DEVELOPING MICROPROPAGATION PROTOCOL FOR *RHAMNUS FRANGULA* CULTIVARS AND EVALUATING ORYZALIN TREATMENTS FOR POLYPLOID DEVELOPMENT

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Todd P. West
Chair

Dr. Wenhao Dai

Dr. Deirdre Voldseth

Approved:

04/19/2024
Date

Richard Horsley
Department Chair

ABSTRACT

Rhamnus frangula is a commonly used landscape plant. The major concern of *R. frangula* is its invasive nature. A means of dealing with invasive landscape plants is the production of sterile triploid cultivars. Development of sterile, triploid *R. frangula* cultivars would prevent its invasiveness. *Rhamnus frangula* is diploid and producing triploid varieties requires the development of tetraploids. These tetraploids could be bred with diploids, which results in triploid offspring. Oryzalin is a mutagen used for inducing chromosome doubling. Chromosome doubling in *R. frangula* is an understudied area, with no information available. The aim of this research was to establish a protocol for a clonal propagation method utilizing tissue of *R. frangula* and to develop a protocol for oryzalin-induced tetraploidy of *R. frangula*. Tetraploidy was confirmed using flow cytometry. Establishing a protocol for micropropagation and polyploid induction for *R. frangula* can assist in the breeding and creation of sterile triploid cultivars.

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DEDICATION

I want to thank my family and many friends. A special feeling of gratitude to my loving parents, Launa Edvall-Doyle and Jaye Edvall, my brother Connor, my sister Bella, and my step-dad Jeff Doyle, whose words of encouragement and push for tenacity helped me continue on.

I also dedicate this thesis to my many friends (Dyson, Chase, Toni, Jade, Jacob, Sakura, and Jarod) who have supported me throughout the process. I will always appreciate all they have done, from emotionally supporting me to helping me relax with their company.

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1. INTRODUCTION

Rhamnus frangula, commonly known "glossy buckthorn", is a plant belonging to the *Rhamnaceae* family. *Rhamnaceae* primarily consists of flowering plants with trees, shrubs, and a few vines. It is commonly known as the "buckthorn family" and contains approximately 55 genera (such as *Rhamnus, Frangula, Helinus, Noltea, Spyridium*, and *Ziziphus*) and 950 species (Christenhusz et al. 2016). Basic features of *Rhamnaceae* include their fruits (mostly berries, fleshy drupes, or nuts), simple leaves, and completely symmetrical flowers. *Rhamnus frangula* is placed either in the genus *Rhamnus* or *Frangula*. According to the USDA-ARS Germplasm Resources Information Network (GRIN), *Rhamnus frangula* is now *Frangula alnus* (GRIN 2023). In the commercial nursery trade, it is still classified as *Rhamnus frangula* because the industry is recalcitrant with accepting plant name changes. For the rest of this project, it will be referred to as *Rhamnus frangula*. The genus *Rhamnus* is comprised of shrubs or small to medium-sized tree species (Archibold et al. 1997).

The most prevalent use of *Rhamnus frangula* is in landscaping as an accent plant for screening or hedging (Catling and Porebski 1994; Dosmann 2001). As early as the 20th century, *R. frangula* was introduced to the United States and Canada from Europe and grown as a landscape shrub (Dosmann 2001). Additionally, *R. frangula* is utilized in mass plantings, border plantings, and framing for pathways and entryways. There are three notable *R. frangula* cultivars: 'Asplenifolia' (or 'Aspleniifolia'), 'Columnaris', and 'Ron Williams' (Fine Line®). 'Asplenifolia', commonly called "fern-leaf" buckthorn, is an upright, spreading, deciduous shrub that typically grows 1.8 to 3 m tall. It is a self-seeding cultivar that has a very fine texture and is slow growing. The most notable part of 'Asplenifolia' is its extremely narrow, glossy, dark green leaves that possess irregular margins and are said to be similar to ferns (Dirr 2009). This cultivar

is said to add a "ferny" texture to the landscape and as a result of the strap-like narrow leaves. 'Asplenifolia' is commonly used as shrub borders, foundation and as background plantings. 'Columnaris' is a deciduous shrub that typically grows in a narrow upright form up to 3.6 to 4.5 m tall but only 1.2 m wide with its branches and stems erect, compact, and close together. 'Columnaris' is typically produced from cuttings and has a rounder leaf compared to 'Asplenifolia'. It is primarily used as hedges and as such is also known as "tallhedge glossy buckthorn" (Dirr 2009). 'Ron Williams' (Fine Line®) is a hybrid of 'Asplenifolia' and 'Columnaris' (Dirr 2009). 'Ron Williams' is an upright, freely branching, columnar, deciduous cultivar with fern-like foliage. It grows slowly to 1.5 to 2.1 m tall and is as wide as 'Columnaris'. It receives its fern-like foliage from its pollen parent ('Asplenifolia') and its columnar shape from its seed parent ('Columnaris'). 'Ron Williams' is generally used in small/narrow areas of the landscape, borders, and foundations. This cultivar is noteworthy for only having a 3% seed viability compared to the 98% seed viability shared by its parents (Sheehan 2007).

Rhamnus frangula is frequently used in medicinal, physiological, and ecological research. Medicinally, it has been established that extracts of *R. frangula* have strong antimicrobial and antioxidant properties. (Manojlovic et al. 2005; Stef et al. 2009). It is also used in laxatives and has been noted as a source of multiple bioactive molecules including anthraquinones. Recent medicinal research of *R. frangula* involves protocols for extracting anthraquinones and the species potential use as a protease inhibitor for bacteria, fungi, and certain viruses (Bacha et al. 2017; Sonchini et al. 2017; Tebrencu et al. 2015). Furthermore, the ecological effects of *R. frangula* have started to gain attention in recent research. The primary ecological topics being focused on are *R. frangula's* physiological competitiveness, invasion potential, and the effects of crown rust on the species (Kalkman et al. 2019; Sulaiman et al. 2023;

Williams & Krock 2012). The most current threat of *Rhamnus frangula* is its invasiveness and the ramifications to native ecosystems if this species is left unchecked.

1.1. *Rhamnus frangula's* Invasiveness

The invasiveness of *Rhamnus frangula* has long since been documented in forest ecosystems (Converse 1984). The presence of invasive plants is alarming for a large number of reasons, including competition with native tree species, reduction of emerging seedlings, reduced growth rates, and increased mortality of native plants. One example is in a study by Fagan & Peart (2004) involving a forest heavily invaded by *R. frangula*. It was estimated that less than 10% of the native tree saplings survived to maturity under high solidities of *R. frangula* (Fagan & Peart 2004). Another well-documented trait that allows *R. frangula* to be a highly invasive species is its ability to grow in a wide range of soil and soil moisture conditions. As such, this promotes the species to invade wetland habitats, sand plains, forest edges, fields, fencerows, and prairies (Possessky et al. 2000). Furthermore, it is theorized that birds are the most likely long-range dispersers of *R. frangula* seeds, quickly releasing seeds due to a laxative chemical contained within the fruit (Catling & Porebski 1994; Wheeler & Starrett 2001). All these factors have contributed to the fast spread of *R. frangula* into various habitats in the northern United States and parts of southern Canada since the 1980s (Fagan & Peart 2004). If ignored, *R. frangula* has the potential to cause immense amounts of damage to native plant life across the United States and Canada. Due to the nonzero seed viability, 'Ron Williams' has been struggling to be removed from some state's restricted plant list. These states include CT, IL, MA, ME, MN, MT, NH, NY, VT, and WI (MDA 2023, MIPN 2023, Sturtevant et al. 2022). This is because regulatory agencies in various U.S. states require that any landscape or commercial nursery trade plant must have a 0% seed viability in those states. Due to its extremely high tolerance for almost

any environmental condition and natural drought resistance, *R. frangula* has remained a staple plant in landscaping. Albeit its invasiveness is a very concerning staple for ecological reasons.

There are a few methods to prevent the spread of *R. frangula*. One successful method is eradication which involves cutting the main stem of the plant and then applying glyphosate herbicide to the cut stump (Reinartz 1997). Another method of eradication is the use of an herbicide called triclopyr at low concentrations mixed with water (DiAllesandro 2012). Besides these eradication methods, there is little research done on preventing *R. frangula* from spreading. While the use of genetic engineering to treat invasiveness has seen an increase in popularity, its usage in woody plants is low (Li et al. 2004; Teem et al. 2020; Nelson 2022). In order to conduct genetic engineering, it is essential to be able to insert DNA into cells. Then, select the modified cells from a large population of unmodified cells, and regenerate those cells into healthy, differentiated plants. This is a process known as transformation. Unfortunately, for the large variety of woody plants, transformation is extremely difficult to do, and for many taxa, it has never been accomplished (Vining et al. 2012). Additionally, this is not viable for plants used in landscaping since there is no way to track and regulate a transgenic landscape plant. Currently, breeding is the most probable and realistic method for dealing with *R. frangula*'s invasiveness, as inducing polyploidy of a woody plant requires little equipment, no genomic information, and little time. Once the polyploid is developed it can take up to 15 years or more to develop a sterile hybrid.

1.2. Induction of Polyploidy

Breeding can be an effective way to deal with a potentially invasive plant species. This can be accomplished through manipulation of a plant's chromosome number, or ploidy to produce sterile cultivars (Vining et al. 2012). Polyploidy is commonly defined as the possession

of three or more complete sets of chromosomes. There are two mechanisms in which to induce polyploids: mitotic polyploidization and meiotic polyploidization. The mitotic method is based on the doubling of somatic tissues, while the meiotic method generates 2n gametes (Ramsey & Schemske 1998). If possible, the meiotic method is typically preferred since the 2n gametes can be used immediately in crossing experiments and reduce breeding by one generation. Diploid refers to organisms with two sets of chromosomes, organisms with three sets are triploid, and those with four sets are tetraploid. During meiosis, an organism's chromosome number is halved, which causes a diploid species to produce gametes with one set of chromosomes. This isn't the case for triploids (or any plant with an odd number of sets of chromosomes). Since triploid plants have three sets of chromosomes, the process of meiosis creates incomplete, infertile gametes, which makes them sterile. The production of these types of sterile triploids can be done in a few steps. First, a diploid plant is treated with some form of a mutagen to alter its ploidy. This turns a diploid into a tetraploid. Next, the tetraploid is then crossed with another diploid plant. Since the tetraploid is producing gametes with two sets of chromosomes, and the diploid with one set, the resulting offspring would have three sets of chromosomes and be known as a triploid. This is usually accomplished with the use of a mutagen such as colchicine or oryzalin.

1.3. Colchicine Versus Oryzalin

Colchicine is an alkaloid, which is derived from the autumn crocus plant (*Colchicum autumnale*) (Eng & Ho 2019). This alkaloid induces chromosome doubling in plants by inhibiting the function of microtubular proteins at a cellular level (Lu et al. 2012). Microtubules are the cell's structure for pulling already copied chromosomes apart during the process of cell division. The disruption of this process leads to cells that have double their usual sets of chromosomes. Colchicine was once a commonly used chemical for manipulating ploidy in

plants. However, it has many disadvantages that has caused its usage to decrease. First, its function as a mutagen can cause unwanted abnormalities in treated tissues and ploidy stabilization issues (Blasco et al. 2015). Second, it is extremely hazardous to humans (Yemets and Blume 2008). Third, the process can be expensive due to a high concentration of the chemical needed to achieve ploidy doubling (Eng and Ho 2019).

Oryzalin is a dinitroaniline herbicide and a preferred substitute for colchicine for polyploid induction. Both have a similar effect on plant cells, disrupting the function of microtubules to produce cells with additional sets of chromosomes (Ebrahimzadeh et al. 2018). The main difference between the two is that oryzalin is less toxic to humans and more cost effective, as only small concentrations of the chemical are necessary to produce the desired chromosome doubling effect (Ebrahimzadeh et al. 2018). In a study of korarima (*Aframomum corrorima* (A. Braun) P.C.M. Jansen) culture by Wannakrairoj and Tefera (2013), it was found that the amount needed to produce chromosome doubling with oryzalin was 1/10th of the amount needed with colchicine. Additionally, oryzalin shows strong binding ability to plant tubulins (Kermani et al. 2003). This makes oryzalin a much more effective herbicide than colchicine for plant polyploidization. There are two common methods of applying oryzalin to plant tissues. These are through tissue culture exposure (Bouvier et al. 1994; Kermani et al. 2003) or through foliar applications to meristematic tissues (Ackerman and Dermen 1972; Pryor and Frazier 1968; Olsen et al. 2006). To date there have not been any studies conducted on oryzalin-induced chromosome doubling in *R. frangula*.

1.4. Steps of Micropropagation

Plant propagation is the multiplication of plant material to form new plants (Davies et al. 2018). There are two types of plant propagation: sexual and asexual. Sexual propagation

involves the fusion of two gametes to create a seed. Asexual or vegetative propagation takes advantage of a plant's theoretical ability to develop into a fully functional plant from a single plant cell (also known as cellular totipotency). Adventitious shoot and root formation let plants develop new tissues from already specialized tissue. Generally, plant cultivars are propagated using asexual methods. Asexual methods of propagation eliminate any form of genetic variation and the resulting plants are genetic clones of the mother plant. This allows nurseries to sell plants that are genetically uniform and will perform consistency in their specific landscapes. There are several methods for asexual propagation including vegetative stem or root cuttings, grafting and tissue culture (micropropagation). Micropropagation is defined as the process of multiplying plant stock by growing plantlets in tissue culture to produce a large number of progeny (clone) plants. The first step to micropropagation involves a disinfestation of plant materials prior to being introduced into an aseptic environment. Disinfestation assists in preventing fungal or bacterial pathogens from attacking the plants. Next, plant tissues are transferred into containers containing a nutrient-rich substrate, referred to as a micropropagation medium. This growing medium commonly contains hormones or plant growth regulators (PGRs), which can stimulate shoot or root development to aid in plant growth. Alongside PGRs, nutrient salts are also added to the medium. Concentration of salts present in the media depends on the particular plant's growth needs. There are varying levels of nutrient salts available for plant tissue culture; Murashige and Skoog formulation (MS; Murashige and Skoog 1962) is considered a high salt concentration, Long and Preece medium (LP; Long et al. 1994) is a medium strength concentration, and Woody plant medium (WPM; Lloyd and McCown 1980) is a low concentration (Greenway 2012; Li et al. 2021). For plants without an already established micropropagation protocol, comparing different levels of nutrient salt concentrations as well as

comparing the effects of PGRs on plant growth is needed to establish a micropropagation protocol (Phillips and Garda 2019). The next step of micropropagation occurs after a 4 to 6-week period in tissue culture with the creation of microshoots, microshoots are shoots initiated during the establishment and multiplication stages of micropropagation. Microshoots are further divided into smaller segments and transferred onto fresh nutrient media, in a process called subculturing. Subculturing allows for a rapid increase in plant tissue populations. Microshoots require varying lengths of time to adjust to the culture conditions, with woody plants, this can be months to even more than a year.

Prior tissue culture of *R. frangula* and its subsequent relatives has been accomplished with varying results. Van den Berg and Labadie (1984) successfully induced callus of *R. frangula* from the cambial zone in a branch using a modified MS medium with kinetin and 2,4-dichloro-phenoxyacetic acid (2,4-D) as plant growth regulators (PGRs). Additionally, Bignami (1983) accomplished shoot proliferation from shoot tips of *R. frangula* utilizing MS and a combination of 6-benzylaminopurine (BA) with indole-3-butyric acid (IBA). Bignami was also successful in rooting with low levels of IBA. However, Bignami (1983) noted that *R. frangula* tends to form callus while in vitro and callus formation cannot be completely avoided.

Additional components can be added depending on desired results such as the use of mutagens like colchicine or oryzalin that causes chromosome doubling. There has been a significant increase of oryzalin usage as a mutagenic agent compared to other mutagens (Islam et al. 2022). This is primarily due to smaller concentrations required to induce polyploidy and being more effective than colchicine (Contreras et al. 2010; Väinölä 2000).

1.5. Determining Ploidy Level

One technique to verify the ploidy level of a plant is utilizing a process called flow cytometry. This method uses a sophisticated machine called a flow cytometer to measure sizes of a cell's chromosomes (Adan et al. 2017). Flow cytometry is widely used in cancer research and diagnosis but has since been applied to plant cells (Yanpaisan et al. 1999). The first step of flow cytometry is to create a suspension of single cells. This can be somewhat difficult as plant cells are surrounded by a thick cell wall. Removing this cell wall creates cells called protoplasts. Creating protoplasts can technically complete the first step but it is frequently an inefficient process. However, plant tissues can be chopped finely with a razor blade and dissolved in a buffer instead. Next, the solution is filtered through nylon, which leaves only small cell fragments (such as the plant's nuclei). Through the use of a laser, the flow cytometer analyzes the nuclei by shining through each individual nucleus. These nuclei pass through the machine in a single-file stream. Light that reflects off the nuclei is used to estimate the size of the chromosomes within, which can be utilized to estimate the ploidy level of the organism. Flow cytometry, while extremely popular, is not without a few disadvantages (Galbraith et al. 2021). One disadvantage is that it is difficult to create cell suspensions that will yield accurate results. The difficulty lies in excluding other debris particles that are similar to the ploidy size from the cell suspension. Another disadvantage is that samples analyzed through flow cytometry only reveal the size of a given nucleus. An extra, already analyzed, sample is needed to compare and obtain an accurate estimate of ploidy level. This makes utilizing flow cytometry to achieve an accurate estimate of ploidy level difficult with species who haven't already been analyzed. There currently is no research done to determine the ploidy level of R. frangula or using flow cytometry with R. frangula. The only study done to determine the ploidy level of R. frangula

determined that the chromosome number of *R. frangula* is 2n = 20 via root squash, making the species a diploid (Tutin et al. 1968).

Another method of determining ploidy level in plants is through a root squash. The first step to this is collecting actively growing root tips from plants (Lattier et al. 2017). Next, the root tip cells are fixed at their current stage of mitosis by placing the root tips in a solution which suspends the action of cell spindle fibers. The root tips are then placed in an acidic solution for a period of time to soften the tissues for squashing. Additionally, a stain is used to better see the cell contents. After the staining, the tissues are hydrolyzed or broken down with a water solution. Finally, root tips are transferred to microscope slides, squashed, and analyzed. As a result of the staining and suspension of mitosis, the chromosomes of each individual plant cell can be observed and counted under the microscope, revealing the plant's chromosome number (Miller 1961). This process is particularly useful in regard to ploidy manipulation studies. Since a root squash could be taken prior to and after the experimental treatment and compared for additional chromosome sets.

1.6. Research Objectives

- Develop a clonal micropropagation protocol for *Rhamnus frangula*.
    - Determine which combination of nutrient salts and PGRs will be the most successful for axillary shoot proliferation in each *Rhamnus frangula* cultivar.
    - Determine which concentration of IBA will be the most successful for microshoot rooting in each *Rhamnus frangula* cultivar.
- Develop a protocol for oryzalin-induced polyploidy of *Rhamnus frangula*.

2. MATERIALS AND METHODS

2.1. In Vitro Axillary Shoot Proliferation

Three different cultivars of *Rhamnus frangula* were to be evaluated including, 'Asplendifolia', 'Columnaris', and 'Ron Williams' (Fine Line®). Potted nursery stock of each of these cultivars were obtained and utilized as source materials. Explants from each cultivar was taken from softwood stem segments in January 2023. Each nodal segment consisted of a stem segment that was approximately 1 ± 0.5 cm in length and consisting of at least one axillary bud being present at each node. All nodal explants were surface sterilized for 15 minutes in a 10% (v/v) Clorox® (0.6% NaClO) bleach solution plus 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween® 20) surfactant with three 2 min sterile deionized distilled water (ddH_20) rinses. After disinfestation, all explants were immediately cut into nodal segments under sterile conditions using a surgical-grade scalpel. All leaves and apical meristems were removed from each explant. Nodal segments were placed upright and vertically onto a standardized establishment stabilization nutrient medium with the use of long-tipped forceps. Standardized establishment stabilization nutrient medium consisted of Murashige and Skoog (MS) nutrient salts and vitamins, 3% (w/v) sucrose, 2 µM 6-benzylaminopurine (BA), pH 5.7± 0.1, and 0.7% (w/v) plant tissue culture grade agar (A110, Phytotechnology Labs; Shawnee Mission, KS, USA). All nutrient media was dispensed into 25 x 150 mm borosilicate glass culture tubes (No. 9820 Pyrex®, Corning, Glendale AZ, USA) capped with autoclavable plastic lids, and autoclaved for 20 min at 121 °C and 1.0 kg cm^{-2}. Nodal segment cultures were placed approximately 30 cm beneath LED lamps that provided a photosynthetic photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod at 25 ± 3 °C. Microshoots that developed from nodal explants were subcultured every 6 w to create a stock population of aseptic plant

material for use in later experiments. ‘Asplenifolia’ was not utilized for experiments due to poor growth when cultured. ‘Columnaris’ was not used for this experiment due to not enough nodal cultures to meet the population size needed for the study.

Once established, nodal segments of the ‘Ron Williams’ (Fine Line®) were further cultured onto differing medium treatments. Differing medium treatments consisted of varying nutrient salt levels and PGR concentrations. MS (high), LP (medium), and WPM (low) were the nutrient salt formulations evaluated. The plant growth regulator evaluated for axillary shoot proliferation was BA with concentrations of 0, 2, 4, and 6 µM. This was done to observe the factorial combination of the nutrient salt formulation with BA concentration for the best axillary shoot proliferation. Nodal segments were utilized as the individual experimental unit and consisted of at least one axillary bud being present at each node with a length of 1 cm. Every 6 w, the uniform nodal segments were subcultured onto the same treatment media as before. This was done to maintain fresh nutrients and minimize any possible residual effects from the standardized establishment stabilization medium used for culture initiation. Leaves were cut off all nodes prior to transfer onto new treatment media. Data was collected after 12 w, after 2 subcultures, on shoot number, shoot length and propagation number. Only axillary shoots that were a minimum of 1 cm in length were counted for data analysis.

2.2. In Vitro Root Proliferation

Maintained elongated microshoots of ‘Ron Williams’ were utilized for the adventitious root evaluation. Microshoots cut under sterile conditions with a surgical grade scalpel. Using long-tipped forceps, approximately 1 cm meristematic microshoots having at least one node were placed upright in 15 mL x 150 borosilicate culture tubes (No. 9820, Pyrex®) containing

treatments. Culture tubes were incubated 30 cm below LED lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16-h photoperiod at 25°C for 6 w.

In vitro microshoot rooting was evaluated by utilizing Long and Preece (LP) nutrient salt formulation, determined from the previous sub-objective, with the addition of 3% (w/v) sucrose, 0.7% (w/v) plant tissue culture grade agar (A111, Phytotechnology Labs), and a pH of 5.7 ± 0.1. Additionally, multiple indole-3-butyric acid (IBA) concentrations of 0, 1, 2, and 4 µM were evaluated for microshoot rooting. 'Asplenifolia' was not utilized for experiments due to poor growth when cultured. 'Columnaris' was not used for this experiment due to not enough meristematic microshoots to meet the population size needed for the study.

There was a minimum of ten replicates of each treatment. Mean root number and mean root length were recorded after 6 w of incubation on treatment media to determine the main effects of IBA concentrations on in vitro rooting. Only roots greater than 1 cm were counted and used for data analysis.

2.3. In Vitro Oryzalin-Induced Polyploidy

Once the ideal micropropagation medium was determined for 'Ron Williams', oryzalin was utilized for in vitro polyploid induction evaluation. The source of oryzalin was from a commercially available herbicide registered as Weed Impede™ (Monterey Lawn & Garden, Fresno, California, USA) which contains 40.4% of oryzalin. Four different concentrations (0, 100, 200 and 400 µM) of oryzalin were evaluated for polyploid induction. These concentrations were made from a stock solution containing 1 ml of oryzalin with 9 ml of ddH_2O in a 50 ml Falcon tube. A nonionic organosilicon wetting and penetrating agent/surfactant (Silenergy®, Brewer International, Vero Beach, Florida, USA) was added at a rate of 1 mlL^{-1} to each treatment to aid in the delivery and penetration of oryzalin to the plant tissue. Oryzalin

concentration treatment solutions were filter sterilized in a laminar flow hood and deposited into autoclaved 100 mL Pyrex bottles with sealable lids.

Plant tissue consisting of nodal segments were taken from elongated microshoots of each *R. frangula* cultivars ('Ron Williams' and 'Columnaris') and exposed to the four different concentrations (0, 100, 200 or 400µM) of oryzalin placed on an orbital shaker at 200 RPM for 0 (control), 1, 2, or 4 days. 'Columnaris' was added for evaluation in this experiment because of the low number of nodal segments needed for oryzalin treatments. Ten nodal segments were used in each of oryzalin concentration treatments. After 1, 2, or 4 d, nodal segments were removed from the oryzalin concentration treatments, rinsed 3x with sterile ddH_20 and placed vertically into 25x150 mm borosilicate glass culture tubes containing growth medium determined from objective 1. Nodal segments in culture tubes were incubated approximately 30 cm beneath LED lamps that provide a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16-h photoperiod at 25°C for a 16-h photoperiod for 6 w. After 6 w, cultures were placed onto fresh growth medium to allow for the continued growth of the oryzalin exposed plant tissues. Leaf tissue from each of the oryzalin exposed and non-exposed microshoots were used to prepare nuclei solutions for ploidy levels via flow cytometry. The presence or absence of ploidy production was noted.

2.4. Flow Cytometry

Flow cytometry was conducted with the assistance of Dr. Scott Hoselton from the North Dakota State University Department of Microbiological Sciences using the CyStain™ PI Absolute P kit (Sysmex, Lincolnshire, Illinois, USA). One leaf approximately 0.5 cm^2 was removed from each of the microshoots, placed in a small petri dish, and covered with 500 µL of nuclei extraction buffer. Microshoots were placed back onto in vitro multiplication medium for

re-culturing after leaves were extracted. Using a sharp razor blade, the samples were chopped for approximately 30 to 60 seconds. After 30 to 90 s of incubation in the nuclei extraction buffer, the samples were filtered through a CellTrics™ (Sysmex, Lincolnshire, Illinois, USA) 50 µM filter into a sample tube. Approximately 1.5 mL of staining solution was added to each sample and allowed to incubate for 30 to 60 min, away from light. Staining solution comprised of 120 µL propidium iodide and 60 µL RNase A stock solution added to 20 mL of staining buffer. Samples were then run through a flow cytometer (Cytoflex S™, Beckman Coulter Life Sciences, Indianapolis, Indiana, USA) to observe for successful polyploid induction.

2.5. Design and Statistical Analysis

All experiments were arranged as completely random designs (CRD) and conducted twice unless otherwise stated. A minimum of 10 replications were used in each run of every experiment unless otherwise noted. All data was analyzed using the General Linear Model (GLM) of SAS 9.4 (SAS Institute, Cary, NC, USA). Mean analysis was conducted using LS means. Mean separation analysis used the LINES method for the multiple comparison test for mean separation of fixed effects.

Experimental design for axillary shoot proliferation contained ten replicates of each three nutrient salt levels and four BA concentrations. It was a factorial design comparing the effects on axillary shoot development and growth using different nutrient salt solutions ranging from high to low salt formulations with (BA) at varying concentrations. Experimental design for in vitro axillary root proliferation contained ten replicates of each four IBA concentrations to determine the main effects of IBA concentrations on in vitro rooting.

3. RESULTS AND DISCUSSION

3.1. In Vitro Axillary Shoot Proliferation

Nutrient salt formulation, presence of BA, and the factorial combination of nutrient salt formulation with BA had a highly significant effect on axillary shoot production (Appendix Table A1). For all main effects, run and replication were not significant and were pooled together for all subsequent analysis. As nutrient salt formulations increased with salt concentrations, shoot number significantly increased. MS (high salt formulation) and LP (medium salt formulation) outperformed WPM (low salt formulation) but did not outperform each other with MS producing 1.86 shoots per nodal segment, LP producing 1.59 shoots per nodal segment and WPM producing 1.27 shoots per nodal segment. (Table 1; Fig. 1). In terms of BA concentration, 4 and 6 µM BA outperformed all other treatments but did not outperform each other with 4 µM producing 2.35 shoots per nodal segment, 2 µM producing 1.89 shoots per nodal segment, 6 µM producing 2.05 shoots per nodal segment, and 0 µM producing 0.02 shoots per nodal segment (Table 2; Fig. 1). There was a highly significant 2-way factorial interaction with nutrient salt formulation and the present of BA in the culture medium with respect to shoot

Table 1. Effect of nutrient salt formulation on 'Ron Williams' (Fine Line®) for axillary shoot number production.

Nutrient Salt Formulation	Mean Shoot Number[1]
WPM	1.27a
LP	1.59b
MS	1.86b

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

Figure 1. Axillary shoot multiplication of 'Ron Williams' after 12 weeks on multiplication media comparing three different salt formulations and four different concentrations of 6-benzylaminopurine (BA) (From left to right: 0, 2, 4, 6 µM). A) MS nutrient salts, B) LP nutrient salts, C) WPM nutrient salts. Scale bar = 2 cm. (0 µM for MS not pictured due to all controls dying before 12 weeks).

Table 2. Effect of 6-benzylaminopurine (BA) concentration on 'Ron Williams' (Fine Line®) for axillary shoot number production.

BA (µM)	Mean Shoot Number[1]
0	0.02a
2	1.89b
4	2.35c
6	2.05bc

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

production of 'Ron Williams' (Fine Line®) (Appendix Table A1; Table 3; Fig. 1, Fig. 2). Two treatments, MS with 2 µM BA and LP with 4 µM BA produced the most axillary shoots as compared to other evaluated treatments. Bignami (1983) reported the best treatment for axillary shoot production in *Rhamnus frangula* (no cultivars) to be MS nutrient salts with 4.4 µM BA. This differs from what was measured in the current study with 'Ron Williams', axillary shoot production was significantly less when cultured on MS nutrient salts supplemented with 2 µM BA. Bignami (1983) only evaluated MS nutrient salts with varying concentrations of BA

Table 3. Effect of nutrient salt formulation and 6-benzylaminopurine (BA) concentration interactions on 'Ron Williams' (Fine Line®) for axillary shoot number production.

Nutrient Salt Formulation	BA (µM)	Mean Shoot Number[1]
WPM	0	0.1a
WPM	2	1.46bc
WPM	4	1.85bcd
WPM	6	1.72bc
LP	0	0.2a
LP	2	1.36b
LP	4	3.11f
LP	6	1.90bcd
MS	0	0.1a
MS	2	2.85ef
MS	4	2.09cd
MS	6	2.51ed

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

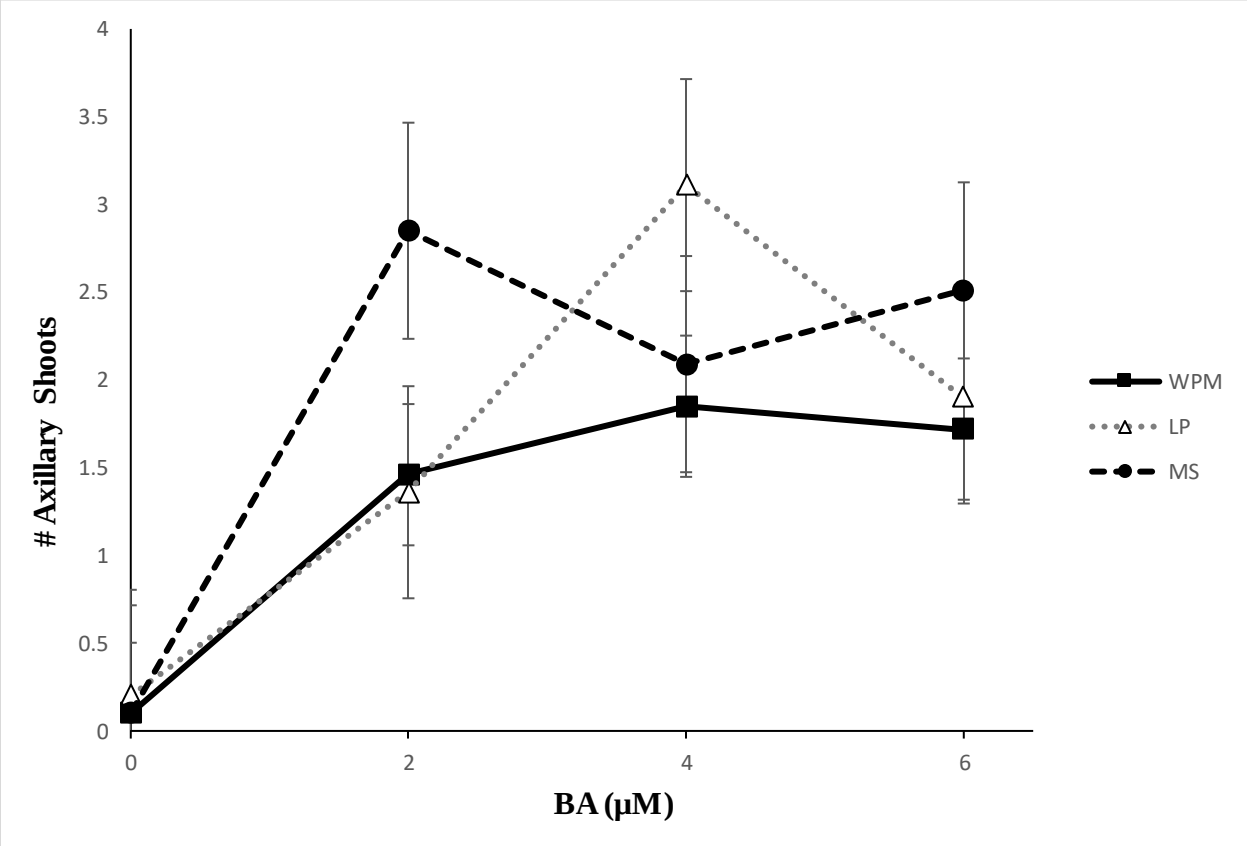

Figure 2. Effect of nutrient salt formulation and 6-benzylaminopurine (BA) concentration interactions on 'Ron Williams' (Fine Line®) for axillary shoot number production.

prohibited making comparisons with nutrient salt formulations. Also, the study did not report data on axillary shoot number, data was only presented on propagation number which will be discussed in a subsequent section (Bignami 1983). The use of different types and concentrations of nutrient salts can drastically alter the number of shoots produced and or the percentage of explants that produce shoots. Additionally, this can have a more dramatic effect than simply altering the PGR concentration in the media (Preece et al. 1995). This can be observed with Mencuccini and Rugini (1993) who found that full-strength MS caused twice as many olive petiole (*Olea europaea* L.) explants to form shoots compared to half-strength MS. Shoot multiplication of *Rhamnus cathartica* proved to be successful using MS medium with combination of 2,4-D and kinetin (Kovačević and Grubišić 2005). Possibly implying that members of *Rhamnus* could thrive with high salt formulation media. This is further demonstrated with the shoot multiplication of *Rhamnus palaestinus* performing the best on MS medium (Zilkah et al. 1999) and DKW (a high salt formulation) with TDZ performing the best for axillary shoot proliferation of *Hibiscus moscheutos* (West and Preece 2004). Additionally, West and Preece (2004) evaluated LP as a potential salt formulation media for *Hibiscus moscheutos* but it did not perform as well as DKW. Conversely, Kovačević and Grubišić (2005) reported successful shoot multiplication of *Frangula alnus* (*R. frangula*) on WPM with 0.57 µM IAA and 4.4 µM BA added. Suggesting that *R. frangula* may not require a medium-to-high salt formulation in tissue culture. However, it should be noted that lower salt formulations tend to promote more rooting, which generally causes less shoot growth (Kyte et al. 2013).

Nutrient salt formulation, presence of BA, and the factorial combination of nutrient salt formulation with BA had a highly significant effect on propagation number. (Appendix Table A2). For all main effects, run and replication were not significant and were pooled together for

all subsequent analysis. As nutrient salt formulations increased in salts availability, propagation number significantly increased. This trend is similar to shoot production with respect to the results. Suggesting that high salt formulations are the best for both shoot production and propagation number of 'Ron Williams'. MS significantly outperformed LP and WPM with MS producing 2.58 nodes per shoot, LP producing 1.82 nodal segments per shoot, and WPM producing 1.07 nodal segments per shoot. (Table 4). In terms of BA concentration, 2 and 4 µM BA significantly outperformed all other treatments producing 2.49 and 2.91 nodal segments per shoot respectively (Table 5). There was a highly significant 2-way factorial interaction with nutrient salt formulation and the present of BA in the culture medium with respect to propagation number of 'Ron Williams' (Fine Line®) (Appendix Table A2; Table 6; Fig. 3). Two treatments, MS with 2 µM BA and LP with 4 µM BA produced the highest propagation number as compared to other evaluated treatments. These results are the same as the previously mentioned shoot

Table 4. Effect of nutrient salt formulation on 'Ron Williams' (Fine Line®) for propagation number.

Nutrient Salt Formulation	Mean Prop Number[1]
WPM	1.07a
LP	1.82b
MS	2.58c

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

Table 5. Effect of 6-benzylaminopurine (BA) concentration on 'Ron Williams' (Fine Line®) for propagation number.

BA (µM)	Mean Prop Number[1]
0	0.02a
2	2.49bc
4	2.91b
6	1.87c

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

Table 6. Effect of nutrient salt formulation and 6-benzylaminopurine (BA) concentration interactions on 'Ron Williams' (Fine Line®) for propagation number.

Nutrient Salt Formulation	BA (µM)	Mean Prop Number[1]
WPM	0	0.1a
WPM	2	1.47b
WPM	4	1.54bc
WPM	6	1.24b
LP	0	0.2a
LP	2	1.31b
LP	4	4.5e
LP	6	1.5bc
MS	0	0.4a
MS	2	4.7e
MS	4	2.71cd
MS	6	2.89d

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

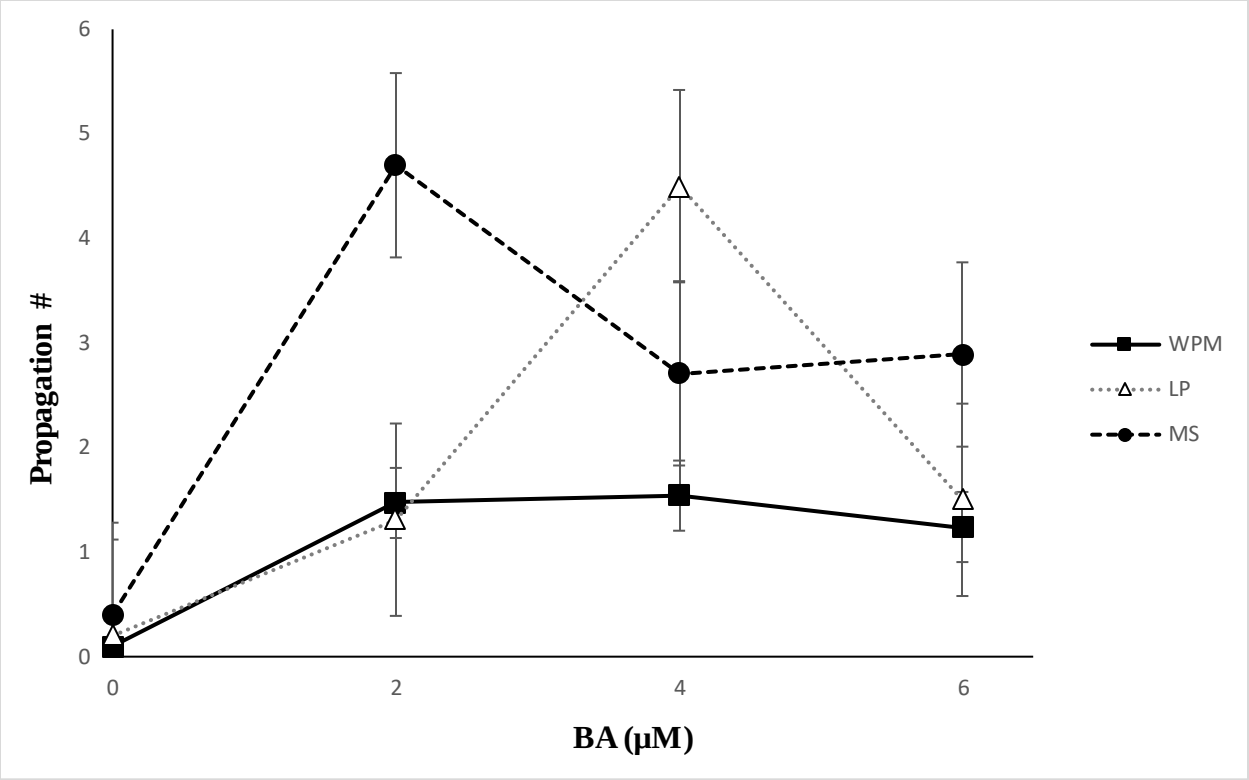

Figure 3. Effect of nutrient salt formulation and 6-benzylaminopurine (BA) concentration interactions on 'Ron Williams' (Fine Line®) for propagation number.

production. With a mean propagation number of 4.7 nodal segments per shoot and 4.5 nodal segments per shoot respectively.

Bignami (1983) reported a propagation ratio of 8:1 on MS media with 4.4 µM BA compared to the current study having a propagation ratio of 4.7:1 on MS with 2 µM and 4.5:1 on LP with 4 µM. This discrepancy could be due to physiological differences in *R. frangula* that Bignami used and the cultivar 'Ron Williams' used in the current study. Sudersan and Hussain (2003) reported that a low concentration of BA (0.044 to 0.44 µM) with MS nutrient salts were optimal for micropropagation of *Ziziphus spinachristi* (Christ's thorn jujube, *Rhamnaceae*). Conversely, *Hagenia abyssinica* (African redwood, *Rosaceae*), a member of the family *Rosaceae* which is closely related to *Rhamnaceae* obtained a propagation rate of 2.1:1 using WPM with 4.4

µM BA and 0.49 µM IBA (Feyissa et al. 2005). This contradicts with results from this study as WPM by itself was significantly outperformed by both LP and MS media in terms of propagation number. Both *Rhamnus* and *Rhamnaceae* are not a commonly tissue cultured family or genera. As such, more research needs to be done on evaluating different types and concentration of PGRs and different nutrient salt formulations regarding propagation of *R. frangula* and its cultivars.

Nutrient salt formulation, presence of BA, and the factorial combination of nutrient salt formulation with BA had a highly significant effect on average shoot length. (Appendix Table A3). For all main effects, run and replication were not significant and were pooled together for all subsequent analysis. As nutrient salt formulations increased in salts availability, average shoot length significantly increased. This trend is similar to shoot number and propagation number with respect to the results. Suggesting that high salt formulations are the best for shoot production, propagation number, and average shoot length of 'Ron Williams'. MS significantly outperformed LP and WPM with MS having an average shoot length of 1.23 cm, LP having an average shoot length of 0.96 cm, and WPM having an average shoot length of 0.85 cm. (Table 7). In terms of BA concentration, 2 and 4 µM BA significantly outperformed all other treatments having an average shoot length of 1.48 and 1.41 cm respectively (Table 8). These two concentrations similarly outperformed all other treatments in propagation number, while 4 and 6 µM outperformed in shoot production. There was a highly significant 2-way factorial interaction

Table 7. Effect of nutrient salt formulation on 'Ron Williams' (Fine Line®) for average shoot length.

Nutrient Salt Formulation	Mean ASL[1] (cm)
WPM	0.85a
LP	0.96a
MS	1.23b

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

Table 8. Effect of 6-benzylaminopurine (BA) concentration on 'Ron Williams' (Fine Line®) for average shoot length.

BA (µM)	Mean ASL[1] (cm)
0	0.02a
2	1.48c
4	1.41c
6	1.16b

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

with nutrient salt formulation and the present of BA in the culture medium with respect to average shoot length of 'Ron Williams' (Fine Line®) (Appendix Table A3; Table 9; Fig. 4). Two treatments, MS with 2 µM BA and LP with 4 µM BA produced the highest average shoot length as compared to other evaluated treatments. With an average shoot length of 2.07 and 1.6 cm respectively. These results are the same as the previously mentioned shoot production and propagation number.

Table 9. Effect of nutrient salt formulation and 6-benzylaminopurine (BA) concentration interactions on 'Ron Williams' (Fine Line®) for average shoot length.

Nutrient Salt Formulation	BA (µM)	Mean ASL[1] (cm)
WPM	0	0.1a
WPM	2	1.12bc
WPM	4	1.14bc
WPM	6	1.02b
LP	0	0.3a
LP	2	1.17b
LP	4	1.6e
LP	6	1.07bc
MS	0	0.3a
MS	2	2.07e
MS	4	1.48cd
MS	6	1.39d

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

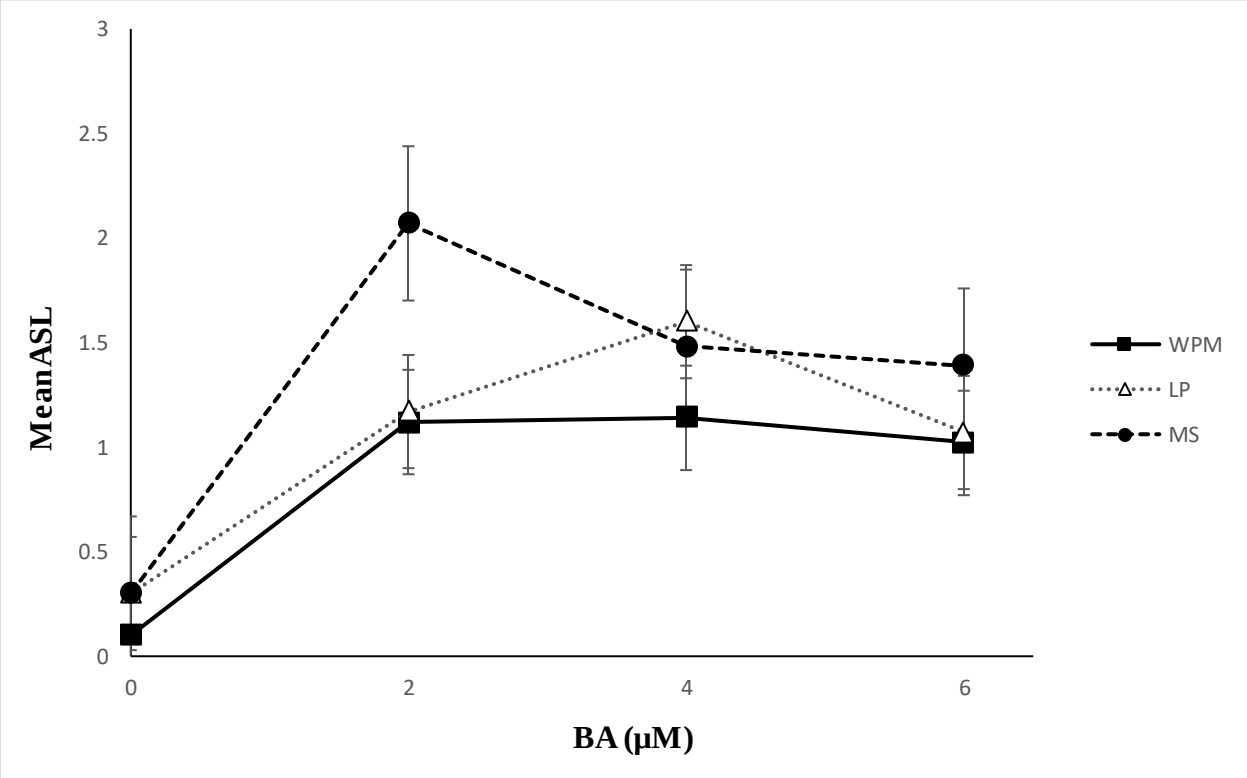

Figure 4. Effect of nutrient salt formulation and 6-benzylaminopurine (BA) concentration interactions on 'Ron Williams' (Fine Line®) for average shoot length.

Sudhersan and Hussain (2003) reported the highest average shoot length of *Ziziphus spina-christi* was obtained via MS and 0 µM BA. However, this treatment also caused 0% of branching to occur as a result. Additionally, the higher the BA concentration, the lower the average shoot length was. As the average shoot length decreased, the higher percentage of branching occurred. This trend did not occur in my experiment as the same treatments that outperformed all others in shoot production and propagation number also outperformed all others in average shoot length. Concentrations of BA beyond the optimal level (2 and 4 µM) appeared to significantly lower the average shoot length (Table 8). This trend is supported by Yepes and Aldwinckle (1994) who reported an increase of BA levels past an optimal level resulted in a decrease of shoot length in *Malus prunifolia* (plumleaf crabapple, *Rosaceae*) cultivars. Haw and Keng (2003) reported the same trend in *Spilanthes acmella* (Szechuan buttons, *Asteraceae*). Due to *Rhamnus* and *Rhamnaceae* not being a commonly tissue cultured genera or family, this current study is unable to conclusively report if these results are typical or abnormal. As such, more research needs to be done on the use of different cytokinins regarding average shoot length of *R. frangula* and its cultivars to see if there's a preference.

There are various potential causes for why 'Asplenifolia' grew poorly when cultured. These can range from the physical qualities of the medium (liquid versus solid), size of explant, light and dark periods, nutrient salt formulation, use of PGRs, and other reasons (Murashige 1974). 'Asplenifolia' was only cultured on a high salt media (MS) and a medium salt media (LP) with two different types of cytokinins (BA and zeatin) and different concentrations were tested (1, 2, 3, and 4 µM). A possible conclusion is that the levels of salt were too high for 'Asplenifolia' to grow. Preece (1995) proposes that with an improper nutrient medium, chances will be very low that explants will respond satisfactory, regardless of the PGR and the

concentrations tested. Another possible strategy would've been to test three different media (a low, medium, and high salt media) (WPM, LP, and MS) to evaluate which media 'Asplenifolia' responds optimally to prior to any testing of PGRs.

3.2. In Vitro Adventitious Root Proliferation

Results indicated that the concentrations of the PGR had a significant effect on both mean root production and mean root length (Appendix Tables A4, A5). For all main effects, run and replication were not significant and were pooled together for all subsequent analysis. As IBA concentration increased, root production and mean root length increased (Table 10, Table 11; Fig. 5). Regarding mean root number, IBA concentration of 2 and 4 µM significantly outperformed other treatments with 2 µM having an average root number of 2.99, 4 µM having an average root number of 2.76, with average root number decreasing as IBA concentration decreased (Table 10). In terms of mean root length, 4 µM IBA significantly outperformed all other concentrations with decreasing root length as IBA concentrations decreased (Table 11).

Table 10. Effect of indole-3-butyric acid (IBA) concentration on 'Ron Williams' (Fine Line®) for mean root number.

IBA (µM)	Mean Root Number[1]
0	0a
1	1.75b
2	2.99c
4	2.76c

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

Table 11. Effect of indole-3-butyric acid (IBA) concentration on 'Ron Williams' (Fine Line®) for mean root length.

IBA (µM)	Mean Root Length[1] (cm)
0	0a
1	2.12b
2	2.37b
4	2.99c

[1] Means with the same letter are not significantly different at $\alpha<0.05$.

Figure 5. Root production of ‘Ron Williams’ after six weeks on rooting media comparing four different concentrations of IBA (from left to right: 0, 1, 2, and 4 µM). Scale bar = 2 cm.

Bignami (1983) reported a best treatment for 100% root production in *Rhamnus frangula* (no cultivars) to be 1/2 strength MS nutrient salts with 0.49 to 1.2 µM IBA. This differs from what was measured in the current study with ‘Ron Williams’, root production was significantly less (65% rooting) when cultured on LP nutrient salts supplemented with 1 µM IBA. The treatment with the highest percentage of rooting (80%) in ‘Ron Williams’ for this experiment was obtained with 4 µM IBA on LP nutrient salts. Additionally, Bignami (1983) noted a trend of IBA concentrations higher than 1.2 µM caused an increased chance for callus production to occur in *R. frangula* before rooting. This callus was unfavorable for *R. frangula* as it rapidly reduced nutrient absorption and turned shoots yellow (Bignami 1983). Callus production did not occur in the current study with ‘Ron Williams’. Also, the study did not report data on mean root number or mean root length, data was only presented on rooting percentage (Bignami 1983). In

this study, as IBA concentration increased, both mean root number and mean root length increased (Table 10, Table 11). This trend is also reported in *Rosa rugosa* (Japanese rose) where it was found that on ½ strength MS, increasing IBA concentration from 0.5 to 5.0 µM increased the number of roots per shoot (Xing et al. 2010). Additionally, the maximum root length and root number of *Prunus dulcis* (sweet almond) was the most significant when cultured on ½ strength MS with 4.92 µM (Kodad et al. 2021). Conversely, ½ MS supplemented with 2.46 µM IBA exhibited the highest average root number and root length for *Rosa hybrida* (Hybrid tea rose) (Maurya et al. 2013).

It is possible that a MS nutrient salt formulation would be better for rooting. This is supported by the best rooting of *Alnus acuminata* (Andean alder) being obtained in MS with 1 µM IBA. It was reported that 88% of *A. acuminata* shoots rooted with 4.6 roots per shoot and had an average root length of 1.5 cm (Enrico et al. 2005). However, WPM could also be utilized instead as reported with the best rooting performance of *Rosa canina* (dog rose) and *Cotoneaster acuminatus* (acuminate cotoneaster) being on WPM medium containing 0.49 µM IBA, with 5.62 roots per explant and the longest roots reaching 4.42 cm (Toma et al. 2014). Future studies should be conducted regarding different types and strengths of nutrient salts on efficiently inducing roots in 'Ron Williams'.

3.3. Measuring Ploidy via Flow Cytometry

Histograms produced using flow cytometry were evaluated to determine the presence or absence of increased ploidy levels for two *R. frangula* cultivars ('Ron Williams' and 'Columnaris'). The two cultivars were treated with three concentrations of oryzalin (100, 200, or 400 µM) and at three different exposure times (1, 2, or 4 days) in aseptic and in vitro conditions. Ten samples for each concentration of oryzalin and exposure time were tested for each *R.*

frangula cultivar. There was a 10% recovery of polyploids in 'Columnaris' based off replicate number per treatment (Table 12) and (Fig. 6). All 'Ron Williams' samples tested resulted in similar histograms to each other (Fig. 7). 'Ron Williams' control samples produced one definitive peak of diploid nuclei (2x) at a Mean PI-A of approximately 33,000 Relative Fluorescent Units (RFU). The samples of 'Ron Williams' treated with oryzalin appeared nearly identical to the controls (Fig. 7). This suggests no increased ploidy on treated samples tested because there were no additional peaks at the histograms of treated plants.

Samples of oryzalin-treated plant material which produced more than one DNA peak in their histograms compared to the control samples were counted as polyploids. Location of the second peak of DNA implies doubling of the DNA profile of the control sample. Which further implies tetraploid conversion. There was only one sample from all the treatments that showed the presence of polyploids, sample 02-09-04 treated with 400 µM of oryzalin and 4-day exposure time (Fig. 6). This suggests that 'Columnaris' is most likely not highly sensitive to oryzalin but polyploid induction is still possible. Sample 02-09-04, displayed two peaks, one diploid (Peak 3) (control, Table 13) and one polyploid from the oryzalin treatment (Peak 4). These two peaks are estimated in Mean PI-A at 34,148 and 64,430 RFU (Table 14). Regarding the 'Columnaris' control, the number of events in P3 (3629) and P4 (163) are vastly different from each other. Even though Mean PI-A doubled between P3 (47,464 RFU) and P4 (89907 RFU), it is not evidence of polyploidy due to large difference in the number of events between the two peaks (Table 13). As well as the wide difference in the percent total between P3 (72.58%) and P4 (3.26%). The percent total is the percentage of events in the peak. For polyploidy to be confirmed, both the number of events and percent total need to be similar to each other in both peaks along with a doubling of Mean PI-A between the two peaks. Sample 02-09-04 had a

similar number of events in P3 (1270) and P4 (1242) (Table 8). Additionally, the percent total was similar in P3 (25.4%) and P4 (24.84%). Alongside the doubling of Mean PI-A between P3 (34,148 RFU) and P4 (64,430 RFU), Sample 02-09-04 was confirmed as a polyploid. There was no data analysis utilizing statistical software done due to low induction rates across the oryzalin treatments evaluated.

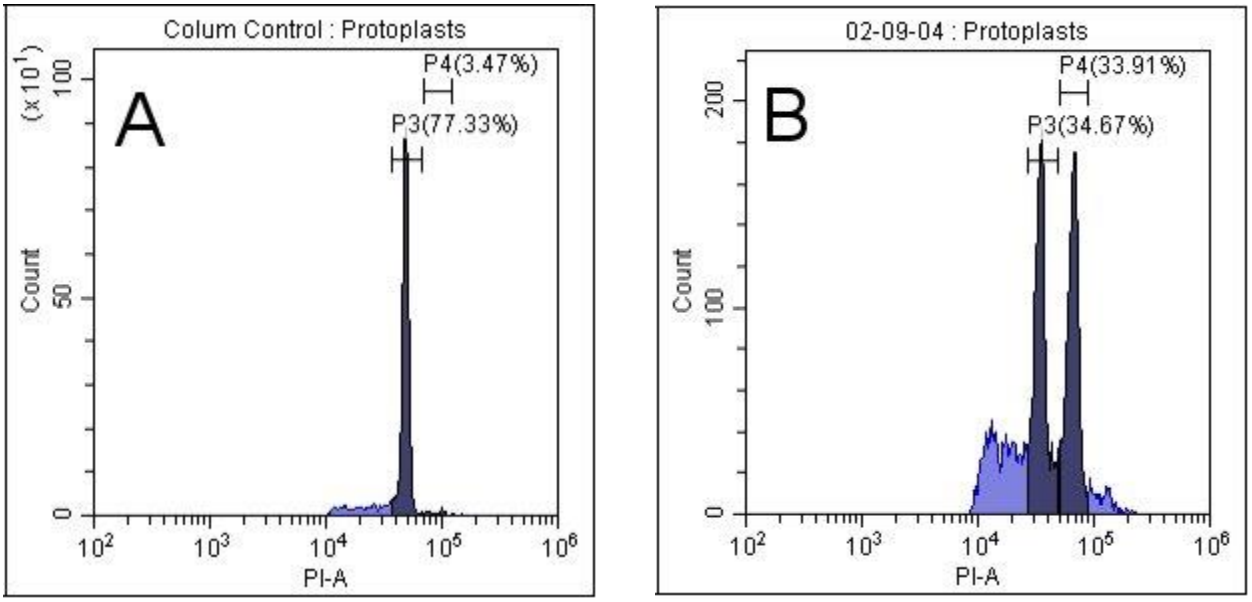

Figure 6. Flow cytometry histograms for a successful polyploid of 'Columnaris'. A) 0 µM Oryzalin control, B) Control (left peak, P3) plus polyploid (right peak, P4) treated with 400 µM Oryzalin and 4 days of exposure time.

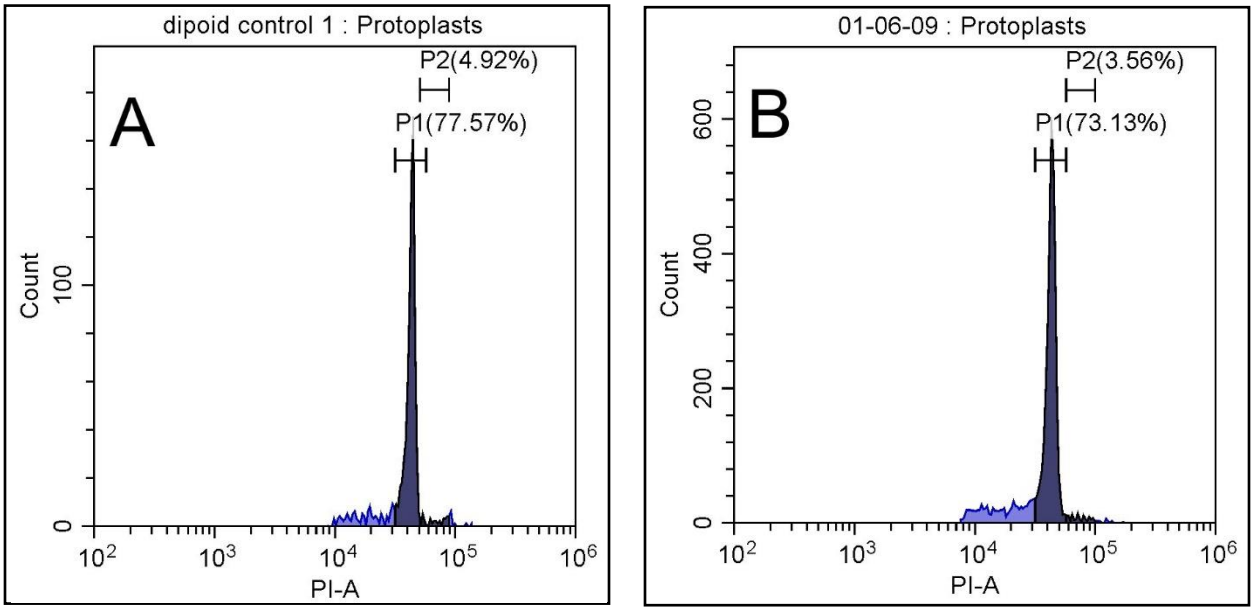

Figure 7. Flow cytometry histograms for unsuccessful polyploid of 'Ron Williams'. A) 0 µM Oryzalin control (left P1), B) Treatment with 400 µM Oryzalin and 4 days of exposure time (right P1).

Table 12. Outputs from flow cytometry to determine the presence or absence of increased ploidy for each *R. frangula* cultivar.

Cultivar	No. of Replicates	Oryzalin Concentration (µM)	Exposure Time (Days)	Ploidy Induction %
Ron Williams	10	100	1	0a
	10	100	2	0a
	10	100	4	0a
	10	200	1	0a
	10	200	2	0a
	10	200	4	0a
	10	400	1	0a
	10	400	2	0a
	10	400	4	0a
Columnaris	10	100	1	0a
	10	100	2	0a
	10	100	4	0a
	10	200	1	0a
	10	200	2	0a
	10	200	4	0a
	10	400	1	0a
	10	400	2	0a
	10	400	4	10b

Table 13. Histogram statistics for 'Columnaris' control (not treated with Oryzalin).

Tube Name	'Columnaris' Control					
Population	Events	% Total	% Parent	Mean PI-A	CV PI-A	Median PI-A
All Events[1]	5000	100.00 %	100.00 %	50987.9	193.85 %	47132
P3	3629	72.58%	77.33%	47464.4	7.45%	47504
P4	163	3.26%	3.47%	89907.2	14.80%	91264.2

[1]No increase in ploidy level noted in mean PI-A between Peak 3 (P3) and Peak 4 (P4) due to low event number in P4.

Table 14. Histogram statistics for ‘Columnaris’ polyploid showing an increase of ploidy level in mean PI-A of P3 and P4.

Tube Name	2-09-04					
Population	Events	% Total	% Parent	Mean PI-A	CV PI-A	Median PI-A
All Events	5000	100.00%	100.00%	78295.5	164.09%	50540.1
P3	1270	25.40%	34.67%	34148.8	12.46%	33735.3
P4	1242	24.84%	33.91%	64430.7	10.63%	64577.9

Although polyploidy was confirmed in one of the three oryzalin concentrations and time exposure between both cultivars, the current tested method of oryzalin application via aseptic conditions (tissue culture) and over multiple exposure times in *R. frangula* tetraploid production needs further research to develop a more efficient protocol. There was only a 10% recovery rate for the highest concentration of oryzalin and highest exposure time. The only successful polyploid demonstrated growth with reduced vigor and only produced one sufficient shoot. As such, establishing a stock population of ‘Columnaris’ polyploids would be difficult and time consuming. With the creation of a possibly more efficient protocol, there could be an increase in recovery rate and successful induction of polyploidy. Another possible strategy for polyploid induction would be to evaluate the efficacy of a foliar oryzalin application on greenhouse-grown plants.

3.4. Conclusions

Two out of three *Rhamnus frangula* cultivars (‘Ron Williams’ and ‘Columnaris’) were successfully propagated in vitro from axillary nodal explants. However, only ‘Ron Williams’ was able to be utilized for the axillary shoot proliferation experiment and rooting experiment. ‘Asplenifolia’ was not utilized for experiments due to poor growth when cultured. ‘Columnaris’ was not used for the axillary shoot proliferation experiment and rooting experiment due to not enough nodal cultures to meet the population size needed for the studies. The use of either 2 µM

BA with MS nutrient salt medium formulation or 4 µM BA with solid LP nutrient salt medium formulation resulted in the best shoot production, highest propagation number, and highest average shoot length for ‘Ron Williams’ as compared to the other treatments in this study. For the highest number of roots and root length in ‘Ron Williams’, 4 µM IBA on LP nutrient salt medium formulation was found to outperform all other IBA concentrations compared in this study. Polyploids can successfully be induced in ‘Columnaris’ using 400 µM of oryzalin for an exposure time of 4 days. More research needs to be conducted to further evaluate polyploid induction for ‘Columnaris’ and to increase the efficiency of oryzalin induced polyploid induction. This study suggests that both micropropagation and polyploid induction is possible for ‘Ron Williams’ and ‘Columnaris’ and can assist in the potential breeding and creation of sterile triploid cultivars of *R. frangula*.

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APPENDIX

Table A1. SAS GLM output using all nutrient salts, BA concentrations, and NS*BA. Dependent variable: Shoot number.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	232.4153243	11.0673964	16.81	<.0001
Error	153	100.7046757	0.6582005		
Corrected Total	174	333.12			

R-Square	Coeff Var	Root MSE	SHOOTN Mean
0.697692	56.33997	0.811296	1.44

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
Run	1	0.1546405	0.1546405	0.23	0.6286
Rep	9	2.3069429	0.256327	0.39	0.9388
Nutrient Salt	2	13.9152739	6.9576369	10.57	<.0001
BA	3	187.0872466	62.3624155	94.75	<.0001
Nutrient Salt*BA	6	28.9512204	4.8252034	7.33	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
Run	1	0.1033594	0.1033594	0.16	0.6925
Rep	9	3.3706144	0.3745127	0.57	0.8209
Nutrient Salt	2	9.2677601	4.63388	7.04	0.0012
BA	3	168.9859077	56.3286359	85.58	<.0001
Nutrient Salt*BA	6	28.9512204	4.8252034	7.33	<.0001

Table A2. SAS GLM output using all nutrient salts, BA concentrations, and NS*BA. Dependent variable: Propagation number.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	510.907508	24.328929	10.56	<.0001
Error	153	352.4867777	2.3038351		
Corrected Total	174	863.3942857			

R-Square	Coeff Var	Root MSE	PROPN Mean
0.591743	85.96175	1.517839	1.765714

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
Run	1	0.1942857	0.1942857	0.08	0.7719
Rep	9	12.5261966	1.3917996	0.6	0.7921
Nutrient Salt	2	69.7625642	34.8812821	15.14	<.0001
BA	3	295.5507817	98.5169272	42.76	<.0001
Nutrient Salt*BA	6	132.8736797	22.1456133	9.61	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
Run	1	0.0628563	0.0628563	0.03	0.869
Rep	9	14.7541253	1.6393473	0.71	0.6977
Nutrient Salt	2	60.1795979	30.0897989	13.06	<.0001
BA	3	244.6163503	81.5387834	35.39	<.0001
Nutrient Salt*BA	6	132.8736797	22.1456133	9.61	<.0001

Table A3. SAS GLM output using all nutrient salts, BA concentrations, and NS*BA. Dependent variable: Average shoot length.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	91.9288327	4.3775635	22.17	<.0001
Error	153	30.210761	0.197456		
Corrected Total	174	122.1395937			

R-Square	Coeff Var	Root MSE	ASL Mean
0.752654	47.47003	0.44436	0.936086

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
Run	1	0.4792837	0.4792837	2.43	0.1213
Rep	9	1.93390235	0.21487804	1.09	0.3746
Nutrient Salt	2	6.05699491	3.02849746	15.34	<.0001
BA	3	76.95439761	25.65146587	129.91	<.0001
Nutrient Salt*BA	6	6.50425411	1.08404235	5.49	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
Run	1	0.46202548	0.46202548	2.34	0.1282
Rep	9	2.06336493	0.22926277	1.16	0.3239
Nutrient Salt	2	4.23884767	2.11942384	10.73	<.0001
BA	3	71.37286498	23.79095499	120.49	<.0001
Nutrient Salt*BA	6	6.50425411	1.08404235	5.49	<.0001

Table A4. SAS GLM output using IBA concentration. Dependent variable: Average root number.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	106.1049101	8.1619162	9.92	<.0001
Error	49	40.3077883	0.8226079		
Corrected Total	62	146.4126984			

R-Square	Coeff Var	Root MSE	ROOTN Mean
0.724697	52.42163	0.906977	1.730159

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
Run	1	0.27936508	0.27936508	0.34	0.8422
Rep	9	5.93673891	0.65963766	0.8	0.903
IBA	3	99.8888061	33.2962687	40.48	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
Run	1	1.10805512	1.10805512	1.35	0.2514
Rep	9	4.46151062	0.4957234	0.6	0.7886
IBA	3	99.8888061	33.2962687	40.48	<.0001

Table A5. SAS GLM output using IBA concentration. Dependent variable: Average root length.

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	94.2353574	7.2488736	21.12	<.0001
Error	49	16.8173315	0.3432108		
Corrected Total	62	111.0526889			

R-Square	Coeff Var	Root MSE	ROOTN Mean
0.848564	34.01663	0.585842	1.722222

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
Run	1	0.00441283	0.00441283	0.01	0.9102
Rep	9	1.29438016	0.14382002	0.42	0.9187
IBA	3	92.93656442	30.97885481	90.26	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
Run	1	0.19138817	0.19138817	0.56	0.4588
Rep	9	0.88180842	0.09797871	0.29	0.9757
IBA	3	92.93656442	30.97885481	90.26	<.0001