous injection but the analysis method followed EPA 547 using HPLC and hypochorite + OPA post-column derivatization with fluorescence detection.

2.4 Statistical analysis

The layout for the water treatments in plant evaluation bed followed a randomized complete block design. SAS (ver. 9.4) was used to conduct statistical analysis and post-hoc mean comparisons were made using Fisher's least significance difference test at $p = 0.05$.

3. Results

3.1. Pesticide concentration in water

Raw groundwater samples collected in 2018 and 2019 were below detection limits for all pesticides included in our sample protocol (data not shown). However, pesticide residues were observed for RR water and RRR water. Concentrations of pesticides found in RR water and RRR water for 2018 and 2019 are listed in Table. 1.

Acephate was the only compound that was sprayed on each pesticide application. In 2018, throughout growing season, acephate concentration varied from 150 µg/L to 1.5 µg/L in RR water that was sampled a day after application (DAA). For the RRR water 1 DAA, acephate concentration varied from 57.3 µg/L to 5.6 µg/L. Approximately 10 days after all four acephate application, the concentration of acephate in the RR water was reduced by 95% to 100% compared to acephate concentration in RR water on 1 DAA. However, for RRR water, the reduction in acephate concentration 10 DAA was only between 83% and 55.76% compared to acephate concentration in RRR water 1 DAA. In 2019, the concentration of acephate in RR water 1 DAA,

was between 137.2 µg/L and 78.7 µg/L and for RRR water on the same day, pesticide concentration was between 24.9 µg/L and 0 µg/L. Unlike 2018, no acephate residue was found in RR water, collected 15 DAA. On two out of four sample dates, acephate residues were higher in RRR water than in RR water collected 1 DAA.

Chlorpyrifos was only sprayed once in 2018. The concentration of chlorpyrifos in the RR water 1 DAA was 41.1 µg/L whereas chlorpyrifos residues in RRR water were below detection limits on the same day. Nine DAA chlorpyrifos concentration in RR and RRR water samples were below the detection limit. Chlorpyrifos was not applied in 2019 due to changes in university regulations.

In 2018, the concentration of isoxaben in RR water and RRR water 1 DAA was 58.5 µg/L and 179 µg/L, respectively. At 11 DAA, isoxaben concentration in RR water decreased by 80.68%, compared to 1 DAA and the concentration in RRR water decreased by 89%. Isoxaben concentration was consistently reduced on subsequent sampling dates for both RR water and RRR water. In 2019, isoxaben concentration in RR water and RRR water was 166.2 µg/L and 8.3 µg/L, respectively, when sampled 1 DAA. Fourteen DAA, isoxaben concentration in RR water was reduced by 96.7% compared to isoxaben concentration in RR water 1 DAA and isoxaben concentration in RRR water increased by 202.4% compared to isoxaben concentration in RRR water 1 DAA.

Glyphosate was sprayed twice in 2018. For the first application, glyphosate was not present in both RR water and RRR water 1 DAA. At 12 DAA, however, 100 µg/L of glyphosate was found in the RR water but residue in the RRR water was below detection. On the second application, 1051 µg/L of glyphosate was detected in RR water and 18.3 µg/L of glyphosate detected in RRR water, 1 DAA. In 2019, glyphosate was only applied once. A day after application, glyphosate concentration in RR water was 1917.5 µg/L while the concentration in RRR water was 520.2 µg/L. Fifteen DAA and thereafter, glyphosate residue was not detected in both RR water and RRR water except 25.2 µg/L of glyphosate in RRR water 45 DAA.

Thiophanate-methyl was sprayed twice in 2018. Thiophanate-methyl was below detection limits for both RR and RRR water 1 DAA and 15 DAA following the first application. However, after the second application of thiophanate-methyl, 5.5 µg/L of thiophanate-methyl in RR water and 1 µg/L of thiophanate-methyl in RRR water were detected 1 DAA. In 2019, 11.9 µg/L and 1.5 µg L-1 of thiophanate-methyl was found in RR water and RRR water 1 DAA. Thiophanate-methyl was below detection in all water sampled 15 DAA.

In 2018, triflumizole was detected in both RR water (13 µg/L) and RRR water (0.4 µg/L) on 1 DAA. At 15 DAA, triflumizole concentration in RR and RRR water were 1.5 µg/L and 0.03 µg/L, respectively. By 20 DAA, triflumizole was still present in RR but not detected in RRR water. In 2019, 16.9 µg/L of triflumizole was detected in RR water sampled 1 DAA and 1.1 µg/L at 15

DAA (93.5% reduction). For RRR water, 1.1 µg/L of triflumizole was present at 1 DAA and not detectable at 15 DAA.

Neither bifenthrin, which was sprayed once in 2018 and twice in 2019, nor prodiamine, which was sprayed once in 2019, were detected in any water samples.

In 2018, 2.5 µg/L and 0.06 µg/L of mefenoxam was detected in RR water 1 DAA and 10 DAA, respectively. In RRR water, the concentration of mefenoxam was 3.9 µg/L, 1 DAA and 1.8 µg/L (53.8% reduction) at 10 DAA. Trace amount (0.3 µg/L) of mefenoxam was detected in both RR and RRR water at 20 DAA. In 2019, mefenoxam was sprayed twice. Mefenoxam residue was not detected in any water samples after the first application, however, after the second application mefenoxam was detected at 9.5 µg/L only in RR water 1 DAA.

In a few instances pesticides were detected prior to application of the compound in a given season. This was observed for triflumizole in 2018 and isoxaben in 2019.

3.2 Plant response to water treatments

3.2.1. Growth index and Pesticide-related visual injury

Irrigation source did not have a consistent effect on GI in the three years of the study. In 2017 water treatments did not affect GI for any of the six taxa. In 2018, water treatments did not affect GI for any of the six taxa except *T. occidentalis*, for which plants receiving RGW and RR

water had similar GI but the GI of plants receiving RRR water was higher compared to plant receiving RGW and RR water (Fig 3). Similar to *T. occidentalis* in 2018, in 2019, GI of *H. paniculata* receiving RGW and RR did not differ but was lower compared to plants receiving RRR water. GI of *J. horizontalis* and *Rosa sp.* was similar for plants receiving RGW and RR water but plants receiving RRR water had lower GI compared to plants receiving RGW and RR water. GI of *T. occidentalis* was higher for RGW compared to both RRR water and RR water (Fig 3). Although the GI of some species differed among water treatments, pesticide related visible injury did not occur on plants of any taxa during the three years of the study (data not shown).

3.2.2. Dry biomass

In 2018, water treatments did not affect the total shoot weight of plants of five of the six taxa (Fig. 4). However, for *T. occidentalis,* shoot weight was higher for plants irrigated with RRR water compared to RGW (Fig 4). On further dividing shoot weight to leaf weight and stem weight, water treatments did not affect leaf weight for four taxa, however, *Rosa sp.* irrigated with RGW had higher leaf weight compared to RR water but not RRR water and *T. occidentalis* had higher leaf weight for RRR water compared to RGW but not RR water. Stem weight of five taxa was similar for all three water treatments; however, for *T. occidentalis* RRR water had higher stem weight compared to RR water. The root weight of all six taxa was similar for all three water treatments (Fig 5). In 2019, shoot weight of *H. arborescens* and *H. paniculata* was higher for plants irrigated with RGW and RRR water compared to RR water. Conversely, for *H. macrophylla,*

plants receiving RR water had higher shoot biomass compared to RGW and RRR water. For *J. horizontalis*, water treatments did not affect shoot biomass and for *R. sp.* and *T. occidentalis* RGW and RR water had similar shoot mass (Fig 4).

3.2.3. Net photosynthesis and fluorescence

Irrigation source did not affect *A* or *Fv'/Fm'* (Fig 6). However, *Fv/Fm* was higher for plants irrigated with RR water compared to RGW and RRR water for *H. arborescens* in 2017 and for all six taxa in 2018. In 2019, irrigation source did not affect *Fv/Fm* for any of the six taxa (Fig 7).

3.2.4. Chlorophyll SPAD index

In 2017 and 2018, water treatments did not affect chlorophyll SPAD index of plants in any taxa except *H. macrophylla* in 2018, which was higher for plants receiving RRR water compared to RGW and RR water. In 2019, chlorophyll SPAD index of *H. arborescens* and *H. macrophylla* plants receiving RRR water was higher compared to RGW and RR water. In the same year for *H. paniculata*, chlorophyll SPAD index was higher for plants receiving RR water compared to RGW and RRR water but for *Rosa sp.* water treatments did not affect chlorophyll SPAD index (Fig 8).

4. Discussion

4.1 Residual pesticide in recycled water varies by compound

Acephate, glyphosate and mefenoxam all have a high water solubility of 850 g/L, 157 g/L and 8400 mg/L, respectively. After application, these pesticides readily mix with irrigation water and RR. Therefore concentrations of these pesticides were relatively higher in recycled water 1 DAA, compared to the other six pesticides. Concentration of acephate and glyphosate in RR water were substantially reduced by 10 or 15 DAA because acephate has a short half-life <3 days and glyphosate half-life in water is somewhere from 7 to 14 days (Giesy et al., 2000; Mamy and Barriuso, 2005; Christiansen et al., 2011; Mesnage et al., 2015). However, mefenoxam is persistent with an average half-life of 58 days (Long Island Pesticide Pollution Prevention Strategy, 2015) and may persist longer than 20 days.

Isoxaben, chlorpyrifos, triflumizole and thiophanate-methyl are moderately soluble in water with a solubility of 1 mg/L, 1.4 mg/L, 10.2 mg/L and 26 mg/L, respectively which is substantially lower compared to acephate, glyphosate and mefenoxam. Therefore maximum detected concentrations of isoxaben, chlorpyrifos, triflumizole and thiophanate-methyl in recycled water were 5 to 25 times lower compared to maximum detected concentration of acephate, glyphosate and mefenoxam, Isoxaben persisted longer than 10 DAA because it has a half-life of approx. six months (Rouchaud et al., 1999; Quali-Pro, 2011). Traces of isoxaben detected in recycled water even before the isoxaben application in 2019, suggest that there was some carry

over effect of isoxaben from last year and occasional desorption of isoxaben from sediments in the reservoir (Walker, 1987). Michigan has relatively colder temperature and less sunshine which can reduce the rate of photo and microbial degradation which and may have accounted for isoxaben persistence and carry-over (Wilson et al., 1995; Camper et al., 2001). Chlorpyrifos and thiophanate-methyl both have a short half-life of <15 days, in addition, chlorpyrifos is degraded by light and microbes (Soeda et al., 1972; Racke, 1993; Mandal et al., 2010; Mugni et al., 2016) hence both of those pesticides were only found in recycled water at 1 DAA. Triflumizole has a half-life of 18 days and is also readily degraded by microbes (Lewis, 2009). Microorganism present in bioreactors can degrade pesticides (Abdi et al., 2020), microorganisms in our bioreactors possibly degraded triflumizole as a result, triflumizole concentration in RRR water, even 1 DAA, was very low. In 2018, traces of triflumizole were detected before triflumizole application both in RR water and RRR water reason for which could not be explained.

Bifenthrin and prodiamine both have a very low (0.1 mg/L and 0.01 mg/L respectively) water solubility and tightly bind to soil organic matter (Koc: 131000 to 3.02000 and 80 to 471000 respectively), also prodiamine rapidly photodegrade (Weber, 1990; Fecko, 1999; Acuña, 2009). Hence both pesticides were not found in RR and RRR water samples.

4.2 Woodchip bioreactor can reduce pesticide concentration

For most of the pesticides, bioreactors reduced the concentration of pesticides in water. In the bioreactors, RR water first passed through heat-expanded shale aggregates. These aggregates

have a predominantly negative charge, and therefore can adsorb pesticides of the opposite charge and have been successfully used to remove pesticides (Fushiwaki and Urano, 2001; Woignier et al., 2015; Marican and Durán-Lara, 2018). However, in a study by Abdi et al., 2019, heat-expanded shale did not reduce the concentration of chlorpyrifos, oxyfluorfen or bifenthrin (Abdi et al., 2020). After passing through heat-expanded shale aggregates, water then flowed into bioreactor tanks half-filled with woodchips. Woodchips facilitate microbial degradation by serving as hosts for microorganisms and can adsorb pesticide with higher organic adsorption coefficient, hence can be used as an inexpensive onsite remediation technique for the removal of pesticides (Brás et al., 1999; Rodriguez-Cruz et al., 2007; Ilhan et al., 2012). Woodchip bioreactors have successfully reduced concentrations of pesticides such as oxyfluorfen, chlorpyrifos, bifenthrin, acetochlor, atrazine and sulfamethazine (Ilhan et al., 2012; Ranaivoson et al., 2019; Abdi et al., 2020). In our study, the concentration of most of the pesticides was reduced by the bioreactors probably because of microbial degradation in woodchip media, pesticide adsorption by woodchips and by greater exposure of RR water for photodegradation and volatilization.

4.3 Recycled water can be used to irrigate ornamental shrubs

Our results indicate that residual pesticides in recycled water had little to no impact on either growth index or plant dry weight of container nursery plants. In 2017, pesticides were applied in three different events and plants were exposed when most of the vegetative growth for the season had already occurred. Also, the frequency of rainy days between first spray and the last

spray was in the order of 2017>2018>2019. The higher frequency of rainfall may have washed off the residual pesticide from plants and diluted pesticides in treatment water hence may be the reason behind no differences in growth for 2017 and 2018. In 2019, growth was higher for *J. horizontalis* and *Rosa sp*. plants irrigated with RGW compared to RRR water but not RR water. The reason for reduced growth under irrigation with the RRR water is unclear. It is unlikely that residual pesticides reduced growth of plants irrigated with RRR water, as plants irrigated with RR, which generally had the highest pesticide concentrations, grew as good as plants irrigated with RGW.

For all three years, the pesticide concentrations found in RR water and RRR water did not cause pesticide-related visual injury. Pesticide concentration in RR water and RRR water was also not high enough to reduce shoot dry mass in 2018. In 2019, all three water treatments had similar shoot dry mass except shoot weight of *Rosa sp.* was reduced by RRR water and shoot weight of *H. arborescens* and *H. paniculata* was reduced by RR water. However, plants receiving RR water also had higher shoot dry mass for *H. macrophylla*. These slight differences in 2018 and 2019 probably are because of variables other than irritation treatments. Similar to shoot weight, in most cases, RGW was not in any way superior for leaf weight, stem weight and root weight in 2018 compared to RRR water and RR water. Pesticide related visual injury, GI and total dry biomass are dependent upon pesticide concentration in water. In a study by Huang et al., 2015, glyphosate applied at the rate of 0.0866 kg a.i./ha d id not reduce plant height and dry weight in soybean but increasing the dose further reduced both, dry weight and plant height, and those reductions were

directly proportional to dose applied (Huang et al., 2015). In another study by Poudyal et al., chlorpyrifos at a residual dose of 0.4 mg/L and isoxaben at 1.4 mg/L did not produce leaf visual injury in *H. paniculata*, *Cornus obliqua* (Powell garden) and *Hosta* (Gold standard) (Poudyal et al., 2019). Similarly, prodiamine at a residual concentration of 6 mg/L also did not produce any visible symptoms on *Prunus persica* (peach seedling) (Lourens et al., 1989). However, herbicides at higher doses have seen to produce visual injury in a wide range of ornamental species (Mathers et al., 2012). Oryzalin at 100 µg/L did not affect fresh root and shoot weight of *Pennisetum rupelli* (fountain grass), but increasing dose to 1000 µg/L reduced both root and shoot weight (Bhandary et al., 1997b) and insecticides such as malathion, permethrin and tetramethrin had dose-dependent effect on biomass production of Sitka spruce (Straw and Fielding, 1998). In our study, the pesticide concentration in RR water and RRR water was probably very diluted by irrigation RR hence did not affect plant growth. When plants are grown in an open field, rain events may wash off pesticide residues from plant parts lowering the risk of phytotoxicity.

Photosynthesis, Fv'/Fm' and Fv/Fm reflect instantaneous plant responses and many pesticides, particularly herbicides, can potentially interfere with those physiological processes (Huang et al., 2012; Silva et al., 2014; Wang et al., 2018). Therefore physiological tools can be used to rapidly assess the physiological impact of pesticides (Petit et al., 2012; Wang et al., 2018; Sharma et al., 2019; Giménez–Moolhuyzen et al., 2020). If the effect of pesticide residue in RR water and remediate water were long-lasting and affected plant physiology, these physiological

parameters would likely reflect it. In the current study, however, there was little evidence that pesticides in the RR water or RRR water impacted physiological function following irrigation with recycled water. Various authors have reported a reduction in photosynthesis and fluorescence parameters by exposure to chlorpyrifos (Xia et al., 2006), isoxaben (Fernandez et al., 1999; Poudyal et al., 2019), acephate (Haile et al., 2009) and glyphosate (Huang et al., 2012) but the concentration they used in all the cases was higher than the pesticide concentration found in RR water and RRR water in our study. Our protocol for physiological measurements was designed to assess potential injury associated with long-term chronic pesticide exposure. However, physiological measurements are plastic and can recover from short-term damage. It is possible that we did not measured reductions in photosynthesis and fluorescence measured immediately after pesticide applications. Nonetheless, the lack of growth impacts associated with RR irrigation suggests any perturbations in photosynthetic function, if they occurred, were minor and transient. Moreover, there were no reductions in chlorophyll SPAD index for plants receiving RR water or RRR water compared to RGW.

Pesticides, mainly herbicides, may produce negative effect on plant growth and physiology. However, a sub-lethal or lower dose of some pesticides may have a positive impact on plants and has been documented in few studies; sub-lethal/lower dose of eleven different herbicide increased root and shoot growth in *Avena sativa* (oat) (Wiedman and Appleby, 1972), glyphosate application at lower that recommend doses stimulated plant growth in a range of plants

species from cereal crops to ornamentals (Velini et al., 2008), lower concentration of chlorpyrifos improved growth and photosynthetic parameters in *Vigna radiata* (mung bean) while higher concentration reduced both, growth and photosynthesis (Parween et al., 2011) and fungicides such as phthalimide and azoles improved growth and photosynthesis in various crops (N. and Türkyilmaz, 2003; Petit et al., 2012). In our study pesticides present in RR water and RRR water were thousands of fold lower compared to general application rates and may have promoted plant growth and physiology, at few instances, instead of hindering it. However, studies on the effect of lower concentrations of pesticides on plant growth and physiology have not extensively published and further confirmation needs to be done before asserting the positive impacts of pesticides at lower concentrations.

5. Conclusion

From the results of our study, we can group pesticides into three different groups based on the likelihood to be detected in recycled water. Pesticides with high detection possibility include acephate, glyphosate and mefenoxam, pesticides with moderate detection possibly include isoxaben, chlorpyrifos, triflumizole and thiophanate-methyl and pesticides with low detection possibility include bifenthrin and prodiamine. In our study pesticide concentration in nursery retention reservoir was thousands of time lower compared to typical application rates and was dependent on pesticide solubility, pesticide adsorption and pesticide persistence. Finding from our

study reveals the possibility of using recycled water for irrigation of various woody ornamental species without impacting the growth and physiology of those plants.

APPENDIX

APPENDIX

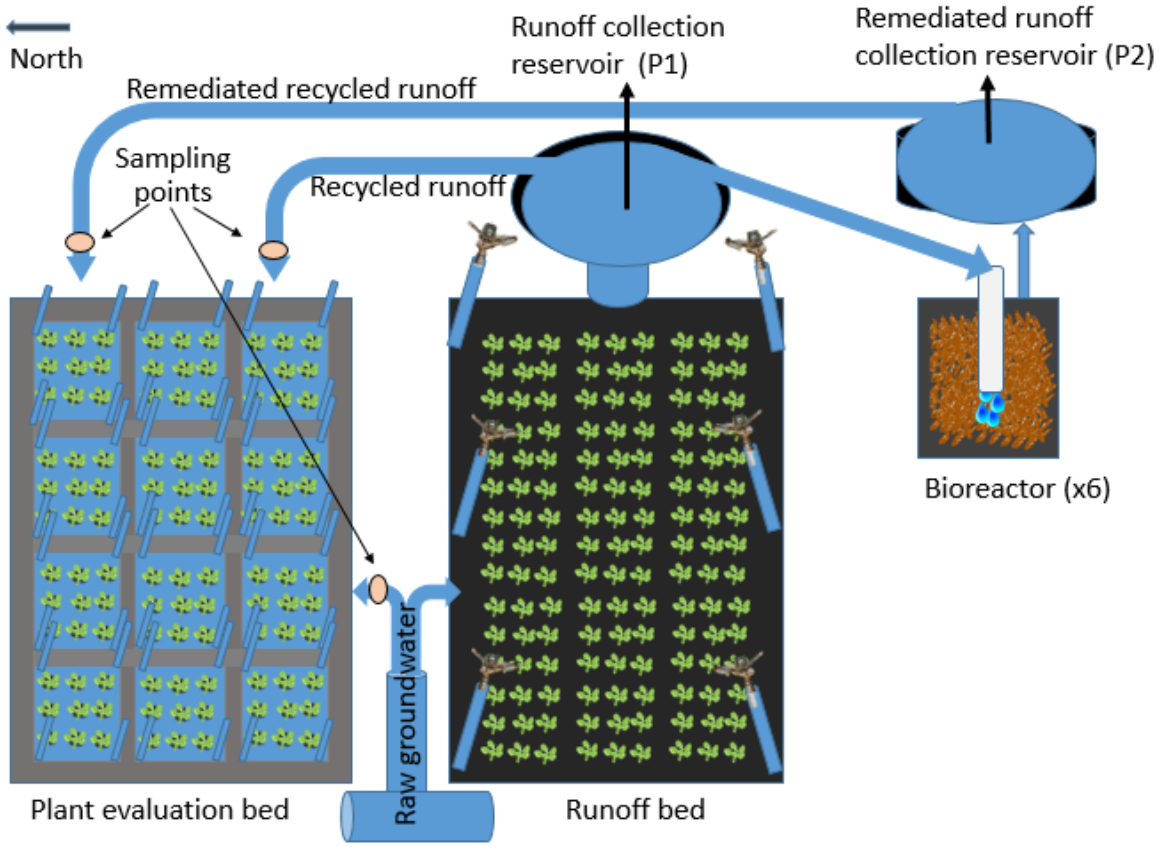

Figure V - 1. Layout of the field study. The plant evaluation bed had four rows and three irrigation zones in each row. Each row had all three water treatment zones that were randomly assigned. Irrigation treatments were water either from raw groundwater (RGW), recycled runoff (RR) from the collection reservoir or remediation recycled runoff (RRR) from the collection reservoir. Figure is not to the scale.

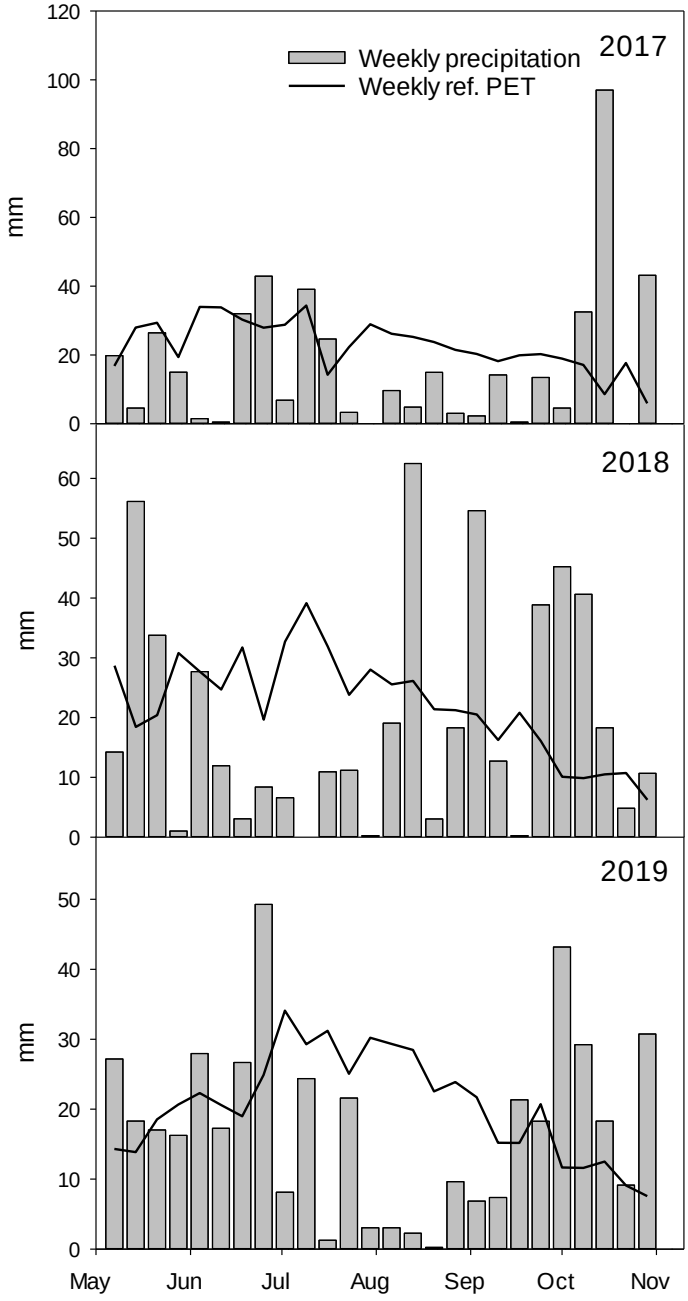

Figure V - 2. Weekly reference potential evapotranspiration (Weekly ref. PET) and weekly precipitation during the treatment application period at the research site from 2017 to 2019. Source: Michigan State University EnviroWeather: https://mawn.geo.msu.edu/station.asp?id=msu

Table V - 1. Pesticides application rates (express as g a.i./ha), concentration of pesticide solution (expressed as g a.i./L), total amount of solution sprayed (expressed as liter) and pesticide concentration in recycled runoff (RR) water and remediated recycled runoff (RRR) water (expressed as µg/L) water during the three year study period. Each water source was sampled twice after each application. First samples were collected a day after pesticide application and the last sample were collected 10 to 15 days after pesticide application. Samples for pesticide concentration were not collected in 2017.

Year 2017 (No pesticide sampling done)

Pesticide sprayed	Rate (g a.i./ha)	Concentration (g a.i./L)	Amount sprayed (L)	7/31/2017	8/14/2017	8/28/2017
Acephate	6.27	0.29	25	x	x	x
Chlorpyrifos	26.25	1.19	25	x		
Prodiamine	198.43	8.93	5			x
Glyphosate	228.26	10.28	5		x	
Thiophanate-methyl	11.28	0.51	25		x	
Triflumizole	6.65	0.3	25	x		
Bifenthrin	3.04	0.14	25			x
Mefenoxam	0.43	0.02	25			x

Year 2018

Pesticide sprayed	Rate (g a.i./ha)	Concentration (g a.i./L)	Amount sprayed (L)	Pesticide sprayed 7/4/2018	Sampled on 7/5/2018 RUW	Sampled on 7/5/2018 REW	Sampled on 7/15/2018 RUW	Sampled on 7/15/2018 REW	Pesticide sprayed 7/16/201	Sampled on 7/17/2018 RUW	Sampled on 7/17/2018 REW	Sampled on 7/25/2018 RUW	Sampled on 7/25/2018 REW	Pesticide sprayed 7/26/201	Sampled on 7/27/2018 RUW	Sampled on 7/27/2018 REW	Sampled on 8/08/2018 RUW	Sampled on 8/08/2018 REW	Pesticide sprayed 8/10/201	Sampled on 8/11/2018 RUW	Sampled on 8/11/2018 REW
Acephate	6.27	0.29	25	x	15.7	57.3	0.7	12	x	102	15.6	3	6.9	x	1.5	5.6	*	0.96	x	150	15.7
Chlorpyrifos	26.25	1.19	25		*	*	*	*	x	41.1	*	*	*		*	*	*	*		*	*
Isoxaben	101.27	4.56	5	x	58.5	179	11.3	19.6		13.8	14	3.4	1.1		*	0.7	0.6	0.4		0.9	0.4
Glyphosate	228.26	10.28	5		*	*	*	*		*	*	*	*	x	*	*	100	*	x	1051	18.3
Thiophanate-methyl	11.28	0.51	25		*	*	*	*		*	*	*	*	x	*	*	*	*	x	5.5	1
Triflumizole	6.65	0.3	25		2.9	0.34	1.5	0.3	x	13	0.4	1.5	0.3		1.6	0.4	0.5	*		0.6	*
Bifenthrin	3.04	0.14	25	x	*	*	*	*		*	*	*	*		*	*	*	*		*	*
Mefenoxam	0.43	0.02	25	x	2.5	3.9	0.6	1.8		0.4	1.5	0.3	*		*	0.3	*	*		*	*

Year 2019

Pesticide sprayed	Rate (g a.i./ha)	Concentration (g a.i./L)	Amount sprayed (L)	Pesticide sprayed 7/15/201	Sampled on 7/16/2019 RUW	Sampled on 7/16/2019 REW	Sampled on 7/29/2019 RUW	Sampled on 7/29/2019 REW	Pesticide sprayed 7/30/201	Sampled on 7/31/2019 RUW	Sampled on 7/31/2019 REW	Sampled on 8/18/2019 RUW	Sampled on 8/18/2019 REW	Pesticide sprayed 8/19/201	Sampled on 8/20/2019 RUW	Sampled on 8/20/2019 REW	Sampled on 9/8/2019 RUW	Sampled on 9/8/2019 REW	Pesticide sprayed 9/9/2019	Sampled on 9/10/2019 RUW	Sampled on 9/10/2019 REW	Sampled on 9/24/2019 RUW	Sampled on 9/24/2019 REW
Acephate	6.27	0.29	25	x	82.2	*	*	*	x	137.2	24.9	*	*	x	87.7	*	*	2.4	x	78.7	7.2	*	8.4
Isoxaben	101.27	4.56	5		*	*	*	*		3.5	6.8	*	*		1.3	*	*	15.1	x	166.2	8.3	5.5	16.8
Prodiamine	198.43	8.93	5		*	*	*	*		*	*	*	*	x	*	*	*	*		*	*	*	*
Glyphosate	228.26	10.28	5	x	1917.5	520.2	*	*		*	*	*	*		*	*	*	25.2		*	*	*	*
Thiophanate-methyl	11.28	0.51	25		*	*	*	*		*	*	*	*		*	*	*	*	x	11.9	1.5	*	*
Triflumizole	6.65	0.3	25		*	*	*	*	x	16.9	1.3	1.1	*		*	*	*	*		*	*	*	*
Bifenthrin	3.04	0.14	25	x	*	*	*	*	x	*	*	*	*	x	*	*	*	*		*	*	*	*
Mefenoxam	0.43	0.02	25	x	*	*	*	*		*	*	*	*	x	9.5	*	*	*		*	*	*	*

RUW = Pesticide residue in nursery runoff water (ug/L); REW = Pesticide residue in remediated runoff water (ug/L) ; * = pesticide below detection

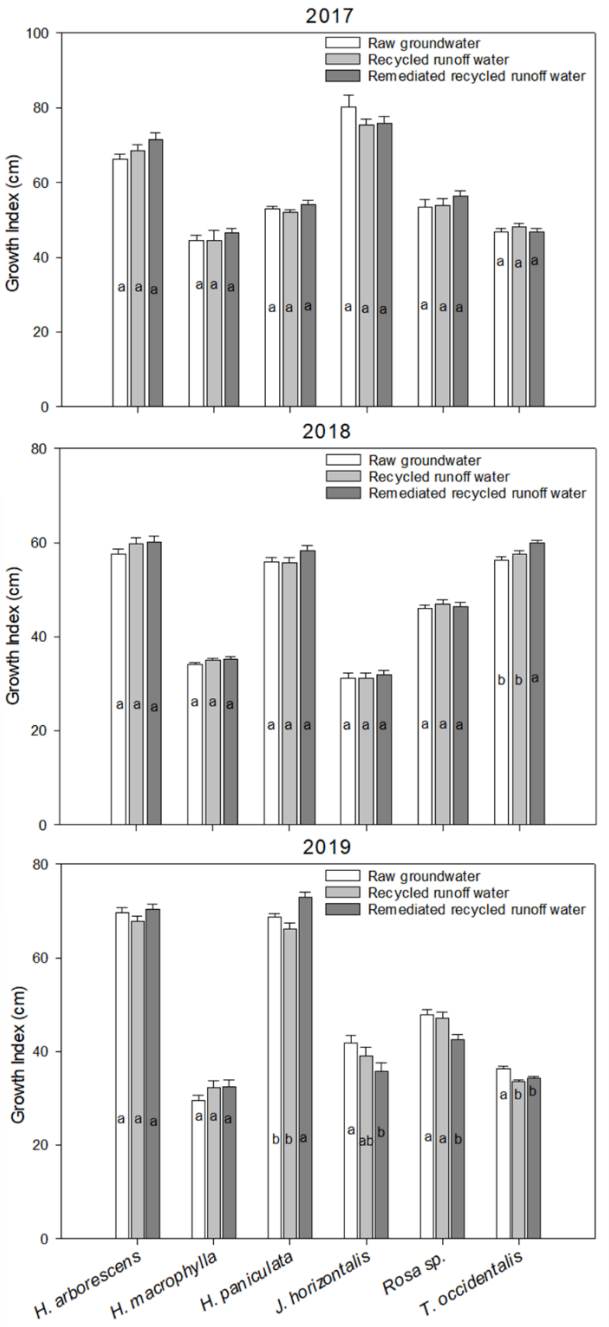

Figure V - 3. Growth index of six ornamental taxa irrigated with raw groundwater (RGW), recycled runoff (RR) water and remediated recycled runoff (RRR) water. Recycled runoff water was captured from a nursery bed managed according to standard nursery practices, including fertilization and pesticide applications, for the region. Remediated recycled runoff water was the recycled runoff water that passed through a heat-expanded shale and woodchip bioreactor system. Means within a taxon that are followed by same letters are not significantly different at p=0.05. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each taxon were carried out using Fisher's Least Significant Difference (LSD) post-hoc test.

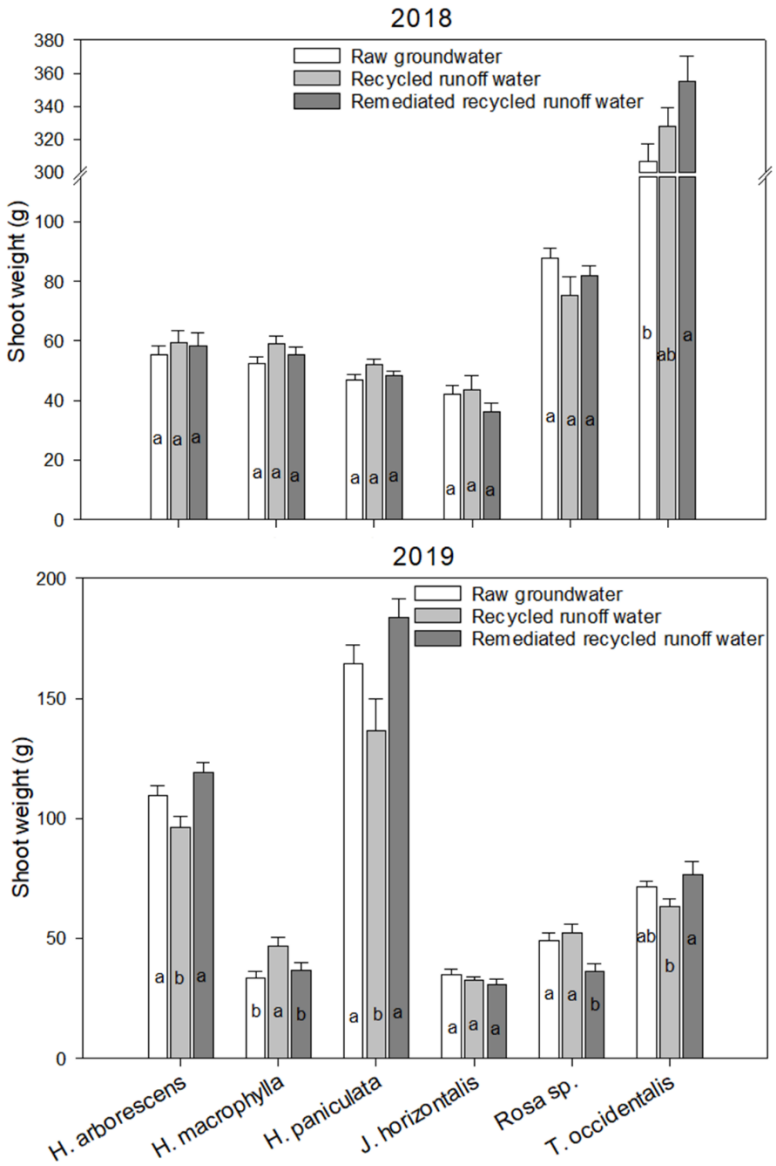

Figure V - 4. Shoot weight of six ornamental taxa irrigated with raw groundwater (RGW), recycled runoff (RR) water and remediated recycled runoff (RRR) water in 2018 and 2019. Recycled runoff water was captured from a nursery bed managed according to standard nursery practices, including fertilization and pesticide applications, for the region. Remediated recycled runoff water was the recycled runoff water that passed through a heat-expanded shale and woodchip bioreactor system. Means within a taxon that are followed by same letters are not significantly different at p=0.05. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each taxon were carried out using Fisher's Least Significant Difference (LSD) post-hoc test.

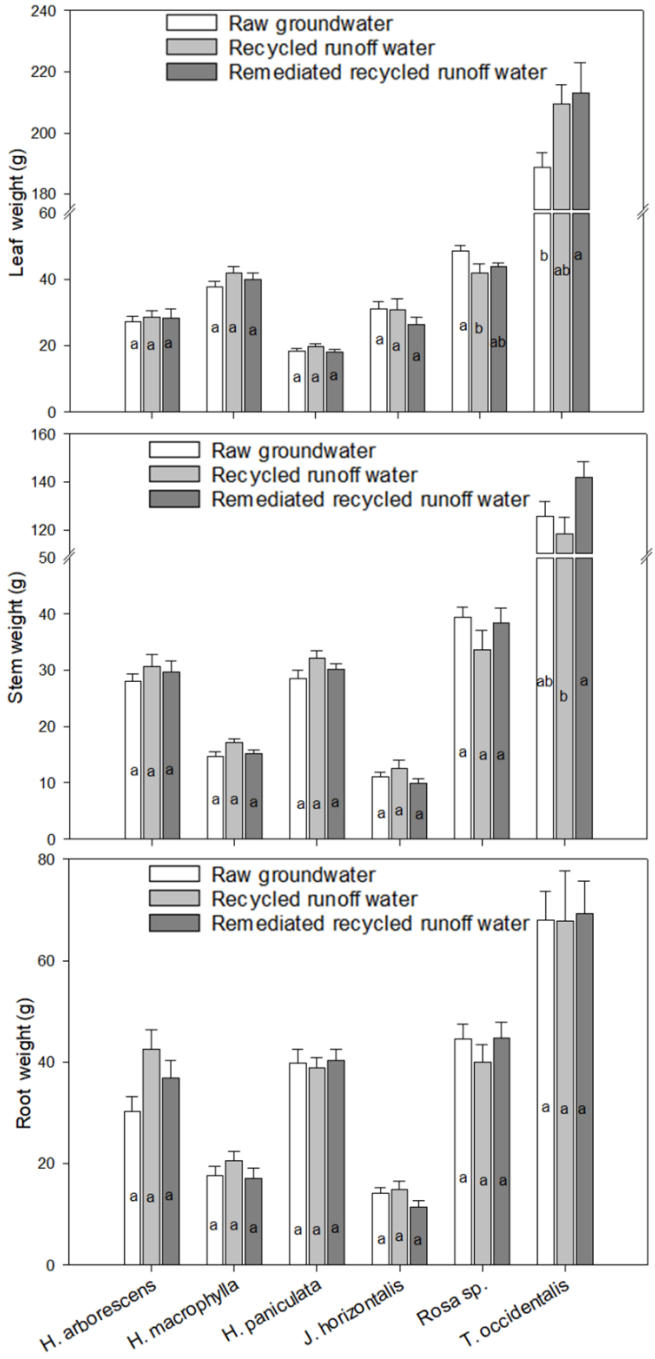

Figure V - 5. Leaf weight, stem weight and root weight of six ornamental taxa irrigated with raw groundwater (RGW), recycled runoff (RR) water and remediated recycled runoff (RRR) water in 2018. Recycled runoff water was captured from a nursery bed managed according to standard nursery practices, including fertilization and pesticide applications, for the region. Remediated recycled runoff water was the recycled runoff water that passed through a heat-expanded shale and woodchip bioreactor system. Means within a taxon that are followed by same letters are not significantly different at p=0.05. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each taxon were carried out using Fisher's Least Significant Difference (LSD) post-hoc test.

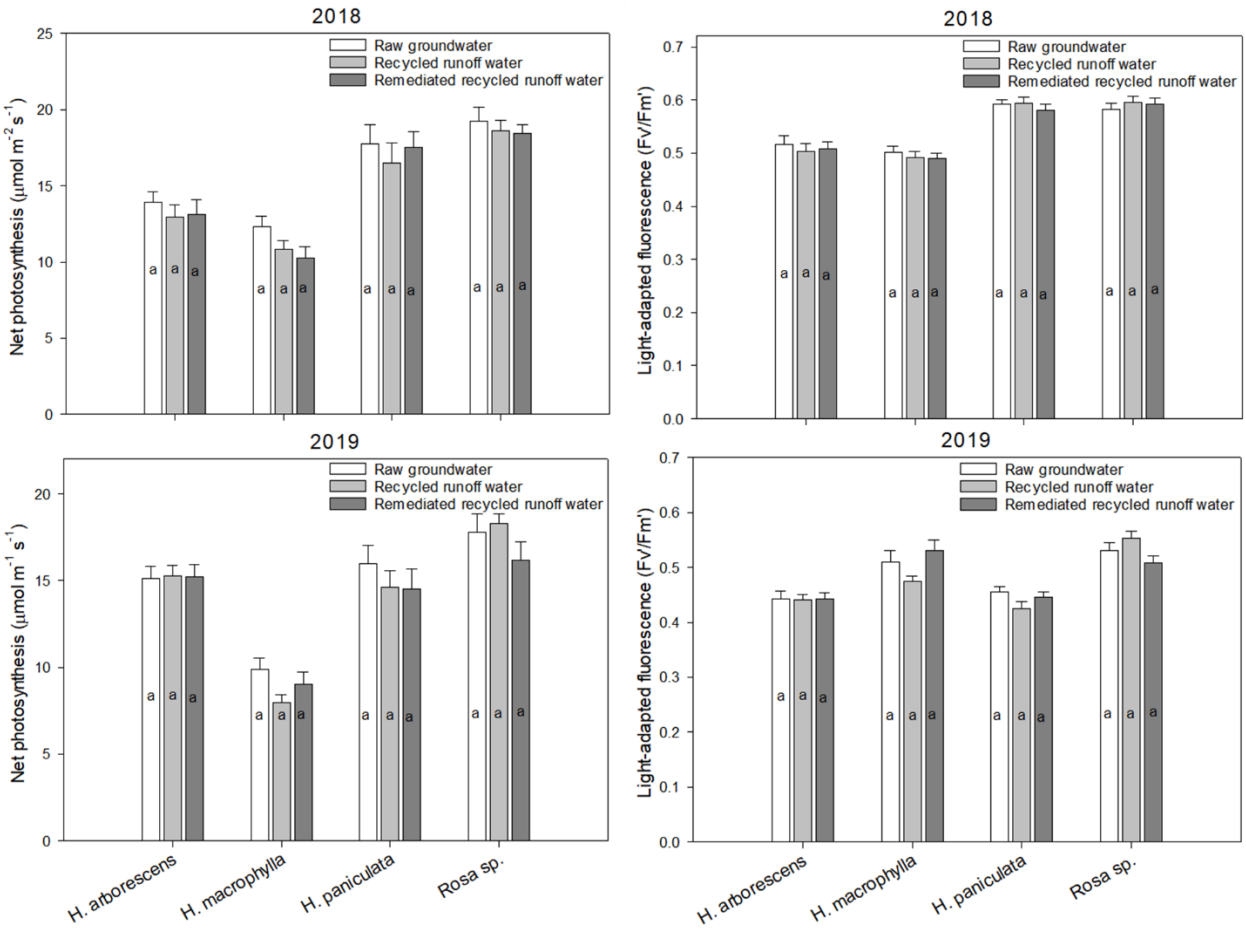

Figure V - 6. Net photosynthesis and Light-adapted fluorescence of four ornamental taxa irrigated with raw groundwater (RGW), recycled runoff (RR) water and remediated recycled runoff (RRR) water in year 2018 and 2019. Recycled runoff water was captured from a nursery bed managed according to standard nursery practices, including fertilization and pesticide applications, for the region. Remediated recycled runoff water was the recycled runoff water that passed through a heat-expanded shale and woodchip bioreactor system. Means within a taxon that are followed by same letters are not significantly different at p=0.05. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each taxon were carried out using Fisher's Least Significant Difference (LSD) post-hoc test.

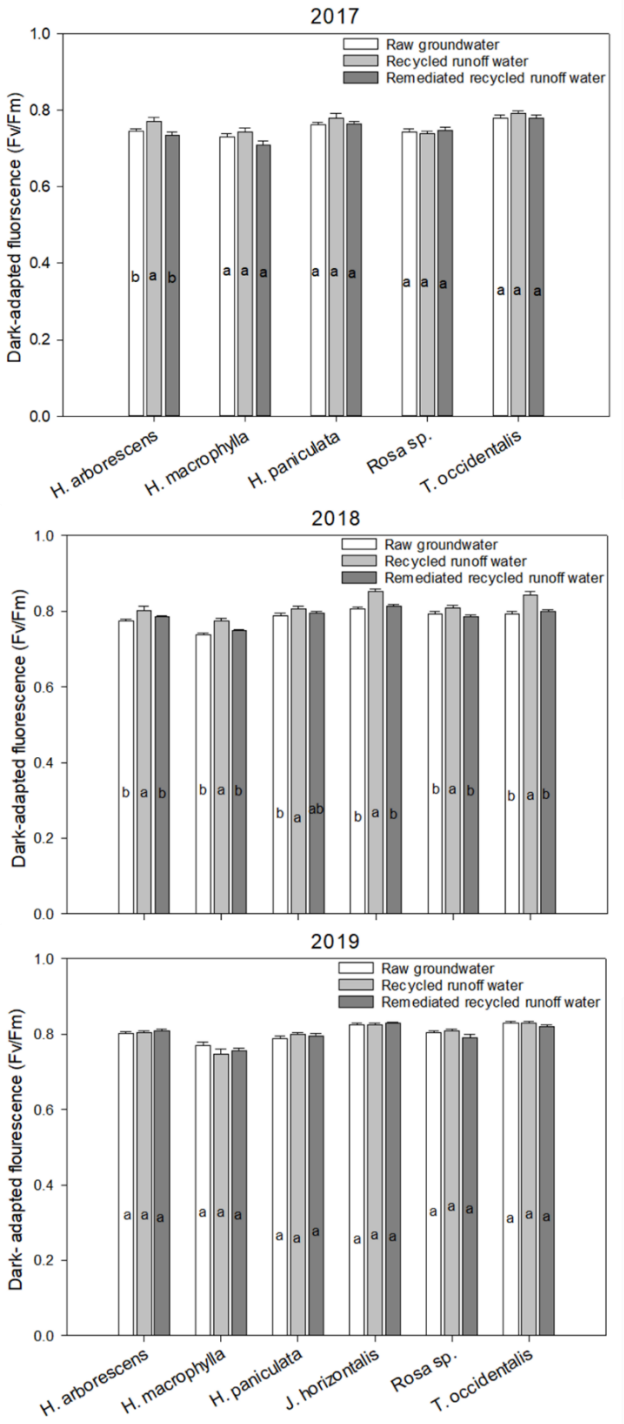

Figure V - 7. Dark-adapted fluorescence of different ornamental taxa irrigated with raw groundwater (RGW), recycled runoff (RR) water and remediated recycled runoff (RRR) water. Recycled runoff water was captured from a nursery bed managed according to standard nursery practices, including fertilization and pesticide applications, for the region. Remediated recycled runoff water was the recycled runoff water that passed through a heat-expanded shale and woodchip bioreactor system. Means within a taxon that are followed by same letters are not significantly different at p=0.05.

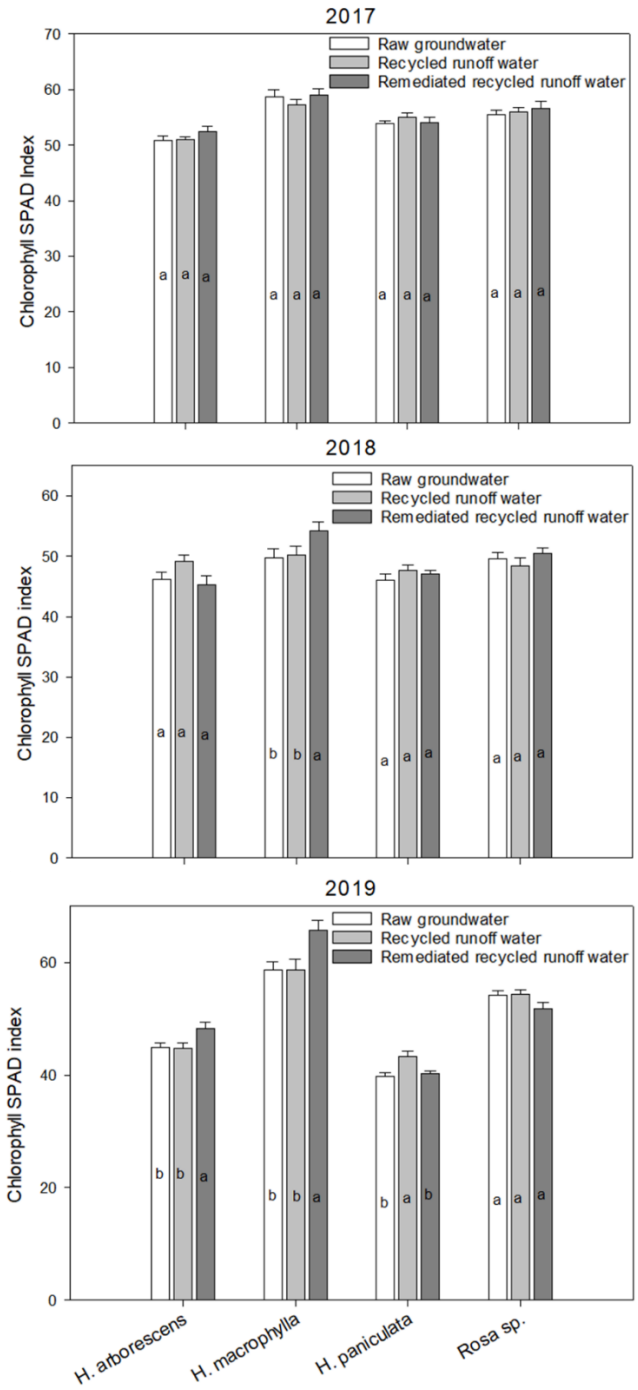

Figure V - 8. Chlorophyll SPAD index of four different ornamental taxa irrigated with raw groundwater (RGW), recycled runoff (RR) water and remediated recycled runoff (RRR) water. Recycled runoff water was captured from a nursery bed managed according to standard nursery practices, including fertilization and pesticide applications, for the region. Remediated recycled runoff water was the recycled runoff water that passed through a heat-expanded shale and woodchip bioreactor system. Means within a taxon that are followed by same letters are not significantly different at p=0.05. Standard errors of the means are denoted by vertical 'T' lines. Mean separations for each taxon were carried out using Fisher's Least Significant Difference (LSD) post-hoc test.

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