Fungal associations and improving micropropagation of native *Rhododendron* spp.

by

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Abstract

Rhododendron is the most abundant genus in the *Ericaceae* family, consisting of over 1000 species that are native to North America, Western Europe, and Northern Asia. With certain Rhododendron varieties being desirable due to flower color or bloom time, propagation of these shrubs is of increased interest. Micropropagation is a technique of multiplying small plant explants, or cultures, in a sterile environment. The overall goal of this study was to improve established micropropagation protocols to increase explant survivability and shoot growth of native deciduous *Rhododendron in vitro.* The specific objectives were: 1) to evaluate fungicide media amendments to increase shoot survivability; 2) to investigate fungal contamination found in micropropagation systems for endophytic associations; and 3) to investigate alternative hormones and container sizes for increasing shoot production. For the first objective, five native deciduous *Rhododendron* nicknamed varieties were established in fungicide amended media. Shoot survival and bud production in each variety was recorded for all fungicide-amendments over 31 days in culture. Thiophanate-methyl-amended media was shown to significantly increase survivability and bud production in four of these varieties. To address the second objective, 20 fungal isolates were identified and analyzed for potential endophytic associations. Of the 20, two isolates were identified from genera *Alternaria* and *Trichoderma*, both known for endophytic activity within *Rhododendron*. For the third objective, Thidiazuron hormone ratios and container sizes were tested in five *Rhododendron* varieties.

Change in explant weight, shoot proliferation, and shoot elongation was evaluated over a 4 week culture period. Shoot performance was significantly improved with the addition of 4.4-8.8 ppm of TDZ stock solution and larger test tube sizes compared to the control.

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List of Abbreviations

2iP	2-isopentenyladenine
TDZ	Phenyl-N'-(1,2,3-thiadiazol-5-yl) urea
TM	Thiophanate-methyl
Triad	Triademifon
Chloro	Chlorothalonil
PGR	Plant Growth Regulator
Ppm	parts per million
MS	Murishage and Skoog media
WPM	Woody Plant Media
E&R	Economou and Read media
PCR	Polymerase Chain Reaction

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Literature Review

Introduction

Green, or plant, biotechnology is based on the confirmed totipotency of plant cells. While biotechnology has been a word commonly associated with genetic engineering, they are not exclusively related to one another. Biotechnology is broadly defined as the process of utilizing biotechnological systems in order to make products. The concept of totipotency is essential in the cell theory of Matthias Schleiden and Theodor Schwann, developing the foundation of modern biology by acknowledging that the cell is the primary unit of all living things. Hermann Vochting noticed that an entire plant could be built up from fragments of plant organs. Up until the 20th century, no real attempts were made to test these theories due to the lack of necessary technologies and understanding of the nutritional requirements of cultured cells (Gautheret 1985).

Experiments were started by Gottlieb Haberlandt to prove the totipotency of plant cells by culturing chloroplast-containing differentiated cells from *Lamium purpureum* leaves, *Eichhornia crassipes* petiole cells, and *Pulmonaria glandular* hairs. The cells did not undergo cell divisions, but they did grow in size. His failings were due to his choice of growing media material, specifically in the lack of nutritional requirements (Thorpe 1995).

More than a century has passed and the culture of leaf cells and other materials used by Haberlandt is difficult or impossible in most species. Nevertheless, Haberlandt laid the

foundation for plant tissue culture with his observations. These observations included but were not limited to: advocating for the use of embryo sac fluids for inducing cell divisions in vegetative cells, and hinting at the possibility of successfully cultivating artificial embryos (Nomura and Komamine 1995). These artificial embryos, known now as somatic embryos, are the predominant means of plant regeneration in a wide range of species (Vasil 1999). For these reasons, Haberlandt is commonly credited with being the founder of plant cell culture.

The discovery of auxin and its identification as indole-3-acetic acid in 1934 (Went and Thimann 1937) allowed increased success in tissue culture (Gautheret 1983). Leaves from dodder host plants produced an extract, and when combined with indole-3-acetic acid (IAA) in culture, yielded varying plant tissue production results soon after discovery of the hormone.

Continued cell division and bud formation of tobacco on media with adenine and high levels of phosphate was reported by Skoog and Tsui in 1951. In 1954, cell divisions were observed when tobacco explants were cultured without vascular tissue. Plant extracts such as coconut milk were added to the media to replace these vascular tissues in hopes to identify the factors responsible for the beneficial effect of increased plant tissue growth. Yeast extract proved to be the most effective and its active compound had purine-like properties. As a result, DNA was added to the media to enhance cell division activity (Vasil 1959). Incorporation of old herring sperm DNA samples to media allowed

for the isolation of kinetin (Miller et al. 1955) and a further understanding of the hormonal regulation of shoot growth in plants.

Later trials led to the successful isolation of synthetic and naturally occurring cytokinins, their role in bud development and cell division, and their use in the micropropagation industry. Furthermore, the development of nutrient solutions, choice of plant material, and focus on importance of aseptic cultures led to long term cultures of tomato roots and cambial tissues of tobacco and carrot (White 1934).

White (1934) believed that the nutrient solution based on Knop's and others didn't provide optimal growth or have stability to be used over a range of pH values. He went on to develop a medium that was used until the 1960s. A study of mineral and other requirements of plant tissues grown *in vitro* was performed, indicating a need for increased levels of mineral salts in White's medium (Ozias-Akins and Vasil 1985). Similarly, an increase of growth was noted by Murashige and Skoog in 1962. Inorganic constituents of extracts added to White's medium led to the first chemically defined and widely used nutrient solution for plant tissue cultures. High levels of organic constituents, such as chelated iron and myintosol, allowed the media to be more stable and accessible during the life of cultures.

Rhododendron genus:

Rhododendron is the most abundant genus in the *Ericaceae* family, consisting of over 1000 species that are native to North America, Western Europe, and Northern Asia. *Rhododendron* spp. include evergreen and deciduous species ranging from shrubs to tree-sized plants with flowers blooming from late winter to early summer. They are acid-loving plants (preferring a pH range of 4.5-6), grow best in cool shady environments, and thrive in a well-drained soil. Some *Rhododendron* species are used as ornamentals in landscapes and woodlands due to their floral display (Zimmerman and Van Eck 2015); however, desirable species can be often difficult to find in retail markets**.**

There is an ongoing discussion about the taxonomy of the *Rhododendron* genus. The latest of these discussions has focused primarily on the subgenera including *Pentanthera* (deciduous azaleas) with about 25 species and *Tsutsusi* (evergreen rhododendron) with about 15 species. These subgenera are divided further into many taxonomic groups including sections and subsections, each of these include one or more species. There is no clear distinction between these two subgenera but there are some generalizations concerning the two. Deciduous azaleas typically do not have lepidote scales underneath leaves and other plant parts while evergreen Rhododendrons do. Lepidote scales are tiny scales found underneath the leaves of some Rhododendron that protect the plant's stomata. Generally, azaleas have one stamen on each of their five lobes and most Rhododendrons have two stamens per lobe for a total of 10 or more

stamens in each flower. Also, the flowers of Rhododendrons are typically bell-shaped as opposed to the funnel-shaped flowers of azaleas (Anonymous 2015).

With certain Rhododendron varieties being desirable due to flower color or bloom time, propagation of these shrubs is of increased interest. Plant propagation is a method of growing new plants and can be done in various ways from a range of sources: seed, cuttings, and other plant parts. Some plants do not grow from seed or are difficult to reproduce vegetatively. Sources such as cuttings are limited by size of the mother plant from which a limited number of cuttings can be taken (Mondal and Chand 2002). Propagating *Rhododendron* by cuttings is the most popular as well as problematic technique one can use; this is particularly true for deciduous azaleas. The main problems are initiating rooting with deciduous azaleas and producing new growth after rooting. To overcome some of these problems in deciduous *Rhododendron*, plants can be produced from a single parent plant by other methods of vegetative cloning without having changing the genetic material (Galle 1979); one method of cloning is micropropagation.

Micropropagation

Micropropagation is defined as the *in vitro* clonal propagation of plants from nodal explants or shoot tips that can originate from a stock plant in the landscape. This process is usually applied to generating a proliferation of shoots during subculturing. It

is typically categorized by 4 stages: initiation of explants, shoot multiplication, root stimulation, and acclimatization of these rooted explants (Shaeffer 1990) (Fig. 1).

Plant tissue culture refers to growing and multiplying cells, tissue, or organs of plants on media under aseptic conditions and a controlled environment. Micropropagation is the technology of plant tissue culture commercially applied in which rapid proliferation of explants from initial stocks is achieved. Micropropagation is a tool utilizing numerous steps in achieving production of a final product as a plant ready for commercial or residential use. These steps include but are not limited to: pre-propagation, establishment of explants, maintenance of explants maximizing proliferation, rooting, and hardening off of the explant to ex-vitro conditions.

The most widely used form of tissue culture is shoot culture to produce disease-free and contaminant-free material (George 1993). Induction of shoots in culture often involves adjusting the media components, specifically plant growth regulators (PGRs), to promote optimal shoot multiplication and root stimulation preceding acclimatization. Difficulty in each of these stages has been documented between species and even between genotypes within a species (Naik et al. 2003). Failure to achieve sterile explants, poor explant performance, and premature explant death are the biggest concerns in establishment during Stage I (Lynch 1999). No response to cytokinin and auxin along with slow and abnormal growth are also concerns that may inhibit optimal shoot multiplication in Stage II (Benson 2000). Lack of response to auxin, excessive

callus, or weakening in shoot quality has been noted in maintaining explants during stage III, especially in woody plants (Lynch 1999).

When transferring explants to soil, they are exposed to a reduction in nutrients, reduced humidity, and higher light levels in some cases (Preece and Sutter 1991). Explants must be able to survive the transition from depending on a carbon source and become photoautotrophic in order to thrive in *ex vitro* conditions (Pospisilova et al. 1999).

Conventional Propagation

There are conflicting reports on the difficulty of native *Rhododendron* propagation through conventional means. Galle (1987) reported that the Florida Flame azalea, *Rhododendron austrinum*, could be easily propagated from softwood cuttings as opposed to the moderate difficulty of propagating Piedmont azalea, *R. canescens*. Bir (1992) reported that native *Rhododendron* cuttings root best from softwood cuttings taken when there is no longer new growth. Bir (1992) also recommended that 0.5-0.8% indole-3-butric acid (IBA) powder or 1000 – 2500 ppm IBA solution be added to medium. Berry (1998) reported that both *R. canescens* and *R. austrinum* can be propagated from new soft growth with the addition of 5000 ppm of potassium salt of IBA in the form of K-IBA. Knight et al. (2005) reported the best rooting for *R. canescens* cuttings occurred between 8000 and 10000 ppm K-IBA; a decline in rooting percentage resulted from exceeding 10000 ppm K-IBA (Knight et al. 2005).

Dirr and Heuser (1987) reported that stoloniferous *Rhododendron* root easier than some non-stoloniferous species. Rooting the cuttings and production of new growth in the spring are noted as the main problems related to native *Rhododendron* propagation from cuttings. Dirr and Heuser (1987) recommended using 4000 ppm IBA and sticking cuttings into a 100% peat moss medium with a fungicide application.

With varying information regarding the best hormone concentration, Knight et al. (2005) undertook the challenge of determining the optimum K-IBA concentration for propagation of *R. canesens* and *R. austrinum*. They found that cuttings of both species rooted when treated with low levels of K-IBA. The use of 10000 ppm K-IBA increased root number, length and quality, which could result in a more marketable product for growers. Both species rooted fairly easily as reported by Galle (1987). However, the final result of poor cutting quality suggests that initiating new growth is as difficult, if not more so, than Dirr and Heuser (1987) originally reported. Knight et al (2005) concluded that 12% more cuttings would not be worth the additional IBA if the greater number of cuttings have inadequate root systems, especially for a producer (Knight et al. 2005).

Similarly, Bir (1996) has noted many specific environmental conditions that are necessary to successfully propagate and root native *Rhododendron* cuttings. Moisture is one of the most important of these environmental factors; thus, taking cuttings in the early morning is the best time since humidity is generally high this time of day. An extra watering of the parent plant may be necessary if the foliage already looks dry. As a rule

of thumb, cuttings should be taken from vigorous and healthy growth; softwood cuttings are preferred for native Rhododendron.

Fog or mist systems have also been implemented in an attempt to achieve optimal environmental conditions for rooting. These systems are usually run in the morning so plants have some time to absorb the moisture in the air before foliage is dried by the sun during the day. Decreasing the time in which there are wet leaves in the evening can prevent diseases from establishing or persisting in the plants. High pressure overhead systems are able to perform better when compared to low pressure due to the stable humid conditions and the ability to cool the microclimate within the plants (Bartok 2009). Mist systems are useful when propagating through cuttings because of their ability to cool the propagation area, hydrate cuttings without drowning them, and to allow the cuttings to maintain turgidity (Sommerville 2014).

Alternative approaches

There are two main approaches to propagate plants *in vitro*: by the multiplication of shoots from axillary buds and by the formation of adventitious shoots from existing explants. Shoot culture and single node culture are two methods used commonly to promote axillary shoots. Robbins (1922) was the first to culture shoot tips on a sugar containing media, however Loo (1945) was able to promote shoot growth from shoot tip explants in dodder plants and *Asparagus*. Loo (1945) made some significant

observations in that growth was dependent on sucrose concentration, explants could be propagated indefinitely, and shoot tip culture was a viable way to propagate plant material. Loo's work did not continue after these observations due to lack of roots formation on the *Asparagus* shoots. The first person to successfully induce root growth from *Tropaeolum* and *Lupinus* was Ball (1946), however, there was no shoot multiplication in these explants.

By 1972, there were numerous successful micropropagation reports through the method developed by Haramaki (1971) using *Gloxinia.* As a result, there was a dramatic increase in papers published on shoot culture and it since has been established as commercial protocol. The technique of node cultures that produce elongated shoots has become more popular in commercial plant propagation due to the increased probability of clonal stability. Furthermore, there is a lower likelihood that callus will develop, so subcultures have lower risk of developing genetic irregularities (George and Debergh 2008).

Adventitious shoot development, or organogenesis, is the formation of organs, such as roots and shoots, on explants from a variety of plant tissues. There is inconsistency in the frequency of organogenesis among varieties and species, suggesting that the proportion of receptive cells vary with *in vitro* conditions. Three distinct phases of organogenesis has been classified and each phase needs a specific balance of PGRs: dedifferentiation, determination, and morphogenesis (Sugiyama 1999).

Dedifferentiation can be defined at the moment when cells are able to respond to the hormone signals of organ induction (Howell et al. 2003). Wounding has been noted to trigger dedifferentiation in micropropagation (Sugiyama 1999). Determination is when the explants' competent cells are determined for forming specific organs and is influenced by the ratio of PGRs in the media. Adventitious shoots form from these competent cells with the help of cytokinin (Gahan and George 2008). Morphogenesis refers to the changes in morphology the micropropagated explants experiences while still *in vitro*, some of which can continue *in vivo*. Factors that are responsible for these changes vary from PGRs to the type of container used (Piqueras and Debergh 1999). There are typically two pathways of adventitious shoot formation, indirect and direct. Regeneration through adventitious shoots has been noted in woody species such as *Liquidambar styraciflua (*Kim et al. 1997*), Salix nigra* (Lyyra et al. 2006*),* and *Cassia angustifolia* (Siddique et al. 2010*).*

Factors affecting shoot regeneration and explant growth

There are a number of factors that affect the growth of an explant in micropropagation and its ability to regenerate shoots. These factors include but are not limited to: type of explant, plant growth regulators (PGRs), pH levels, media, and carbon source.

Studies have shown that characteristics of the explant (type, genotype, and source) affect the success and feasibility of micropropagation systems (Chan and Chang 2002).

Juvenile plants or juvenile growth in general have been a successful source for propagation material. Juvenile node cuttings have been used to propagate woody plants such as *Vitex negundo* (Ahmad and Anis 2011) and *Sapindus trifoliatus* (Asthana et al. 2011). Nodal segments are typically used due to the availability of axillary buds that only require a bud break trigger as opposed to adventitious buds from leaves or shoots that require initiation of buds before shoots can regenerate (Lombardi et al. 2007). Plants of different ages may have varied levels of endogenous hormones, influencing the success of regeneration of shoots. This has been noted in plants such as *Prunus* (Mante et al. 1989), *Malus* (Famiani et al. 1994), and *Cercis canadensis* (Distabanjong and Geneve 1997). Tang and Guo (2001) also determined genotype as a factor in successful regenerating shoots in micropropagation.

Plant growth regulators are chemical compounds that occur naturally in tissues of plants and regulate growth and development within the plant. These compounds are low in concentration and make up several classes; the most important include auxins and cytokinins. It has been noted that hormones involved in tissue culture can have synergistic or antagonistic roles (Skoog and Miller 1957). Altering ratios of these two types of hormones can favor meristematic growth through shoots or roots (Sugimoto et al. 2011). Increasing the ratio of cytokinin to auxin in media results in explant growth more shoots than roots.

Auxins and cytokinins are able to regulate morphogenesis and growth in general in plant tissue systems. Growth of callus and plant organs can be achieved when combining auxin with cytokinin in the media. Auxins have the ability to control processes within the plant such as cell elongation and division. With the capability of initiating cell division, auxins are involved in developing meristems that can produce unorganized tissue such as callus, or defined organs such as roots and shoots. Auxins are primarily involved in establishment, along with maintaining polarity and apical dominance within the plant (Friml 2003). The activity and success of individual auxins on growth is highly variable from plant to plant, specifically differing from cell to cell and age or physiological state of the plant exogenously (Davies 2004).

Auxins were first documented as a key part of tissue culture systems by the discovery of organogenesis regulation in vitro through different hormone rations in media (Skoog and Miller 1957). Li et al. (1994) has supported these findings through studies of the auxin/cytokinin relationship in regards to the response from various plants. Since each tissue culture system is unique to the host plant, the effects of plant growth hormones should be tested for each situation.

Cytokinins have been noted as being the most important factor affecting shoot regeneration, leading many to believe this is related to the histological changes in affected tissues (Magyar-Tabori et al. 2010). Cytokinins are defined as "N^6- substituted adenines with growth regulatory activity in plants that promote cell division and play a

role in cell differentiation" (McGaw and Burch 1995). When cytokinin is added to the medium, it can induce division and organogenesis along with other physiological and developmental processes. Specific cytokinins, like other hormones, differ in the rate of uptake, transport, and metabolism within the plant such that each variety and species should be investigated separately. Cytokinins administered in media have also been seen to interact with naturally occurring cytokinins of an explant (Van Staden et al. 2008).

There are a number of cytokinins available on the market such as benzyladenine (BA), thidiazuron (TDZ), and kinetin (Kn); however, 2-isopentenyladenine (2-iP) has been regarded as the best option for multiplication of shoots in *Vaccinium* (Cohen 1980). Furthermore, it was found in *Rhododendron* that the highest rate of shoot multiplication was seen on MS medium amended with 2-iP in studies when compared to BA or Kn (Singh and Gurung 2009; Vejsadova 2008).

Synthetic cytokinins such as TDZ have been shown to have varying effects depending on host plant, concentration, and the duration of exposure to the hormone (Murthy et al. 1988) and has been effective in promoting explant growth at extremely low concentrations for certain species (Lu 1993). Additionally, studies have shown the induction of bud regeneration is relatively high in explants administered with TDZ when compared to purine-based cytokinins such as 2-iP. (Mok et al. 2005).

While TDZ can be beneficial in some tissue culture systems, it can cause side effects that are undesirable such as hyperhydricity and inhibited shoot elongation. Overall, TDZ has been shown to be beneficial for regeneration of recalcitrant species of a select number of higher plants (Heutteman and Preece 1993) and has been successful in inducing adventitious shoot proliferation in herbaceous, perennial, and tree species such as *Cassia angustifolia* (Siddique and Anis 2007).

The essential components that make up plant tissue culture medium are mineral nutrients. The amount of nutrients supplied by the media can facilitate tissue growth over time and impact the quality of plant response to the nutrient ratio. There are 13 mineral elements essential for promotion of plant growth (Epstein and Bloom 2005). Determining optimum nutrient levels through trials can vary due to a high number of nutrient combinations that can be made depending on the plant genus. The development of MS media (Murashige and Skoog 1962) was elemental in creating a revised medium with high nitrate, ammonium, and potassium concentrations. The MS media was not optimal for all tissues, but it provided a starting point. With *Rhododendron* species, it was seen that MS media produced adverse effects such as browning and eventual death. After modification of woody plant media (WPM) in 1984, *Rhododendron* explants survived the duration of the culturing process. This modification was accomplished by reducing nitrogen and potassium, increasing ammonium and nitrate to 1:1 ratio, adding ammonium sulfate, replacing Na_2EDTA and $FeSO_4$ with FeNaDTPA, omitting KI, and eliminating most organic components (Economou and Read

1984). Explants did not turn brown in the modified medium, appearing healthy, with bud break occurring with the influence of 2-iP. Similar results were seen by others (Ma and Wang 1977) using ½ - 1/3 MS major elements in the medium.

Plants growing under tissue culture conditions are semiautotrophic (Hazarika 2003), preventing leaves formed during in vitro growth from attaining photosynthetic competence (Van Huylenbroeck and Debergh 1996). Plants under *in vitro* conditions have limited accessibility to CO_2 inside the vessel (Hazarika 2003). Therefore, sugar is often supplemented as a carbon source to maintain multiplication and growth of plant cells, organs, or whole plantlets. The addition of sugar to the media also helps maintain osmotic potential of cells and conservation of water (Hazarika 2003). The conservation of water is critical for settlement of *ex vitro* plants due to the lack of a well-developed cuticle and epicuticular wax *in vitro* grown plants (Van Huylenbroeck et al. 2000). Moreover, an exogenous supply of sugar increases starch and sucrose reserves in plants and could be a factor in favorable *ex vitro* acclimatization (Pospisilova et al. 1999). However it has been shown that the addition of sugar has a negative correlation with growth (Kwa et al. 1995) and photosynthesis (Hazarika 2003).

Endophytes in micropropagation

Endophytes are classified as microorganisms that have the ability to internally colonize a plant without having adverse effects on the host plant (Bacon et al. 2002). These

microorganisms stay inside the plant for a portion or the duration of their life cycle and do not seem to cause any symptoms of diseases. In some cases, endophytes can provide benefits to the plant. Endophytic fungi and bacteria are able to enter through plant stomata, root systems, and open wounds (Hallman et al. 1997). Endophytes can be isolated from tissue that has been surface sterilized, and can include prokaryotic bacteria, eukaryotic fungi and yeasts. The isolation from sterilized tissue denotes that disinfection procedures followed during initiation of plant tissue cultures does not eliminate these organisms.

Early reports stated that endophytes are present in low proportions and were isolated more frequently from roots as opposed to leaf or stem cuttings (Hallman 2001). Shoot tips from stem cuttings taken from the landscape that are used for establishing plant tissue cultures have been considered to have fewer microorganisms from other plant sources. However, further evidence suggests that plants can have many endophytes in significant numbers in different organs. Endophytes that have been found include culturable and non-culturable microorganisms, implying that their introduction *in vitro* through the explant and survival in the culture are highly probable (Thomas and Soly 2009).

While endophytes are considered to be harmless to the host they colonize, in modified conditions in plant tissue culture they can overtake the stock cultures and can become detrimental to maintaining stock populations. The term “vitropath” was given to the

designation of microorganisms that have the ability to cause harm *in vitro* from those that are pathogenic to *ex vitro* plants acclimated to the environment (Herman 1997). Endophytic and epiphytic microorganisms can cause significant losses to micropropagation *in vitro* during every stage of growth (Cassels 1991). Some bacterial contamination can be difficult to detect if they remain inside plant tissue due to failure of elimination through surface sterilization (Debergh and Vanderschaghe 1988).

Endophytic microorganisms found in plants can include arbuscular mycorrhizal fungi, latent plant pathogens, nitrogen fixing bacteria, and other symbionts that have yet to be discovered (Hyde and Soytong 2008). These organisms have the ability to affect functional roles of the host plant they colonize in a profound way in terms of plant fitness, interactions with their environment, and the diversity of associated organisms (Rodriguez et al. 2009). It is thought that the development of these functional roles has evolved with plants for 400+ million years (Redecker et al. 2000). The most noticeable benefit to plants can be seen in their protective quality or in inducing systemic resistance and increasing drought tolerance (Hubbard et al. 2014).

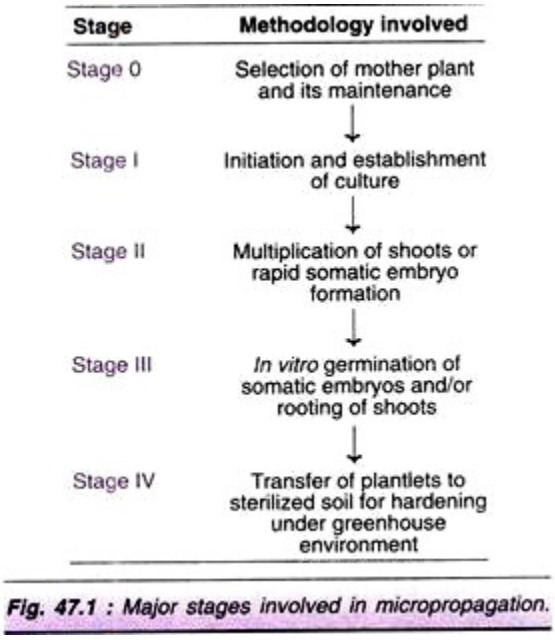
Biocontrol strains that are established in plants as endophytes and are applied as a seed treatment can offer a unique potential in crop protection. It is estimated the annual market for seed treatments with microbes represents $1.5 Billion and is growing rapidly (Reisch 2014).The low cost and easy incorporation of these seed treatments into

existing agronomic practices is appealing for introducing biocontrol agents into row crop systems (Coombs and Franco 2003).

Project goals

The overall goals of this project were to increase knowledge and improve micopropagation protocols, focusing on establishment and maintenance of native deciduous *Rhododendron in vitro* cultures. The aim is to add knowledge to the tested protocols used in establishment and maintenance of *Rhododendron* and other woody plants into micropropagation systems. Micropropagation offers the ability to use clonal propagation to increase reproduction rate in plants, especially in recalcitrant species, and allows production year-round without the limitations of changing seasons.

Figure 1: Stages of micropropagation technique[a]



Stage	Methodology involved
Stage 0	Selection of mother plant and its maintenance
↓	
Stage I	Initiation and establishment of culture
↓	
Stage II	Multiplication of shoots or rapid somatic embryo formation
↓	
Stage III	*In vitro* germination of somatic embryos and/or rooting of shoots
↓	
Stage IV	Transfer of plantlets to sterilized soil for hardening under greenhouse environment

Fig. 47.1 : Major stages involved in micropropagation.

[a] Biologydiscussion.com/biotechnology/clonal-propagation

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Chapter 1: The use of fungicides for increasing success of micropropagation of native deciduous *Rhododendron* spp.

Abstract: *Rhododendron* is the most abundant genus in the *Ericaceae* family, consisting of over 1000 species that are native to North America, Western Europe, and Northern Asia. With certain Rhododendron varieties being desirable due to flower color or bloom time, propagation of these shrubs is of increasing interest. Micropropagation is a technique of multiplying small plant explants, or cultures, and involves a sterile environment. In this process, clones of desirable plants are produced in a relatively short period of time from stock cultures. Since cuttings can originate from the landscape, they could bring a number of fungal pathogens with them and shoot survivability can suffer as seen by others. Three fungicides (thiophanate-methyl, chlorothalonil, and triademifon) were amended into media in an effort to decrease contamination in culture to increase survivability in five native deciduous *Rhododendron* varieties and hybrids listed by their nickname ("Coral", "Easter Pink", Cumberland, F-L, and Oconee). Thiophanate methyl- amended media increased survival *in vitro* significantly in all varieties when compared to the non-amended control except for "F-L" in which all treatments were significant. Bud production while establishing *Rhododendron* species was further increased in fungicide amended media when compared to the non-amended control.

Keywords: Fungicide-amendments, explant survivability

Introduction

Rhododendron is the most abundant genus in the *Ericaceae* family, consisting of over 1000 species that are native to North America, Western Europe, and Northern Asia. *Rhododendron* spp. includes evergreen and deciduous species ranging from shrubs to tree-sized plants with flowers blooming from late winter to early summer. They are acid-loving plants (preferring a pH range of 4.5-6), grow best in cool shady environments, and thrive in a well-drained soil. Some *Rhododendron* species are used as ornamentals in landscapes and woodlands due to their floral display (Zimmerman and Van Eck 2015); however, desirable species can be often difficult to find in retail markets**.** With certain Rhododendron varieties and hybrids being desirable due to flower color or bloom time, propagation of these shrubs is of increasing interest. Plant propagation is a method of growing new plants and can be done in various ways from a range of sources: seed, cuttings, and other plant parts. Some plants do not grow from seed at all or are difficult to reproduce vegetatively. Sources such as cuttings are limited by size of the mother plant from which a limited number of cuttings can be taken (Mondal and Chand 2002). Propagating *Rhododendron* by cuttings is the most popular as well as problematic technique one can use; this is particularly true for deciduous azaleas. The main problems with propagation by cuttings with deciduous azaleas are initiating rooting and producing new growth after rooting. To overcome these problems, plants can be produced from a single parent plant by other methods vegetative cloning without

having changing genetic material (Galle 1979); one method of cloning is micropropagation.

Micropropagation is a technique of multiplying small plant explants, or cultures, and involves a sterile environment. In this process, clones of desirable plants are produced in a relatively short period of time from stock cultures (Akin-Idowu et al. 2009). Often, these stock cultures start from stem cuttings taken from the landscape. With deciduous azaleas, cuttings are taken from the parent plant when the new growth is soft and flexible, generally in mid-spring. The cuttings from deciduous azaleas are made earlier than are cuttings for evergreen rhododendrons, usually at the end of May, at first growth flush. This flush of growth is known as Lammas growth, referred to as a period of renewed growth of leaves and stems during summer months. This name comes from the Celtic Harvest Day, known as Lammas Day (Battey 2003). However, this flush of growth can be forced during other months by fertilizer applications and pruning to achieve new growth. Deciduous cuttings generally require and exogenous application of rooting hormone to aid in rooting. Using a more potent hormone will increase the chance of initial roots but can also decrease incidence of breaking dormancy and producing new growth due to its increased strength.

Since cuttings can originate from the landscape, they could bring a number of fungal pathogens with them and shoot survivability can suffer as seen by others (Cassels 1991,

Economou and Read 1984).The aim of this study is to evaluate methods of fungicide amendments that could inhibit or eliminate some fungal contaminants when establishing shoot cultures.

Materials and Methods

Three fungicides were used in this study including thiophanate-methyl (TM) (Makhteshim-Agan of North America, Raleigh) at 586 ppm, chlorothalonil (Chloro) (Daconil®, Syngenta Crop Protection, Greensboro) at 1378 ppm, and triadimefon (Triad) (Bayleton®, Bayer Environmental Science, Research Triangle PK) at 75 ppm. These rates were based on recommended rates of application (Alabama Cooperative Extension System 2013). For the initial trial, a wide range of fungicides were selected: non-amended control, TM, propiconazole (Tilt® Syngenta Crop Protection, Greensboro), chlorothalonil, triadimefon, azoxystrobin (Quadris®, Syngenta Crop Protection, Greensboro), pyraclostrobin (Headline®, BASF Ag Products, Florham Park), and tebuconazole (Makhteshim-Agan of North America, Raleigh). Certain fungicides were chosen because of their success in preliminary trials, such as Chloro (Brown and Bowen 2016), and stability through autoclaving at high temperatures. All fungicides used in these evaluations are labeled for fungal disease control in *Rhododendron* (Alabama Cooperative Extension System 2013).

The medium used in these trials was a modified E&R (Economou and Read) medium supplemented with 2 grams of sucrose and 0.6 grams of agar per 100 ml media

(Economou and Read 1984). Each of the three fungicides was added to the media and the pH was adjusted to 5.0 prior to autoclaving; media was dispensed into sterile 18 mm test tubes after autoclaving.

This study was done on varieties and hybrids of deciduous Pentanthera azaleas, nicknamed “Coral” (*R. canescens* X), Oconee (*R. flammeum*), “Easter Pink” (*R. cumberlandense* X), Cumberland (*R. cumberlandense*), and “F-L” (*R. cumberlandense* X). The stock materials came from a private property in Lee County, Alabama. Stem cuttings, 4-6 inches long, were taken from stock plants in the landscape during the first spring flush of growth before hardening of stems. The stem cuttings were put into moist chambers until their transfer to media. Transfer of stem cuttings was done within 2 hours of taking cuttings in the morning. Stem cuttings were taken from the same plant for each variety.

All leaves were removed from cuttings to expose the bud node. Cuttings were put into a 0.82% sodium hypochlorite solution with 0.001% Tween (Sigma Aldrich) for 15 minutes under agitation for surface sterilization. Tween is detergent widely used in biochemical applications. Each of the stem cuttings were cut into 1-1.5 cm pieces, each with at least one bud node; these were maintained and established in order of removal from the cutting. These node pieces were then submersed in a 0.82% sodium hypochlorite solution for no more than 2 minutes and rinsed in sterile water with 0.001% Tween for 1 minute, three times. This second round of sterilization was done once the node cuttings

were cut prior to establishment, for sufficient sterilization. Once these cuttings were rinsed, they were placed onto fungicide amended media. Stem node pieces (apical to basal) were arranged such that, for example, tips would be placed on each fungicide amendment. If the tip of the first stem cutting was started on non-amended control, the next stem cutting tip would start on thiophanate-methyl (TM). This continued until all stem pieces were in each treatment with one bud on each node established

"Coral" shoots were established on April 30th. "Easter Pink", "F-L", and Cumberland shoots were established on May 13th. Oconee shoots were established May 18th during the first flush and July 1st during the second flush.

Cultures were kept at ambient indoor conditions under fluorescent lamps (13 hour day/11 hour night). Data were taken for 31 days, at 5 to 7 day intervals, to monitor development of buds or contamination. Survival data for each variety were taken at 5-7 day intervals in which bud nodes survived. Notes were taken on the state of the node cutting: contaminated, browning, or having bud emergence.

Data were analyzed separately for each *Rhododendron* variety and hybrid in culture. Data for days to death, or survival, in culture was analyzed with generalized linear mixed model procedures. Treatment means were separated using Fisher's protected least square mean separation at $p \leq 0.10$ level of significance. All treatments were repeated three times and at least three node cuttings were included in one repetition.

Results

In establishing “Coral” shoots, one or two pieces on the control and on TM, Chloro, and Triad amendments survived through 31 days (Table 1). Stem pieces on the control as well as on TM, Triad, and Chloro-amendments had the first bud break on day 17. TM-amendment was the first amendment to allow production of explant leaves on day 24 while Chloro-amendment produced leaves on day 31. Browning of shoot pieces was not observed until day 17 with the Triad amendment. Of the four original shoots in three treatments, one produced a bud break in the non-amended control, one in Chloro, and two in Triad. Of the three original shoots in TM, two produced a bud at the end of 31 days. The amendment that achieved the highest survival, at 67% of shoot pieces, was TM.

In establishing “Easter Pink” shoots, stem pieces on the TM and Triad-amended media produced the first buds on day 7. After day 21, browning of stem pieces was observed on these same two amendments, but two of the stem pieces on TM had produced buds on day 31 (Table 2). Stem pieces of this variety on non-amended control media had contamination by day 7 of culture. Of the eight original shoots on the TM, two produced buds by day 31. Among all tested fungicides, the highest percent survivability was 38% with TM-amended media.

In establishing Cumberland shoots, stem pieces on TM, Triad, and Chloro amendments had the first bud break on day 5. However, only stem pieces on the TM amended media

retained a bud through day 31. Stem pieces of this variety on the non-amended control media were contaminated by day 5. Of the 5 original shoots in the TM, two produced buds at the end of the 31 days (Table 2). The amendment that resulted in the highest percent survivability (40%) was TM.

In establishing “F-L” shoots, all shoot pieces produced a bud by day 5 regardless of the amendment, and at least one remained without contamination until day 31 (Table 2). The control was the first to produce leaves on day 21 while the TM amendment produced leaves on day 31. Browning of shoot pieces was not observed until day 21 with the Triad amendment. Of the four original shoots in each amendment; one produced a bud in the non-amended control, three in the TM, two in the Chloro, and one in the Triad. Of the four original shoots with TM, three produced shoots at the end of the 31 days. The amendment that achieved the highest survival, at 75% of shoot pieces, was TM.

Oconee shoots were taken from stems after the first flush of growth. All shoot pieces had noticeable browning by day 13 but the control and amendments of TM and Chloro started to produce at least one bud by day 13 in culture (Table 1). However, by day 21, these buds were contaminated or underwent excess browning that prevented further bud growth. Of the ten original shoots with non-amended control, none survived until day 31. Of the nine original shoots with TM-amendment and twelve with Chloro amendment, six of each survived till day 31. While the shoots survived till the end of the

31 day period, none successfully produced a bud. This failure led to another attempt of taking stem cuttings after the second flush of growth.

Oconee shoots were taken from stems after the second flush of growth. No browning was observed in the shoots with stems taken at this later date. All shoot pieces produced at least one bud in media except for the TM-amendment. Of the 12 original shoots on the non-amended control and Chloro-amendment, two and four buds respectively were produced by day 31 (Table 3). Of the 14 original shoots with Triad-amendment, three buds were produced at the end of 31 days. The amendment that resulted in the highest percent survivability (64%) was Triad.

A consistent trend was observed with bud development on TM-amended media on five *Rhododendron* varieties—"Coral" (0.0816), "Easter Pink" (0.0164), "F-L" (0.119), Cumberland (0.0545), and Oconee (<0.0001) with a $p \leq 0.1$. When analyzing data through generalized mixed model procedures, TM-amended media was shown to significantly increase shoot survivability based on a culture period of 31 days in these four *Rhododendron* varieties compared to the non-amended control. TM-amended media allowed survival of "Coral" shoots for an average of 28.3 days, compared to the control without amendments allowing survival average of 15.75 days (Fig. 2). The same amendment allowed survival of "Easter Pink" shoots for 20.74 days compared to 9.73 days in non-amended media. Triademifon- amended media allowed average days for survival, while not significant; it increased survivability in "Easter Pink" shoots (Fig. 3).

Similarly, Cumberland shoots had significant survival in TM-amended media with 19 days of survival compared to 8.56 days in non-amended control. Chlorothalonil-amended media allowed 11.57 days of survival, while not significant; it increased survivability in Cumberland shoots (Fig. 4). “F-L” was the only variety in which fungicide amendments did not significantly increase days of survival when compared to the control. However, TM-amended media allowed 25 days of survival while non-amended media allowed 18 days of survival in “F-L” shoots (Fig. 5). Finally, TM-amended media allowed significant survival of Oconee shoots for 25 days, compared to the control allowing survival for 21.11 days. Similar to “Easter Pink” and Cumberland varieties, chlorothalonil and triademifon-amended media allowed 23.86 and 22.85 days of survival. While not significant, these amendments increased survivability in Oconee shoots when compared to non-amended control (Fig. 6).

Oconee initially did not produce buds from stem cuttings taken from the first flush of growth. Another attempt of establishment was made with the second flush of growth due to the desirability in this particular *Rhododendron* variety. This second flush of growth was forced through fertilizer applications and pruning to achieve new stem growth. Use of stem cuttings taken from the second flush of growth has not been documented before for Oconee and should be noted for other *Rhododendron* varieties that are difficult to establish into micropropagation systems.

Discussion

The present study investigates the use of fungicides to increase survivability in establishing native deciduous *Rhododendron* in micropropagation systems. The aim of this work was to amend media with various fungicides labeled for use on *Rhododendron*, and assess survival of bud nodes and ultimately bud production compared to media without amendments.

A crucial part of developing the micropropagation procedures described was successful sterilization of shoot/stem cuttings taken from the stock plants in the landscape. It has been noted in the literature that contamination can cause loss of shoot tips depending on the species being established (Cassels 1991). During establishment of *Rhododendron calendulaceum*, 4 out of 20 shoot tips placed in culture were lost due to contamination issues within 4 weeks of culture (Blazich and Acedo 1988). Experience has shown that a failure to sufficiently sterilize shoot cuttings before being placed onto media can and will present problems, leading to explant death. Similarly, Economou and Read (1984) found contamination in establishment of Minnesota deciduous azaleas to be problematic in establishing shoot cultures from stock plants. They took cuttings from plants under a "protected" environment in the green house, which is recommended to increase chance of success in establishment. While deciduous *Rhododendron* native to Alabama have been investigated herein, similar methods can be applied to other deciduous *Rhododendron* native to other areas in the U.S.

This study has shown that incorporation of fungicides in media for woody plants such as *Rhododendron* can increase survivability of stem cuttings, allowing more time for buds to develop. The main finding of this chapter is that media-amendments are successful in increasing survival in micropropagation systems and should be used in establishing other woody plants.

There were no statistical data taken to compare survival of tip and basal stem cuttings. Observations were made through establishment that tip stem cuttings typically had more than one bud node on them, allowing more than one shoot to develop from one stem node cutting. The tip of the stem cutting was the most juvenile growth when compared to basal stem cuttings

The tip of stem cutting were the first section of the stem, which could have affected survivability compared to basal stem cuttings that were the last section of the stem to be established.

Furthermore, the objective of incorporating fungicide amendments into media was to prolong survival of stem cuttings *in vitro* to allow for an increased chance of bud node production. While control treatments were able to successfully allow production of bud nodes, treatments with fungicide-amendments allowed longer survival in certain varieties which allowed more time for bud development to occur on stem cuttings. With

more buds produced, more explants can then be sub cultured quickly to attain the desired explant production in commercial settings.

Differences among varieties were noted relative to the different fungicide amendments. Depending on the variety of *Rhododendron*, TM and Chloro were the most successful fungicides in these trials based on the results displaying the highest survivability percentage on day 31. While there were similarities between some varieties, consistency with micropropagation success with any one fungicide was not observed for these five varieties.

The addition of fungicides in media was successful in preventing contamination that can inhibit bud and explant development taken from stem cuttings in the landscape. While this has been confirmed in our results for species native to certain areas of the U.S., more varieties should be investigated including a wider range of fungicide amendments to determine the best treatments according to *Rhododendron* species. Further studies are required to established protocols for establishing other *Rhododendron* species and other woody plant species.

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Table 1. Numbers of shoot pieces of “Coral”and Oconee *Rhododendron* surviving without contamination on fungicide-amended media at varying intervals.

		- - - - - - - - - - - - - - - Days after start - - - - - -						
Variety/Hybrid	Amendment	0	5	10	17	24	33	Buds
“Coral”	Control	4	3	2	1	1	1	1
	thiophanate-methyl	3	3	2	2	2	2	2
	chlorothalonil	4	3	2	2	1	1	1
	triadimefon	4	2	2	2	2	2	2
Oconee1[a]	Control	10	5	5	1	0	0	0
	thiophanate-methyl	9	9	9	8	6	6	0
	chlorothalonil	12	12	10	8	6	6	0

[a] Oconee Shoots taken from first flush of growth

Table 2. Numbers of shoot pieces of “Easter Pink”, Cumberland, “F-L”, and Oconee *Rhododendron* surviving without contamination on fungicide-amended media at varying intervals.

		Days after start						
Variety/Hybrid	Amendment	0	7	13	21	26	31	Buds
“Easter Pink”	Control	6	0	0	0	0	0	0
	thiophanate-methyl	8	5	5	3	3	3	2
	chlorothalonil	9	2	1	1	1	1	0
	triadimefon	6	2	2	2	0	0	0
Cumberland	Control	4	0	0	0	0	0	0
	thiophanate-methyl	5	4	2	2	2	2	2
	chlorothalonil	4	2	0	0	0	0	0
	triadimefon	4	1	0	0	0	0	0
“F-L”	Control	4	3	2	1	1	1	1
	thiophanate-methyl	4	3	3	3	3	3	3
	chlorothalonil	4	3	2	2	2	2	2
	triadimefon	4	4	3	2	2	2	1
Oconee2[b]	Control	12	10	7	4	4	4	2
	thiophanate-methyl	14	12	10	10	10	10	0
	chlorothalonil	12	10	8	7	6	6	4
	triadimefon	14	10	9	9	9	9	3

[b] Oconee shoots taken from 2nd flush of growth

Figure 2: Average survivability of “Coral” shoots in Fungicide-amended media.

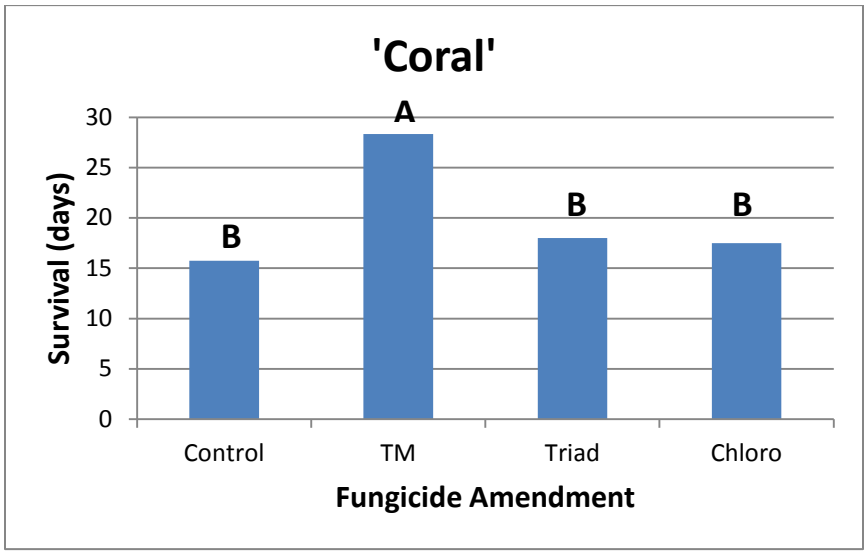


Figure 3: Average survivability of “Easter Pink” shoots in fungicide-amended media.

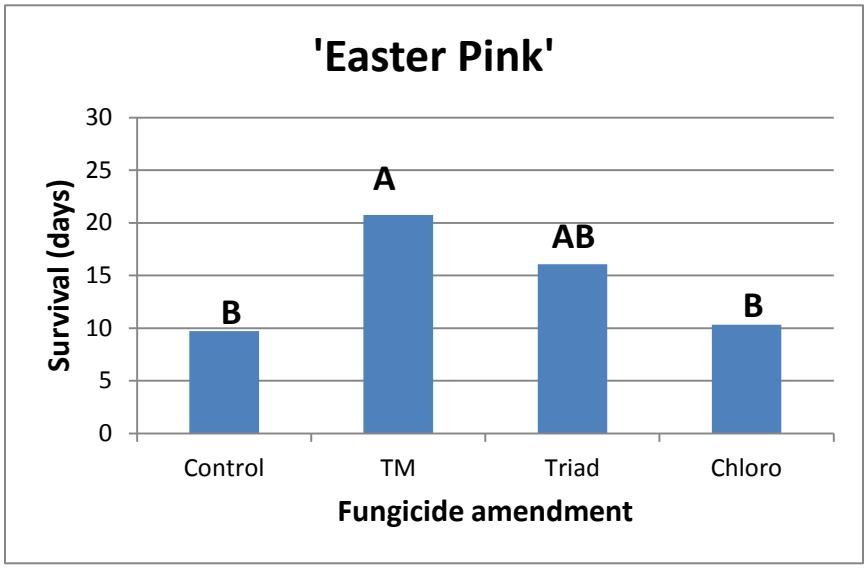


Figure 4: Average survivability of Cumberland shoots in Fungicide-amended media.

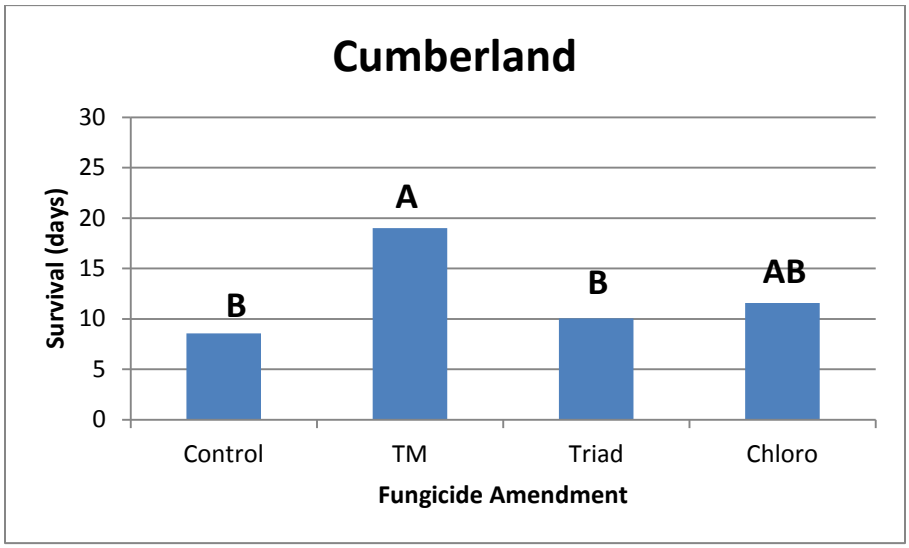


Figure 5. Average survivability of "F-L" shoots in fungicide amended media.

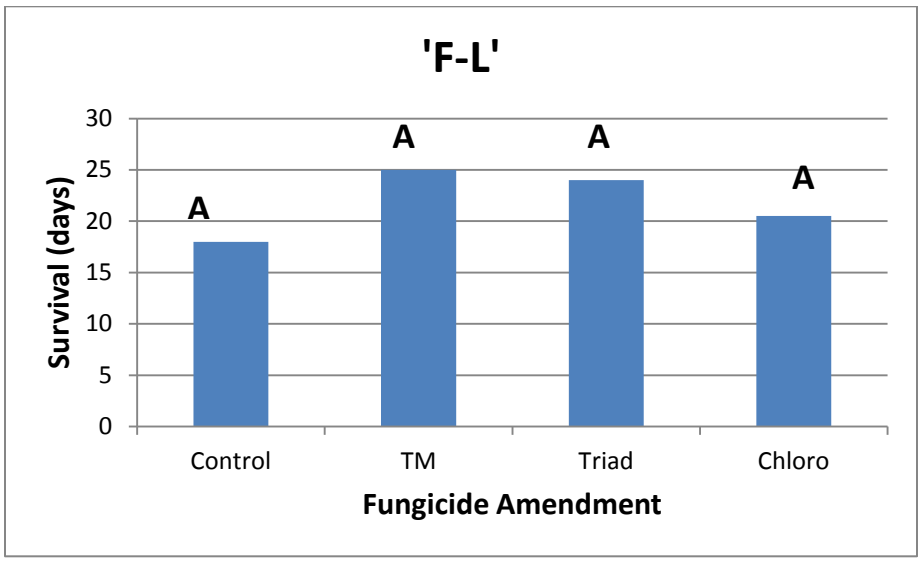
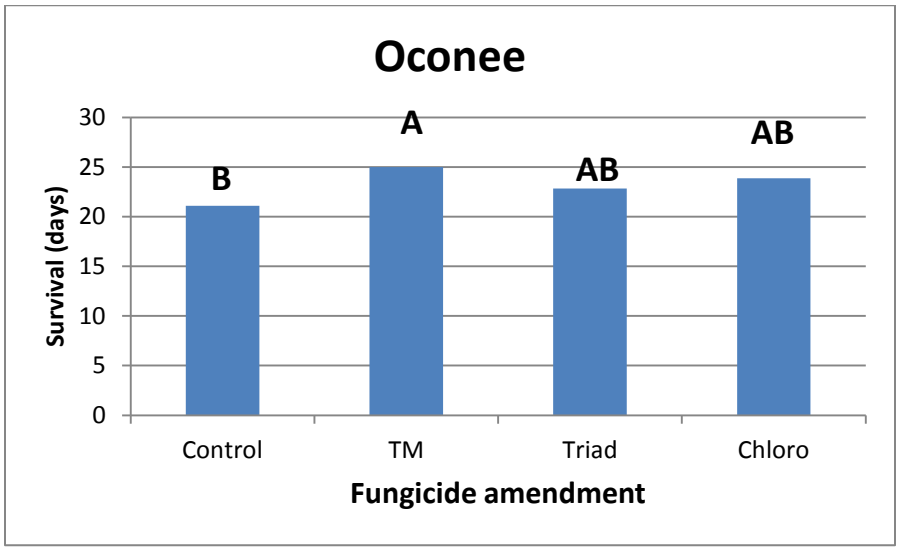


Figure 6: Average survivability of Oconee shoots from second flush of growth in fungicide-amended media.



Chapter 2: Investigation of fungal contaminants in micropropagation systems for endophytic associations.

Abstract: Micropropagation is the technology of plant tissue culture commercially applied in which rapid proliferation of explants from initial stocks is achieved. When establishing these *Rhododendron* shoots, contamination can come from a variety of sources inside and outside the lab. To combat this, extensive surface sterilization is performed on the shoots to limit the contamination in the micropropagation process. Even with thorough surface sterilization, contamination can still persist when establishing shoot cultures. DNA from fungal contaminants was extracted and PCR was performed to identify two genera, *Trichoderma* and *Alternaria,* which have been known to have endophytic properties.

Keywords: Contamination, endophyte

Introduction

Plant tissue culture refers to growing and multiplying cells, tissue, or organs of plants on media under aseptic conditions and in a controlled environment. Micropropagation is the technology of plant tissue culture commercially applied in which rapid proliferation of explants from initial stocks is achieved. Micropropagation is a technique used by utilizing numerous steps in achieving production of a final product such as a plant ready for commercial use. These steps include but are not limited to: pre-propagation, establishment of explants, maintenance of explants maximizing proliferation, rooting,

and hardening of the explant to *ex-vitro* conditions. Compared to rooting cuttings, this alternate type of propagation has many advantages such as allowing clonal production of desirable plants in a relatively short time and allowing production to be year-round since it is not dependent on the seasons (Akin-Indowu et al. 2009).

When establishing these *Rhododendron* shoots, contamination can come from a variety of sources inside and outside the lab (Purmale et al. 2012). These shoots were taken from a private nursery in Lee County, Alabama. Since these shoots were taken from the landscape, any number of pathogens could be brought into the sterile environment needed for micropropagation to propagate. To combat this, extensive surface sterilization is done on the shoots to limit the contamination in the micropropagation process. Another way to combat contaminants from persisting in the cultures is to include fungicides in the media in which they are grown.

Even with thorough surface sterilization, contamination can still persist when establishing shoot cultures (Cassels 1991, Purmale et al. 2012). In preliminary studies, even after several processes of sterilization and re-culturing, there was still fungal growth from shoots With this much persistence there could be a chance that some of these contaminants were endophytic fungi that had come from the landscape. The main purpose of this chapter was to identify these fungal cultures to determine whether they were endophytic fungi. This was done through DNA extraction and PCR for molecular

identification. Once identified, sequences were analyzed to determine if certain fungal genera preferred a particular fungicide-amended media.

Endophytic microorganisms found in plants can include arbuscular mycorrhizal fungi, latent plant pathogens, nitrogen fixing bacteria, and other symbionts that have yet to be discovered (Hyde and Soytong 2008). These organisms have the ability to affect functional roles of their host plant by affecting plant fitness, interactions with the environment, and the diversity of associated organisms (Rodriguez et al. 2009). It is thought that the development of these functional roles has evolved with plants for 400+ million years (Redecker et al. 2000). The most noticeable benefit to plants can be seen in their protective quality it terms of inducing systemic resistance and increasing drought tolerance (Hubbard et al. 2014).

Biocontrol strains that are established in plants as endophytes have the ability to be applied as a seed treatment, offering a unique potential in crop protection. Some of these strains are not found established in plant tissue and can benefit from applications. It is estimated the annual market for seed treatments with microbes represents $1.5 Billion and is growing rapidly (Reisch 2014). The low cost and easy incorporation of seed treatments into existing agronomic practices is appealing for introducing biocontrol agents into row crop systems (Coombs and Franco 2003).

Polymerase Chain Reaction (PCR) is a method that uses the DNA polymerase ability to synthesize new DNA strands complementary to the template DNA strand. Primers are

needed in this process to delineate a specific area in the DNA sequence to amplify. The purpose of this chapter was to identify the fungi through extracting DNA from a number of samples taken from contaminants in establishing *Rhododendron* shoots in plant tissue culture. PCR was performed in order to determine gene sequences of fungal samples.

Materials and Methods

Surface sterilization prior to establishment of shoot segments including at least one bud node was performed by washing the stem cuttings in a 0.82% sodium hyperchlorite solution for 15 minutes while under agitation, soaking in a 0.82% sodium hyperchlorite solution for 2 minutes, and rinsing three times in sterile water before establishment of the stem segments. Once these cuttings were rinsed, they were placed onto fungicide amended media. Stem node pieces (apical to basal) were arranged such that, for example, tips would be placed on each fungicide amendment. If the tip of the first stem cutting was started on non-amended control, the next stem cutting tip would start on thiophanate-methyl (TM). This continued until all stem pieces were in each treatment. Rate of fungal development varied with each fungicide-amended media and was first observed 7 days after establishment of stem pieces.

For this trial, there were a total of 20 fungal contaminants investigated that were cultured from fungicide amended media. There were three different fungicides used at varying rates; including thiophanate-methyl, chlorothalonil, and triadimefon, in the establishment of *Rhododendron* shoot cultures. The rates of each fungicide included

into media were 586 ppm, 1378 ppm, and 75 ppm respectively. These rates used were label rates taken by referring to the 2nd Volume of Alabama Pest Management handbook in which a section of products were labeled for *Rhododendron* (Azalea) (Alabama Cooperative Extension System, 2013).

These fungal cultures were initially grown on solid Economou and Read (E&R) (1984) media without hormones since they came from the shoots growing in the same media. Once grown on the plate, hyphal tipping was done to isolate a single colony. When a single colony was achieved, it was placed into a liquid media of the same E&R recipe and grown for approximately 1 week.

When the cultures were sufficiently grown, they were vacuumed to a mucus consistency to begin the DNA extraction process. A small portion of mycelium was placed into a centrifuge tube with 750 µL DNA extraction solution. After bead beating, protease K was added to the sample to degrade proteins in the cells. After centrifuging, samples were placed in 55 °C water for 15 minutes, NH_4Ac was added to bind the carbohydrates for removal. The samples are then placed into ice for 20 minutes and centrifuged for 10 minutes. Finally, the supernatant was removed and put into a new micro centrifuge tube with equal amount of isopropanol.

The supernatant was left overnight in a -80 °C freezer, then 200 µL of water and 5 µL of RNase were added each sample. After a centrifuge "burst" of 10 seconds, 200 µL water and 600 µL phenol was added. Samples were placed on ice for 10 minutes, centrifuged

for 1 minute, and the aqueous layer was placed in a new tube. Equal volume chloroform was added to each sample along with 1/10 the volume of sodium acetate and 20 times the volume of ethanol. The final steps of DNA extraction included pouring out of the tube as before, centrifuging for 15 minutes, and adding 25 µL of water.

Once DNA extraction was complete, concentrations were obtained through the Nanodrop 2000 spectofluorometer. This instrument provides information about concentration and chemical environment of samples administered. Once the nanodrop is complete, calculate the ng/µL needed was calculated from each sample when the final volume is 100 µL of 75 ng/µL of DNA. PCR tubes will need, in this order: 13 µL water, 25 µL of master mix, 5 µL of ITS primers, and 2 µL of DNA template made from the previous calculation. This will come to a total volume of 50 µL in the PCR tubes which will be placed into the thermal cycler to run at varying temperatures throughout the four hour process.

Once PCR was complete, gel electrophoresis was done in order to detect the PCR products. This is accomplished with a 0.8% agarose gel and a sample of DNA template, dye, and water. If the PCR products are clear when comparing to the 1kb band standard, the DNA needs to be cleaned up through the protocol in the green PCR box using CP buffer and elution buffer. A final nanodrop is performed followed by a final calculation for diluting the DNA to 30 ng/µL since the samples were ~600 base pairs. The last step is

to include 8 µL of DNA and 4 µL of ITS primer in a new tube and send it off to be sequenced by Eurofins Genomics.

Results

Results were analyzed using the Chromas program. The sequence was further analyzed using the nucleotide BLAST application on the NCBI website. The sequence was taken from the points at which there were no N's in the readings and copied to the site for further identification through comparison with genomes already in the system.

Of the 20 samples sent off for sequencing, only two had pure DNA in the samples. One of these samples was *Alternaria* (Fig. 7) while the other was *Trichoderma*. While both of these genera have been known to be endophytes, the sequence outlined *Alternaria* having certain endophyte associations when analyzed through NCBI BLAST.

Table 3: Endophyte associations in establishing *Rhododendron* shoots.

***Rhododendron* Hybrid**	**Fungicide amendment**	**Endophyte**
"Fragrant Late"	Bayleton	*Alternaria*
"Raspberry"	Bayleton	*Trichoderma*

Although there were no official results taken regarding media, similar fungal cultures were grown from two or three of their respective fungicide-amended media *in vitro*

(Table 3). This could signify that these two genera are more persistent in plant tissue taken as stem cuttings from the landscape when compared to other fungal genera.

These two samples that could be identified did both come from a Bayleton fungicide-amended media. This outcome suggests that these endophytes could grow better on this fungicide-amended media when compared to the other fungicide-amended media used when establishing shoots

Discussion

The aim of this chapter was to identify these fungal isolates and determine if they have been known recognized as endophytes. The study set out to assess the consistency of endophytic fungal contaminants in micropropagation systems.

The results of this study are broadly consistent with others that have reported that microorganisms are harbored in micropropagated plants and can be problem-causing contaminants (Cassels 1991). These finding concur with other studies that show *Alternaria* has been seen to have endophytic activity within plants in *Rhododendron* (Leifert et al. 1994). *Trichoderma* has also been confirmed to have the ability of colonizing above ground plant tissues and can be used as a biocontrol agent (Bae et al. 2011). Our observations that these two genera may be endophytes are not new,

however, this is the first study to look at the endophytic activity in contaminants found in establishing native deciduous *Rhododendron* in micropropagation systems.

There were a number of possible limitations relative to the results of this work. One problem is that only five *Rhododendron* varieties/hybrids were established on fungicide amended media. Many other varieties and cultivars in *Rhododendron* and other woody plants need to be investigated to determine what fungal contaminants can persist in each genus and species. The main limitation was that 20 fungal isolates could be isolated from the five *Rhododendron* varieties on fungicide-amended media. For an appropriate representation of endophytic fungi found in *Rhododendron*, a wider spectrum of potential contaminants should be screened in future studies.

The results obtained through these experiments were not expected. The original hypothesis was that since there was persistent contamination, a significant amount of this contamination should by endophytic since thorough surface sterilization was performed. However, this was not the case since two of the twenty samples isolated came back with positive results.

In preliminary trials, there was some contamination which caused all the sequences to read as *Fusarium oxysporum.* This could have been caused by something in the lab or a failure to sterilize thoroughly before working. The sterile water and stock solutions were changed, and care was taken when changing out pipette tips in between each sample.

While there wasn't a high number of endophytes with the results, that doesn't mean that endophytes aren't persistent in ornamental landscape plants such as *Rhododendron*. Ultimately, endophytic fungi have the ability to positively affect plant growth in a number of ways. Increased plant biomass (Kapoor et al. 2008), better rooting *in vitro* (Larraburu and Llorente 2015) better acclimatization in survival rate after transfer *ex vitro* and overall plant performance (Duffy et al. 1999), induction of stress resistance (Nowak and Shulaev 2003), and biocontrol effects (Harish et al 2008) have been reported as beneficial functions of endophytic fungi found in plants. Improving *in vitro* acclimatization of explants has been noted to be difficult in the final stage of micropropagation depending on genus. Incorporation of endophytic AMF mycorrhizae was reported to have beneficial effects with rooting micropropagated explants through allowance of high transpiration rates, improved absorption of nutrients and water, and increased stress tolerance (Azcon-Aguilar and Barea 1996, Jaizme-Vega et al. 1997). Similarly, it was demonstrated that AMF in micropropagated banana explants alleviated transplantation shock and improved successful establishment of explants (Yano-Melo et al. 1999).

In future studies, more varieties can be established into plant tissue culture. From these established cultures, contaminants will certainly grow. From these, additional experiments of DNA extraction and PCR can be accomplished to further outline the relationship between endophytic fungi and the plant hosts they colonize. Better

understanding the role of endophytic fungi in plants will allow for proper utilization in commercial micropropagation production industries.

This study has shown that some contamination in micropropagation systems is associated with genera of endophytic fungi. The main finding of this chapter is that of the fungal contaminants that survive surface sterilization, some can be related to endophytic genera. This study demonstrates that endophytes have the ability persist through extensive surface sterilization.

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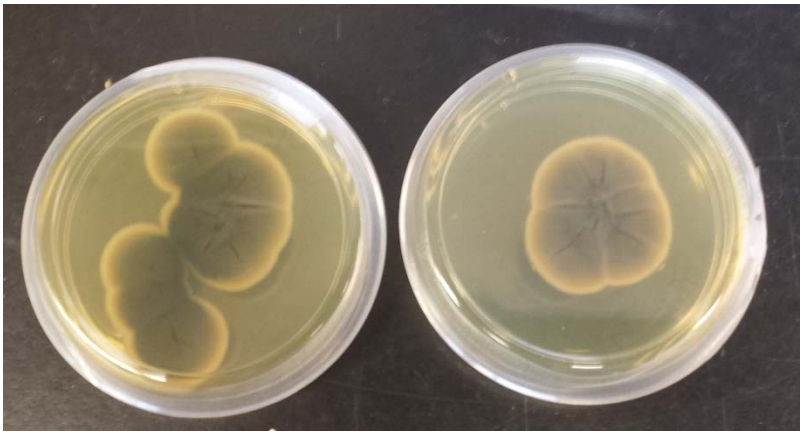
Figure 7: *Alternaria* fungal contamination isolated from established *Rhododendron* bud nodes.



Chapter 3: The use of alternate hormones and container sizes for increasing micropropagation of native deciduous *Rhododendron* spp.

Abstract: Micropropagation is a technique of multiplying plant explants, or cultures, in a sterile environment. In this process, clones of desirable plants are produced in a relatively short period of time from stock cultures. One of these hormones that was explored in maintaining *Rhododendron* explants was phenyl-N'-(1,2,3-thiadiazol-5-yl) urea (TDZ), a potent cytokinin. In addition to alternate hormones, varying vessel sizes were investigated in regards to maximizing growth while maintaining cultures. TDZ has shown the ability to significantly increase explant weight and shoot proliferation in micropropagated *Rhododendron.* Larger container sizes allow more media to be accessed by microropagated *Rhododendron,* significantly increasing explant weight, shoot proliferation, and shoot elongation depending on variety.

Keywords: TDZ, 2iP, plant growth hormones

Introduction

Rhododendron is the most abundant genus in the *Ericaceae* family, consisting of over 1000 species that are native to North America, Western Europe, and Northern Asia. *Rhododendron* spp. includes evergreen and deciduous species ranging from shrubs to tree-sized plants with flowers blooming from late winter to early summer. They are acid-loving plants (preferring a pH range of 4.5-6), grow best in cool shady

environments, and thrive in a well-drained soil. Some *Rhododendron* species are used as ornamentals in landscapes and woodlands due to their floral display (Zimmerman and Van Eck 2015); however, desirable species can be often difficult to find in retail markets**.**

With certain Rhododendron varieties being desirable due to flower color or bloom time, propagation of these shrubs is of increased interest. Plant propagation is a method of growing new plants and can be done in various ways from a range of sources: seed, cuttings, and other plant parts. Some plants do not grow from seed or are difficult to reproduce vegetatively. Sources such as cuttings are limited by size of the mother plant from which a limited number of cuttings can be taken (Mondal and Chand 2002).). Propagating *Rhododendron* by cuttings is one of the most popular and can be a problematic technique one can use; this is particularly true for deciduous azaleas. The main problems are initiating rooting with deciduous azaleas and producing new growth after rooting. To overcome some of these problems in deciduous *Rhododendron*, plants can be produced from a single parent plant by other methods of vegetative cloning without having changing the genetic material (Galle 1979); one method of cloning is micropropagation.

Micropropagation is a technique of multiplying plant explants, or cultures, in a sterile environment. In this process, clones of desirable plants are produced in a relatively short period of time from stock cultures (Akin-Idowu et al. 2009). Often, these stock

cultures start from stem cuttings taken from the landscape. Since these stocks are initially from the landscape, they could bring a number of fungal pathogens with them and shoot survivability can suffer. The aim of this study is to investigate alternate hormones and varying vessel sizes used to increase production in explant weight, shoot proliferation, and shoot elongation of native deciduous *Rhododendron* in micropropagation systems.

With deciduous azaleas, cuttings should be taken from the parent plant when the new growth is soft and flexible, generally in mid-spring. The cuttings from deciduous azaleas should be taken earlier than the procedure for evergreen rhododendrons, usually at the end of May, following the first growth flush. Deciduous cuttings often require rooting hormones; it can be difficult to produce roots without this aid (Konrad 1984). Including stronger hormones in maintaining cultures will increase the chance of initial roots but can also decrease incidence of breaking dormancy and producing new growth. One of these hormones that was tested in maintaining *Rhododendron* explants was phenyl-N'-(1,2,3-thiadiazol-5-yl) urea (TDZ), a potent cytokinin.

Shoot length, rate of proliferation and fresh weights of micropropagated cultures can vary according to culture vessel type and size. It was demonstrated that the ratio of explant number to volume of media causes fresh weight and proliferation rates to vary (Mackay and Kitto 1988). Further investigations included five genera, *Amelanchier*, *Acer*, *Forsythia*, *Malus*, and *Betula*, grown in 350 ml Magenta vessels (GA7), 200 ml glass baby

food jars, and 60 ml glass culture tubes (McClellend and Smith 1990). Explants were established in a vertical or horizontal orientation and cultures were maintained for 4-7 weeks depending on the genera. After the respective growth period, data was collected on shoot length and proliferation rates through video image analysis. Shoots were found to have significantly greater based on image density when cultured in the two larger vessels. The production of shoots in larger vessels leads to increased shoot leaf area and can impact rooting capacity (Macdonald 1986).

Materials and Methods

Shoots obtained from micropropagated plants of *Rhododendron* varieties and hybrids including Alabama (*R. alabamense*), "Coral" (*R. canescens* X), Cumberland (*R. cumberlandense*), Oconee (*R. flammeum*), and "Fragrant-Late" *(R. cumberlandense* X) were grown on Economou and Read (E&R) basal medium modified from Woody Plant Media (WPM) (Economou and Read 1984). This media was adjusted to 5.00±0.01 pH before supplementing with 2% sucrose and 0.6% agar. The media was autoclaved at 120 °C for 25 min before dispensing into sterile 18 mm test tubes.

Hormone Assay

The explants were kept in TDZ-free media for 4 weeks and then transferred to E&R medium supplemented with 2% sucrose, 0.6% agar, and a range of 1 to 10 µM TDZ depending on treatment. The control medium consisted of transferring the explant to

fresh E&R media with 3.84 µM N^6-(2-Isopentenyl) adenine, commonly known as 2iP. This was calculated into 4.8 ppm 2iP in culture. Explants were grown in 18 mm test tubes for 4 weeks.

Treatments were organized in a range of 1 to 10 µM TDZ for a total of seven treatments, including the control of 2iP. Seven explants were included in each treatment. The experiment included TDZ rates of 1, 2, 4, 6, 8, and 10 ml increments of stock solution made to include 0.022 g of TDZ in 100 ml total volume. This was calculated into 2.2, 4.4, 8.8, 13.2, 17.6 and 22 ppm of TDZ in culture. Explants were established from 4 week old established cultures of each variety.

Explants were kept in culture for 4 weeks. Fresh weight was calculated by weighing test tube, cap, and explant following 4 weeks culture period before and after transfer of culture to treatments. Shoot elongation and proliferation were quantified using measurements in centimeters and counted by hand, respectively. Shoot number and length of explants were recorded at time of establishment to compare treatments in regards to proliferation and elongation at time of culture and after 4 weeks of culture. Shoot weight data were taken before and after culture to compare treatments over 4 weeks. “F-L” and Oconee varieties had treatments omitted in data analysis due to contamination.

Vessel size evaluation

The stock explants were kept in 18 mm and 25 mm test tubes with E&R medium supplemented with 2% sucrose, and 0.6% agar for four weeks. The experimental design allowed for the change of growth over time when transferred to each of these vessel sizes to be investigated. Explants from each size were analyzed separately to compare growth of explants in sterile 18, 20, and 25 mm test tubes from explants originating 18 and 25 mm test tubes. Five explants were included in each vessel size per treatment.

Transfer of explants was performed in a laminar flow hood after proper surface sterilization with 70% ethanol to limit contamination from persisting in culture. Shoot length was recorded as explants were established into media. Shoot number for each explant in varying containers were recorded after establishment into media.

After culture of explants for 4 weeks, explant weight was recorded before and after the transplant of explants to determine the change in weight over the culture period. The tube, explant, and cap were weighed before transplant of culture while the tube and cap was weighed after transplant of culture to determine change in weight. Shoot number was recorded again before the transplant process to determine shoot proliferation in culture for each container size. Shoot length was recorded again during the transplant process to determine the change in shoot elongation while in culture for each container size.

Treatments were separated according to the vessel size from which explants originated from. Explants taken from 25 mm stock cultures were treatment 1 while explants taken from 18 mm stock cultures were treatment 2. All Cultures were kept at ambient indoor conditions under fluorescent lamps (13 hr day/11 hr night).

Data analysis

Data were analyzed separately for each *Rhododendron* variety in culture. Data for fresh weight, shoot proliferation, and shoot elongation were analyzed with generalized linear mixed model and regression procedures. Treatment means were separated using Fisher's protected least square mean separation at P≤ 0.10 level of significance. All TDZ treatments were repeated twice and seven explants were included in one repetition. All container treatments were repeated twice and five explants were included in one repetition.

Results

TDZ trials:

Cumberland explants were established March 10th. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least four weeks in TDZ free media (Fig. 8). The addition of TDZ hormones at various ratios significantly affected weight, shoot proliferation, and shoot elongation with *p* values of 0.0007, 0.008, and 0.0001, respectively (Table 4).

"Coral" explants were established March 23rd. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least four weeks in TDZ free media (Fig. 9). The addition of TDZ hormones at various ratios significantly affected explant weight, shoot proliferation, and shoot elongation with *p* values of 0.0031, 0.0778, and 0.0006, respectively (Table 4).

Oconee explants were established April 7th. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least four weeks in TDZ free media (Fig. 10). The addition of TDZ hormones at various ratios significantly affected explant weight, shoot proliferation, and shoot elongation with *p* values of 0.0612, 0.0056, and 0.0008 respectively (Table 4).

"F-L" explants were established May 1st. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least four weeks in TDZ free media (Fig. 11). The addition of TDZ hormones at various ratios significantly affected explant weight and shoot proliferation with *p* values of 0.0673 and 0.036, respectively. The addition of TDZ hormones did not significantly affect shoot elongation with a *p* value of 0.2318 (Table 4).

Alabama explants were established May 1st. Explants from 1-2 cm in length were taken from stocks previously maintained for at least four weeks in TDZ free media (Fig. 12).

The addition of TDZ hormones at various ratios significantly affected explant weight, shoot proliferation, and shoot elongation with *p* values of <0.0001, 0.0005, and 0.083, respectively (Table 4)

Container trials:

Alabama explants were established March 31st. Explants from 0.5-2.5 cm in length were taken from stocks previously maintained for at least 4 weeks. Each treatment was analyzed separately to compare growth of explants in sterile 18, 20, and 25 mm test tubes from plants originating from 18 and 25 mm tubes. In treatment 1, Alabama shoots gained significantly more weight with a *p*-value of 0.0035 over the culture period in 25 mm tubes at 90 mg compared to weight in 20 or 18 mm tubes. Shoot proliferation was also significantly affected and was greater in 25 and 20 mm tubes compared to 18 mm tubes with a *p*-value of 0.0139. Shoot elongation was significantly affected also and better in 25 mm tubes when compared to 20 and 18 mm tubes over 4 weeks in culture with a *p*-value of 0.0015 (Table 5).

For the 2nd treatment, Alabama shoots gained significantly more weight over the culture period in 25 mm test tubes compared to 18 mm tubes with a *p*-value of 0.099. Shoot proliferation was significantly higher in 25 mm tubes over 4 weeks in culture compared to in 18 mm tubes with a *p*-value of 0.0636. Shoot elongation was significantly higher in 25 mm tubes compared to 18 mm tubes with a *p*-value of 0.0497 (Table 5).

Cumberland explants were established April 10th. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least 4 weeks in 18 and 25 mm test tubes (Fig. 13). In treatment 1, Cumberland shoots gained significantly more weight over the culture period in 25 mm tubes than in 20 or 18 tubes with a *p*-value of 0.0012. Shoot proliferation was not significantly affected in any size of container in treatment 1 with a *p*-value of 0.1656. While shoot elongation was not significantly changed, shoots in 25 mm tubes had better growth compared to 18 mm tubes with a *p*-value of 0.0215(Table 5).

For the 2nd treatment, Cumberland shoots gained significantly more weight over the culture period in 25 and 20 mm tubes than in 18 mm tubes with a *p*-value of 0.0009. While shoot number was not significantly affected in any size of containers, shoots in 25 and 20 mm tubes more new shoots compared to 18 mm tubes with a *p*-value of 0.0196. Shoot elongation was significantly higher in 25 mm tubes compared to 18 mm tubes with a *p*-value of 0.0001 (Table 5).

"Coral" explants were established April 26th. Explants from 0.5-1.5 cm in length were taken from stocks previously maintained for at least 4 weeks in 18 and 25 mm test tubes. In treatment 1, "Coral" shoots gained significantly more weight over the culture period in 25 and 20 mm tubes compared to 18 mm tubes with a *p*-value of 0.0095 (Table

5). Shoot proliferation was also significantly better in 25 and 20 mm tubes then in 18 mm tubes with a *p*-value of 0.0012. Shoot elongation was significantly better in 25 and 20 mm tubes compared to 18 mm tubes over 4 weeks in culture with a *p*-value of 0.0428 (Table 5).

For the 2nd treatment, "Coral" shoots gained significantly more weight over the culture period in 25 and 20 mm tubes compared to 18 mm tubes with a *p*-value of 0.0208. Shoot proliferation was significantly better in 25 and 20 mm tubes than in 18 mm tubes with a *p*-value of 0.0015. Shoot elongation was significantly higher in 25 and 20 mm tubes than 18 mm tubes with a *p*-value of 0.0053 (Table 5).

Oconee explants were established June 10th. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least 4 weeks in 18 and 25 mm test tubes (Fig. 14). In treatment 1, Oconee shoots gained significantly more weight over the culture period in 25 mm tubes compared to 18 mm tubes with a *p*-value of 0.0046 (Table 5). Shoot proliferation was also significantly better in 25 and 20 mm tubes compared 18 mm tubes with a *p*-value of 0.0011. While shoot elongation was not significantly affected in any size of containers, shoots in 25 and 20 mm tubes trended better compared to 18 mm tubes with a *p*-value of 0.1991.

For the 2nd treatment, Oconee shoots gained significantly more weight over the culture period in 25 mm tubes compared to 18 mm and 20 mm tubes with a *p*-value of 0.0080. Shoot proliferation was significantly better in 25 and 20 mm tubes than in 18 mm tubes with a *p*-value of 0.0008. Shoot elongation was significantly higher in 25 mm compared to 18 mm tubes with a *p*-value of 0.0191 (Table 5).

"F-L" explants were established June 1st. Explants from 0.5-1 cm in length were taken from stocks previously maintained for at least 4 weeks in 18 and 25 mm test tubes. In treatment 1, Oconee shoots gained significantly more weight over the culture period in 25 mm tubes compared to 18 mm with a *p*-value of 0.0247. Shoot proliferation was also significantly affected in 25 mm compared to 18 mm with a *p*-value of 0.0039. While shoot elongation was not significantly affected by size of containers with a *p*-value of 0.1544, shoots in 25 and 20 mm tubes were about three times longer than in 18 mm tubes (Table 5).

For the 2nd treatment, "F-L" shoots gained significantly more weight over the culture period in 25 at 139 mg compared 18 mm tubes with a *p*-value of 0.0008. Shoot proliferation was significantly better in 25 and 20 mm tubes compared to 18 mm tubes with a *p*-value of <0.0001. Shoot elongation was significantly higher in 20 mm tubes compared to 18 mm tubes with a *p*-value of 0.0962 (Table 5).

Discussion

The objectives of this chapter were to investigate and test a range of TDZ hormone concentrations and container sizes in maintaining and increasing native deciduous *Rhododendron* through micropropagation. The purpose of investigating these two parameters in maintaining explants in a micropropagation system improve the process through determining the change of explant weight, shoot proliferation, and shoot elongation over 4 week culture periods. This study set out to assess the impact of TDZ incorporation into media instead of 2iP and the effect of test tubes size on weight, proliferation, and elongation in explants.

This study has shown that TDZ has the ability to increase overall shoot proliferation and explant growth over 4 weeks in culture when compared to 2iP alone in some species of native deciduous *Rhododendron*. This work also has shown that increased container size, and thus overall media amount in containers, can increase explant growth, proliferation, and elongation in some species of native deciduous *Rhododendron.* The main finding of this work is that all *Rhododendron* species must be investigated individually since the impact of hormone concentration and container size can vary. The study demonstrated that replacing TDZ with 2iP as the cytokinin in media and increasing the container size for micropropagating *Rhododendron* can increase proliferation of shoots, ultimately decreasing the time in culture or the amount of subcultures required to achieve desired production levels of stock explants in commercial settings.

The results are broadly consistent with others that have included alternate hormones and varying container sizes in an effort to increase shoot production (Rahimi et al. 2013). Our observations were that TDZ stimulated the production of adventitious shoots and at times inhibited shoot length. The control treatment of 2iP only produced adventitious shoots but the replacement of 2iP with TDZ allowed for a significant increase in shoot proliferation in all varieties investigated. The combination of low concentrations of 2iP and TDZ was reported (Rahimi et al. 2013), and should be included in further experiments to determine the shoot elongation response by native deciduous *Rhododendron.* Briggs et al. (1988) reported that shoots of deciduous *Rhododendron* were compact and hyperhydric in growth on medium with 4.5 µM TDZ (Briggs et al. 1988).

Future larger studies including more treatments in terms of TDZ ratios and container sizes with statistical analyses are important to determine optimal ratios and sizes for other species within *Rhododendren* as well as other genera of woody plants in micropropagation systems. Several questions can be answered; in particular if deciduous and evergreen varieties respond similarly to each other. More research in this area is necessary before making recommendations to growers in commercial settings. Further studies are required to pinpoint optimal hormone ratios and container sizes to maximize production of native deciduous *Rhododendron* explants.

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Table 4: *P*-value effects of TDZ on maintaining *Rhododendron in vitro*

Variety/Hybrid[a]	Weight	Shoot Proliferation	Shoot Elongation
Alabama	<.0001	0.0006	0.083
Cumberland	0.0007	0.008	0.0001
"Coral"	0.0031	0.0778	0.0006
Oconee	0.0612	0.0056	0.0008
"F-L"	0.0673	0.0536	0.2318

[a] Data were analyzed using PROC GLIMMIX (SAS Institute, Cary, NC).

Table 5: Effects of container sizes on maintaining *Rhododendron in vitro*

Container			
Variety/Hybrid	Explant Weight (mg)	Shoot Proliferation	Shoot Elongation (mm)
Alabama1[a]			
18 mm	47 b	3.38 b	3.53 b
20 mm	51 b	5.4 ab	1.5 b
25 mm	90 a	8 a	6.35 a
Pr<F[b]	0.0035	0.0139	0.0015
Alabama2			
18 mm	67 b	3.22 b	2.7 b
20 mm	84 ab	5.48 ab	5.89 a
25 mm	99 a	7.58 a	4 ab
Pr<F	0.0990	0.0636	0.0497
Cumberland1			
18 mm	24 b	3 a	1 b
20 mm	32 b	5.55 a	2.22 ab
25 mm	58 a	5.43 a	2.87 a
Pr<F	0.0012	0.1656	0.0215
Cumberland2			
18 mm	24 b	3.09 b	0.769 b
20 mm	53 a	6.11 a	1.23 b
25 mm	60 a	5.93 a	3.375 a
Pr<F	0.0009	0.0196	0.0001
Coral1			
18 mm	52 b	2.4 b	1.67 b
20 mm	124 a	7.58 a	3.58 ab
25 mm	125 a	7.6 a	3.88 a
Pr<F	0.0095	0.0012	0.0428
Coral2			
18 mm	50 b	2.2 b	0.46 b

20 mm	54 b	5.2 a	1.9 a
25 mm	157 a	6.571 a	2.786 a
Pr<F	0.0208	0.0015	0.0053
Oconee1			
18 mm	45 b	1.25 b	1 a
20 mm	67 b	3.727 a	2 a
25 mm	107 a	5.125 a	2.27 a
Pr<F	0.0046	0.0011	0.1991
Oconee2			
18 mm	54 b	0.656 b	0.444 b
20 mm	78 b	4.767 a	1.667 ab
25 mm	119 a	4.8 a	2 a
Pr<F	0.0208	0.0015	0.0053
“F-L”1			
18 mm	46 b	0.56 b	0.63 a
20 mm	116 b	1.66 b	1.73 a
25 mm	123 a	3.16 a	1.83 a
Pr<F	0.0247	0.0039	0.1544
“F-L”2			
18 mm	66 b	1.3 b	1.25 b
20 mm	79 b	2.3 a	2.35 a
25 mm	139 a	4.25 a	2.08 ab
Pr<F	0.0008	<.0001	0.0962

[a] Alabama 1 originated from 18 mm tubes and Alabama 2 originated from 25 mm tubes. Other varieties/hybrids were separated similarly.

[b] Data were analyzed using PROC GLIMMIX (SAS Institute, Cary, NC).

Figure 8. Cumberland explants after culture period with 2iP and 2.2-22 ppm TDZ hormones.

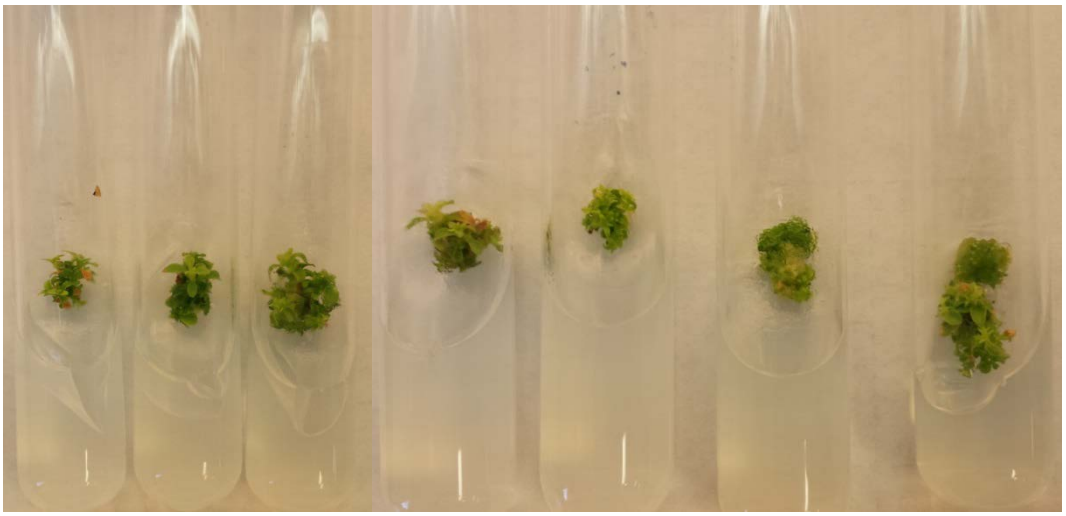

Figure 9: “Coral” explants after culture period with 2iP and 2.2-22 ppm TDZ hormones.

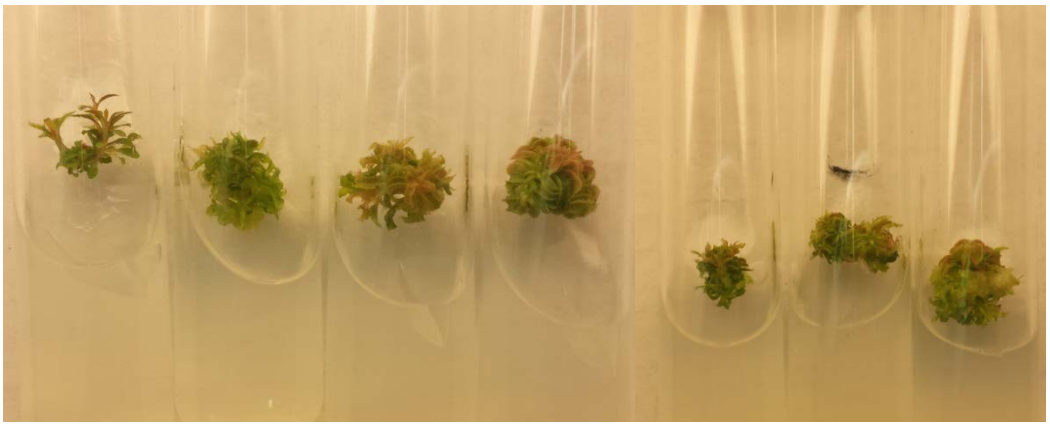

Figure 10: Oconee explants after culture period with 2iP and 2.2-22 ppm TDZ hormones.

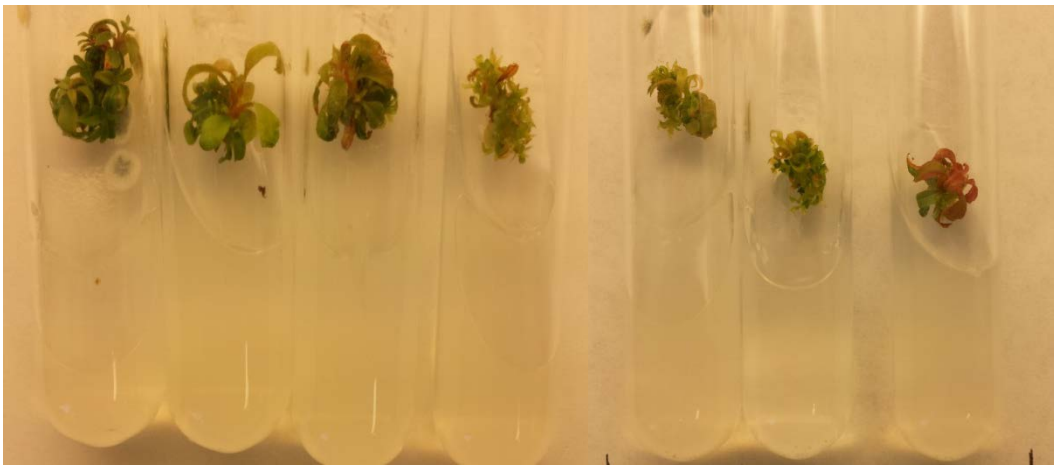

Figure 11: “F-L” explants after culture period with 2iP and 2.2- 13.2 TDZ hormones.

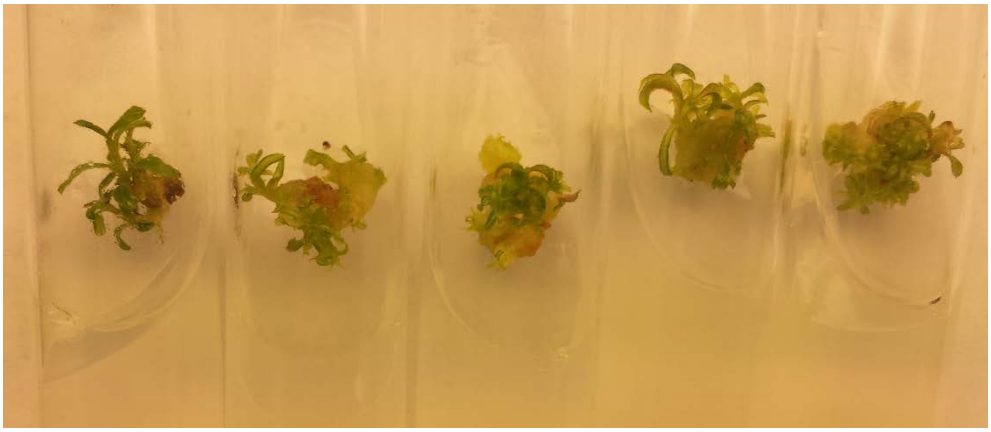

Figure 12: Alabama explants after culture period with 2iP and 2.2 and 22 ppm TDZ hormones

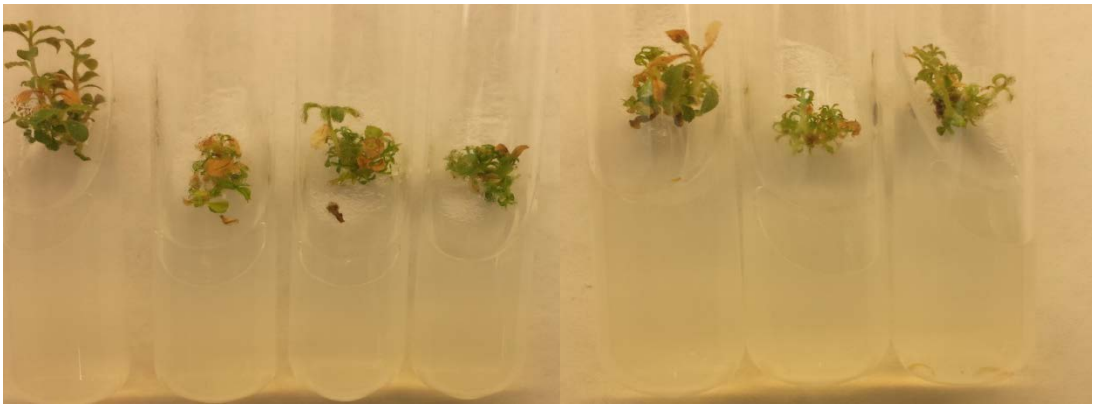

Figure 13: Cumberland explants after culture period in 18, 20 and 25 mm test tubes.

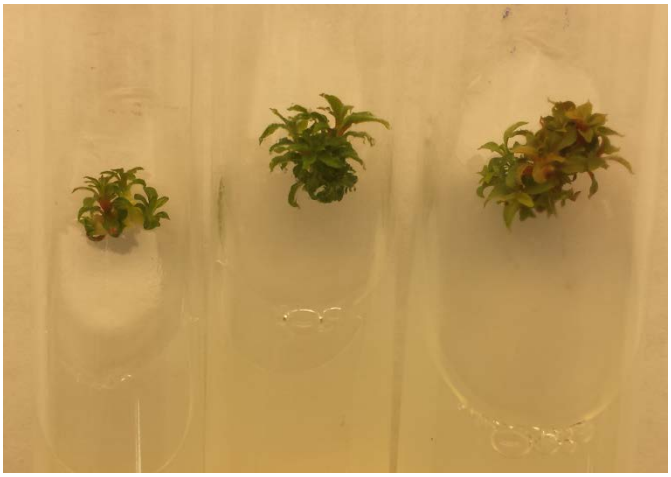

Figure 14: Oconee explants after culture period in 18, 20 and 25 mm test tubes.