DEVELOPING MICROPROPAGATION PROTOCOL FOR *EUONYMUS* SPECIES AND EVALUATING ORYZALIN TREATMENTS FOR POLYPLOID DEVELOPMENT

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Developing Micropropagation Protocol for Euonymus Species 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

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ABSTRACT

Euonymus has invasive traits such as high seed production and good seed viability. Development of sterile, polyploid euonymus cultivars could prevent euonymus from invading native ecosystems. Many common euonymus cultivars are diploid. Oryzalin is a commonly used mutagen for chromosome doubling which disrupts the function of cellular microtubules to produce cells with additional sets of chromosomes. Chromosome doubling in euonymus is an understudied area. There is also a lack of published information in the literature about asexual propagation, specifically micropropagation, with respect to several species within this genus. The aims of this research were to develop a micropropagation protocol and to establish a protocol for inducing polyploids (tetraploids) for two euonymus species, *E. bungeanus* and *E. turkestanicus*. Micropropagation was successful with both species. The addition of 1 µM of BA to the MS culture medium performed better for *E. turkestanicus* with respect to axillary shoot production and propagation number. For propagation number, MS with 1 or 2 µM BA outperformed the other treatments for both species. Adventitious roots were successfully produced with the addition of IBA, but root initiation was not consistent with the evaluated treatment concentrations. Oryzalin was successful in polyploid induction of euonymus. The presence of tetraploids in *E. turkestanicus* using 50 µM and 100 µM of oryzalin was confirmed using flow cytometry techniques. No tetraploids were detected with *E. bungeanus* suggesting that the concentrations of oryzalin were not effective and may require increased concentrations for polyploid induction.

ACKNOWLEDGMENT

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DEDICATION

To my dear husband Samir Hammal, thank you for giving me the support to reach my dream, accomplishing this would hopefully make you proud of me as much as I am proud of having you as my soulmate.

To my child Anir Hammal, thank you for being the shining gleam in the sky of dark times, you were my motivation to be the best version of myself during this study.

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1. INTRODUCTION

Euonymus is a genus of about 175 species of shrubs, woody climbers, and small trees, in the staff tree family *Celastraceae*. Members of the *Euonymus* genera are commonly referred to as euonymus. Euonymus is originally from northeastern Asia including most of the provinces of China (Ding et al., 2006). It was introduced into the United States during the 1860s and was quickly recognized as being a desirable horticultural specimen exhibiting an attractive growth habit, autumn leaves of brilliant red color, dark purple or dark green with white veins, and attractive fruits (Dirr, 2009; Ebinger, 1996).

Euonymus species can be deciduous or evergreen shrub or small trees, generally with superb fall color, the inconspicuous flowers occur in small groups, and can be green, yellow, pink or maroon in color depending on species. The leaves have opposite (rarely alternate) arrangement and are simple ovoid, typically 2 to 15 cm long, and usually with a finely serrated margin. The fruit is a pink or white four- or five-valved pod-like berry, which splits open to reveal the fleshy-coated orange or red seeds. Species of euonymus are highly versatile, excellent for hedging, as specimen plants, for borders and screening. Easy to grow, they tolerate most soils and can grow in full sun or heavy shades (Burnie, 2004).

Euonymus species have medicinal importance whereas more than 230 chemical constituents have been isolated and identified from *E. alatus* (winged euonymus or burning bush), including sesquiterpenoids, diterpenoids, triterpenoids, flavonoids, phenylpropanoids, lignans, steroids, alkaloids and other compounds (Fan et al., 2020). Numerous studies also have shown that extracts and compounds from *E. alatus* exert a wide spectrum of pharmacological effects, including antidiabetic effect, anti-tumor effects, anti-inflammatory effects, hepatoprotective effects, antioxidant effects, antibacterial effects, as well as other effects (Fan et

al., 2020). *Euonymus alatus* is considered to elicit various beneficial effects against cancer, hyperglycemia, menstrual discomfort, diabetic complications, and detoxification (Woo et al., 2020). The young leaves of this plant are utilized as food and for traditional medicine in East Asian countries, including Korea and China (Woo et al., 2020), the same study demonstrated that ethanolic extract from the *E. alatus* leaf (EAE) exhibited the strongest antioxidant enzyme-inducing activity among more than 100 kinds of edible tree leaf extracts.

Euonymus japonicus, also called Japanese spindle tree, is one of the most prevalent and important species in *Celastraceae* in northern Chinese cities such as Beijing, the capital of China. As a species of evergreen shrub, *E. japonicus* has strong resistance to the dry and cold conditions in Beijing and can efficiently reduce particulate matter in winter. Furthermore, roots, stems, and leaves of the shrub have a high capacity to enrich heavy metals (Lin et al., 2023). Also, Cai et al. (2022) indicated that *E. japonicus* leaves have certain cold resistance and can maintain their viability during wintering. Compared to other *Celastraceae* species, the variation in the chloroplast genome sequence was lower, and the gene structure was more stable. A total of 11 functional positive selected genes were identified, which may have played an important role in the process of *Celastraceae* species adapting to environmental changes (Cai et al., 2022).

Several species of euonymus are important members in landscaping generally because of their outstanding fall foliage color typically ranging from pink to red. Two such species, *Euonymus bungeanus* (winterberry euonymus) and *Euonymus turkestanicus* (dwarf euonymus) have unique ornamental pink capsuled fruits along with outstanding fall foliage color (Eisel and Gaudett, 1974).

Euonymus bungeanus is a small tree or large rounded shrub that grows to 4 to 7 m tall with semi-pendulous branches (Dirr, 2009). Fall leaf colors may be muted, depending on

location but are often in the yellow, pink to bronze range. Showy pink fruits, opening to expose reddish seeds. Attractive gray bark. Used for patio trees, border plantings, screen plantings, or single specimen. Wood is used in making pipestems, charcoal and gunpowder. Medicinally, used as a laxative, appetite stimulant, hepatic stimulant, heart medicine, and diuretic (TLC Garden Centers, 2023).

Euonymus turkestanicus is a sprawling, somewhat upright shrub that grows to 1 m tall with narrow semi-evergreen leaves that may turn reddish purple in fall; main attribute is showy orange and red seeds in late summer and fall (Dirr, 2009). This shrub performs well in both full sun and full shade. It is very adaptable to both dry and moist locations and does fine under typical garden conditions. It is not particular as to soil type or pH. It is highly tolerant of urban pollution and will even thrive in inner city environments (TLC Garden Centers, 2023).

Plant propagation is the multiplication of plant material to form new plants (Davies et al., 2018). It is the process by which new plants grow from a variety of sources: seeds, cuttings, and other plant parts. Plant propagation can be divided into two basic types: sexual (seeds) and asexual (vegetative, non-seed or apomictic). Seeds are typically produced from sexual reproduction within a species because genetic recombination has occurred. A plant grown from seeds may have different characteristics from its parents. Asexual or vegetative propagation involves the use of plants that are produced using material from a single parent and as such, there is no exchange of genetic material. Vegetative propagation methods generally produce plants that are identical to the parent and typically uses plant parts such as roots, stems, and leaves. Asexual propagation allows for genetic cloning of a parent plant to retain all its best characteristics in its offspring which is essential when working with a cultivar. The three most

common asexual propagation methods for woody plants include cuttings, grafting, and tissue culture (micropropagation) (Chauhan et al., 2021).

Production of genetically identical copies of a plant without the utilization of a sex organ is called clonal propagation. The most widely used “ex vitro” methods of cloning agricultural crops include cuttings of vegetative parts, layering, grafting and budding. Clonal propagation through tissue culture is popularly known as micropropagation was initiated by G. Morel (1960) who found this as the only commercially viable approach for orchid propagation. Since then, several crop species have been micropropagated and protocols are now available which can be adopted by growers trained in aseptic manipulation and plant husbandry. Clonal propagation “in vitro” appears to have a permanent advantage in cases in which serious problems with disease occur. This is because through “in vitro” propagation methods, such as meristematic culture, the propagation unit (propagule) is genetically identical and virus-free plants can be produced and maintained economically, which is important to the horticulture trade. The primary method of increase in woody plant micropropagation is by axillary shoot proliferation. There are concern issues with micropropagation including adventitious shoot proliferation which may occur and produce genetic variation off-types. Axillary shoot proliferation is preferred to avoid the potential of these off-types (somaclonal variation) from occurring (Suttle, 1996).

Micropropagation is used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds or does not respond well to other asexual propagation methods (Kehie et al., 2012).

Euonymus propagation can be achieved utilizing seeds, cuttings and micropropagation. Both species in this project, winterberry and dwarf euonymus, can be propagated by seeds (non-

clonal and not with cultivars) and cuttings (clonal and with cultivars) but there is no published information about micropropagation for those two species, although some resources include tissue culture propagation for *E. bungeanus* through adventitious buds but not through actively growing axillary buds (Ning et al., 2020).

Nutrient media for plant tissue culture are designed to enable explants to grow in a totally artificial environment. For plants to grow in vitro, scientists have devised growth media that provide the nutrients that are usually available in soil. Plant tissues and organs are grown in vitro on artificial media, which supply the nutrients necessary for growth. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used (George et al., 2007). Each plant species has its own characteristic elementary composition which can be used to adapt the medium formulation. These media result often in a much-improved growth (Rugini, 1984; Pullman et al., 2003; Bouman and Tiekstra, 2005; Nas and Read, 2004; Gonçalves et al., 2005). Plant tissue culture media provide not only these inorganic nutrients, but usually a carbohydrate (sucrose is most common) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. To improve growth, many media also include trace amounts of certain organic compounds, notably vitamins, and plant growth regulators (George et al., 2007). Ramage and Williams (2002) also argue that minerals appear to have an important role in the regulation of plant morphogenesis as opposed to just growth.

There are different commonly available tissue culture media recipes. Some are high in salt such as Murashige and Skoog medium (MS; Murashige and Skoog, 1962) and Kuniyaki Walnut (DKW; Driver and Suttle, 1987), some are medium in salt such as LP medium (average of DKW and WPM; Preece et al., 1995), and some are low in salt such as Woody Plant Medium (WPM; Lloyd and McCown, 1980). In addition to mineral elements that make up the macro-and

micronutrients present in soils and fertilizers, nutrient media also contain organic compounds such as vitamins, PGRs and a carbon source. Macronutrients consist of calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K) and sulfur (S). Micronutrients used in tissue culture consist of boron (B), cobalt (Co), copper (Cu), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn). A variety of organic compounds are added to plant culture media. Such as sugars, are required for growth, whereas others, such as vitamins, undefined compounds, and organic acids, may be useful because they enhance growth significantly.

Supporting systems are required to keep plant culture from being submerged in the medium. Liquid medium is used for many plants, but it must often be agitated to provide sufficient oxygen. Among supporting systems are gelling agents, such as agar, Gelrite® (gellan gum), agarose and gelatin, and mechanical supports, such as filter paper bridges and a variety of commercially available polypropylene membrane raft systems ((Trigiano and Gray, 2018)).

Plant tissue culture is generally dependent for its success on the inclusion of plant hormones and plant growth regulators (PGRs) and/or other growth active substances, as one of the main required substances, in the medium (Gamborg et al., 1976). The major classes of PGRs used in tissue culture are auxin, cytokinin, gibberellins and abscisic acid. Cytokinins such as benzylaminopurine (BA) and kinetin are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants in banana (Madhulatha et al., 2004). BA has a marked effect in stimulating the growth of axillary and adventitious buds and foliar development of shoot tip cultures (Abeyarante and Lathiff, 2001; Buah et al., 2010). The concentration of PGRs range from 0.1 to 100 µM depending on the specific PGR and the situation in which it is used ((Trigiano and Gray, 2018)).

Most tissue cultures are grown at pH 5.2 to 5.8 with pH adjustments being made prior to autoclaving. There are significant differences between initial pH levels and pH levels following autoclaving, particularly in the pH range of 5.7 to 8.5 (Skirvin et al, 1986).

Tissue culture will be analyzed and the best micropropagation protocol for this species will be determined and the best treatment for polyploid development will be evaluated. The results of these experiments will be relayed to nursery producers to expedite this superior tree into the market. Incorporating this plant adds biodiversity, increases aesthetics of areas that would otherwise not be well suited to growing Euonymus, and has potential to be a strong seller as an important member in landscaping in North Dakota and across the country.

Euonymus has been classified as an invasive plant due to invasive traits such as high seed production and good seed viability, seed is dispersed by birds and other berry feeding animals over great distances, easy vegetative or asexual reproduction, predator avoidance and shade/sun tolerance (Dirr, 1990; Searcy et al., 2007) and therefore the importation, distribution, trade, and sale of euonymus have been banned in several states in the United States.

The use of non-native species has the potential to contribute to invasive plant introductions (Reichard and White, 2001). To reduce the risk of invasiveness for new nursery crops derived from non-native species, ornamental plant breeders are developing methodologies and breeding strategies to create non-invasive cultivars for the nursery industry. These strategies include developing polyploids that are sterile which are often triploids. Triploids are obtained by developing a tetraploid (polyploid) from the plant of interest and backcrossing it to the original plant. Various methods have been developed in woody crops for inducing polyploidy, most involving somatic polyploidization of meristematic tissue, including the application of mitotic inhibitors to actively growing buds in vivo (Ackerman and Dermen, 1972; Olsen et al., 2006;

Pryor and Frazier, 1968) or in vitro treatment of microshoots or nodal explants (Bouvier et al., 1994; Väinölä, 2000). There are negative effects to polyploid development. Park et al (2002) reported that induced autotetraploid grapes have often shown poor fruitfulness, low vigor, brittle shoots and decreased cold hardiness.

Oryzalin (4-(dipropylamino)-3,5-dinitrobenzenesulfonamide) is a chemical that has shown to be effective with polyploid induction. Oryzalin is a mitotic inhibitor that has been used for polyploidization of woody plants, when used at concentrations ranging from 5 to 300 M (Bouvier et al., 1994, 2002; Olsen et al., 2006; Väinölä, 2000). Oryzalin is a dinitroaniline herbicide that acts as a chromosome doubling mutagen by inhibiting the cellular function of microtubular proteins (Bartels and Hilton, 1973). Microtubules are the cell's apparatus for pulling copied chromosomes apart during the process of cell division or mitosis, so disruption leads to cells that have twice their typical sets of chromosomes. Two common methods of applying oryzalin to plant tissues are through tissue culture exposure or through foliar applications to meristematic tissues (Ascough et al., 2008). After a 4 to 6-week period in tissue culture, plants can be further divided into smaller segments and transferred into additional media, in a process called subculturing. Once a plant has been subcultured and is acclimated to tissue culture conditions, additional components may be added to the media, such as mutagens like oryzalin that induce chromosome doubling. For example, Väinölä (2000) placed subcultured segments of *Rhodendron* hybrids into a liquid plant tissue culture media with oryzalin to induce the production of polyploid plants. While polyploid plants have been created in other woody taxa in the family *Fabaceae* (Harbard et al., 2012), there are no published reports for inducing polyploidy in *Euonymus* spp. Therefore, a micropropagation protocol is essential to evaluate in vitro oryzalin treatments with subsequent microshoot recovery.

One of the methods to determine a plant's ploidy level is a process called flow cytometry. This uses a specialized machine called a flow cytometer to measure sizes of a cell's chromosomes (Galbraith et al., 1983). First, a suspension of single cells must be created. This can prove to be difficult as plant cells are arranged mostly in tissues, with each cell surrounded by a thick cell wall. Removal of this cell wall (which creates cells called "protoplasts") can complete this first necessary step but is typically an inefficient and cumbersome process. Instead, plant tissues (such as roots or leaves) can be placed under ice, chopped finely with a razor blade, and dissolved in a buffer. This solution is then filtered through nylon, leaving only small cell fragments, such as the plant's nuclei. The flow cytometer analyzes the nuclei by use of a laser, which shines through each individual nucleus as it passes through the machine in a single-file stream. The light reflected off the nuclei is then used to estimate the size of the chromosomes within, which can be used to estimate the ploidy level of the organism (Galbraith et al., 2021). Results then must be properly analyzed to include only the particles relevant to ploidy size, excluding other organelles or debris that may be present in the suspension. Finally, samples analyzed through flow cytometry only reveal the size of a given nucleus; a comparison must then be made from another, previously analyzed sample to determine an accurate estimate of ploidy level (Galbraith et al., 2021).

1.1 Research Objectives

The objectives of this research project were:

1. to develop micropropagation protocol for *Euonymus* spp. by evaluating nutrient salt formulations, plant growth regulators, and concentrations,
2. and to determine if oryzalin can be utilized for polyploid production in *Euonymus*.

2. MATERIALS AND METHODS

2.1 Source of Explant Materials for In Vitro and Polyploid Induction Experiments

Euonymus spp. (*E. bungeanus* and *E. turkestanicus*) parent plants were located at the North Dakota State University main campus (NDSU; Fargo, ND, USA) and were utilized as the primary source of plant materials for this study. Both species were evaluated and compared in each of the subsequent experiments.

2.2 Aseptic Nutrient Media and Proliferation Stock Cultures

Nodal explants from soft wood cuttings from parent plants of the two species were placed in vitro in early spring 2021. Each nodal segment consisted of a stem segment that was approximately 2 ± 0.5 cm in length and consisting of two axillary buds being present at each node. All nodal explants were surface sterilized for 15 minutes in a 10% (v/v) Clorox® (0.75% NaClO) bleach solution plus 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween® 20) surfactant with three subsequent 2 min sterile deionized distilled water (ddH_20) rinses. After disinfestation, all explants were freshly cut under sterile conditions using a surgical-grade scalpel. Using a long-tipped forceps, Nodal segments were placed onto a nutrient medium. All nutrient media contained Murashige and Skoog (MS) minerals and vitamins (Murashige and Skoog 1962), 3% (w/v) sucrose, and 2 µM 6-benzylaminopurine (BA). The pH was adjusted to 5.8 ± 0.1 with 1.0 N KOH or 1.0 N HCl prior to adding 7.0 g L^{-1} plant tissue culture grade agar (Phytotechnology Labs; Shawnee Mission, KS, USA), dispensing into 25 x 150 mm borosilicate glass culture tubes (Pyrex®, No. 9820) capped with autoclavable plastic lids, and autoclaving for 20 min at 121 °C and 1.0 kg cm^{-2}. In vitro nodal segment cultures were placed ~30 cm beneath cool white fluorescent lamps that provided a photosynthetic photon flux of ~40 µmol m^{-2} s^{-1} for a 16 h photoperiod at 25 ± 3 °C. Microshoots that developed from nodal explants were subcultured

every 4 weeks to establish a stock population of aseptic plant material for use in subsequent experiments.

2.3 Axillary Shoot Proliferation Micropropagation Experiment

The micropropagation experiment consisted of a factorial design comparing the effect on growth using different nutrient salt solutions ranging from high to medium salt formulations with selected cytokinin (6-benzylaminopurine, BA) plant growth regulators (PGRs) at varying concentrations. Nodal segments utilized in this experiment were excised from proliferation stock cultures. Nodal segments (consisting of one node containing two axillary buds and approximately one cm long in length) were inserted into the different media treatments media under aseptic conditions in a laminar flow hood.

One cytokinin form, 6-benzylaminopurine (BA), was evaluated. The effect of BA concentration (0, 1, 2, and 4 μM) was also measured. The nutrient salt solutions used in this experiment varied in strength from medium to high concentration. Murashige and Skoog (MS) and Driver and Kuniyuk Walnut medium (DKW) represent formulations high concentrations. Long and Preece medium (LP) represents a medium strength concentration.

There was a minimum of five replicates of each treatment. After 12 weeks (two 6-week subculture periods) on the different treatments, shoot length, shoot number and propagation number were measured to determine the factorial effects of nutrient salts, cytokinin, and cytokinin concentration. Propagation number is a factor of both the total shoot number and total shoot length. Propagation number factors the number of "nodal segments" that can be produced per treatment. Reporting just shoot length and number does not give an accurate measurement on if a treatment produced significant number of propagules (propagation units). If shoots are

elongated, fewer nodal segments may result. Similarly, if multiple shoots are produced but are not adequately elongated, few propagules are produced.

2.4 Microshoot In Vitro Rooting Experiment

Maintained elongated microshoots were excised under sterile conditions with a surgical-grade scalpel. Using long-tipped forceps, ~2 cm meristematic microshoots were placed in 15 mL x 150 borosilicate Pyrex® (No. 9820) culture tubes, containing treatment compositions, upright and vertically, 5 mm below axillary buds. Culture tubes were incubated 30 cm below cool white florescent lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod at 25ºC for 4 weeks.

Initiates were excised from elongated microshoots on MS basal salts and nutrients, with the addition of 4 different concentrations (0, 1, 2 and 4 µM) of indole-3-butyric acid (IBA), 30g of sucrose, 7.0 g L^{-1} agar (No. A111; PhytoTech Labs, Lenexa, KS, USA), with the pH adjusted using a pH meter (Accumet® Basic, AB15; Fisher Scientific, Hampton, NH, USA) to 5.7 ± 0.01 with 1.0 N NaOH.

There was a minimum of five replicates of each treatment. After 4 weeks, root number and average root length were measured to determine the factorial effects of IBA concentrations on in vitro rooting.

2.5 In vitro Polyploid Induction – Oryzalin Treatment

An herbicide, oryzalin, was evaluated for polyploid induction. The source of oryzalin was from a commercially available herbicide registered as Weed Impede® (Oryzalin, Monterey Lawn & Garden; Fresno, California, USA). Four different concentrations of oryzalin were evaluated: 25, 50, 100 and 200 µM. 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, FL, USA) was added at a rate of 1 ml L^{-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 flasks with sealable lids. Plant tissue, consisted of nodal segments, were excised from elongated microshoots of *E. turkestanicus* and *E. bungeanus* and exposed to 4 different concentrations (25, 50, 100 and 200 µM) of oryzalin placed in a shaker for 1, 2 and 4 days. A minimum of 10 nodal segments were used in each of the oryzalin concentration treatments. After either 1, 2 or 4 days, nodal segments were removed from the oryzalin concentration treatments, rinsed with sterile ddH_20 and placed vertically into glass culture tubes (15 x 150 ml; Pyrex®, No. 9820) containing growth medium consisting of MS nutrients, 2 µM BA, 30 g sucrose, 7 g of agar and pH of 5.7. Culture tubes were incubated 30 cm below cool white florescent lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod at 25ºC for 6 weeks. After 6 weeks, cultures were placed onto fresh growth medium to allow for the continued growth of the oryzalin exposed axillary buds into microshoots.

The factorial of oryzalin concentration and exposure time were evaluated for polyploid induction. This was done by utilizing flow cytometry. Oryzalin exposed microshoots from each concentration treatment including non-exposed (control) were sent to Next Stage Labs (Kalamazoo, MI, USA) for flow cytometry polyploid analysis. Leaf tissue from each of the exposed and non-exposed microshoots were used to prepare nuclei solutions for determining ploidy levels. Samples with increased ploidy levels were determined by comparing histograms with the non-exposed control samples. Histograms of samples which produced multiple peaks of

DNA (representing presence of both non-exposed control and polyploid sample) were counted as polyploid.

2.6 Experimental Design, Data Collection and Statistical Analysis

All experiments were arranged as random complete block designs and conducted twice (two runs). There was a minimum of five replications per experimental unit unless otherwise noted. All data were analyzed using the General Linear Model (GLM) of SAS 9.4 (SAS Institute, Cary, NC, USA). Mean separation analysis used the Least Square Means (LSMeans) method for comparison.

3. RESULTS AND DISCUSSION

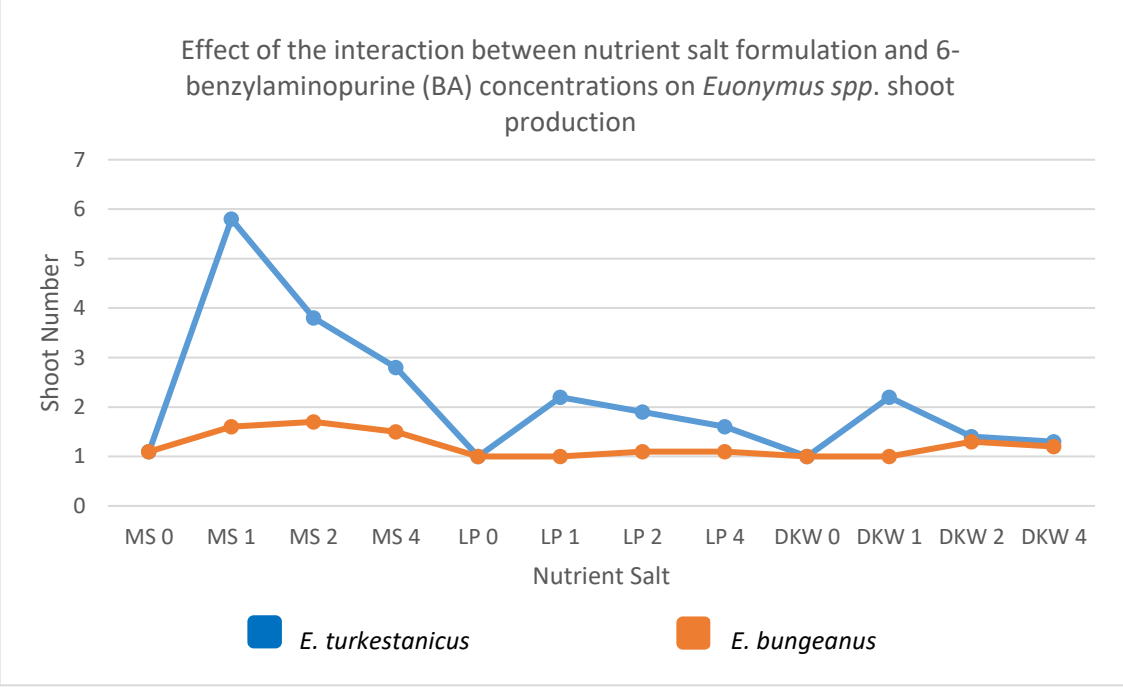
3.1 Evaluating Nutrient Salt Formulations, Plant Growth Regulators and Concentrations for Micropropagation Protocol

There was a significant 2-way interaction between the nutrient salt formulation and BA concentration with MS and 1 µM BA producing the highest propagation numbers for both species. For shoot production, the 2-way interaction between the nutrient salt formulation and BA concentration was only significant for *E. turkestanicus* (Appendix Tables A-1, A-2, A-3, Table 1). Gerald et al. (2006) reported similar findings for *Celastrus paniculatus*, another genera in the same family (Celastraceae) as Euonymus, that maximum in vitro shoot induction (five shoots per explant) was achieved with MS medium supplemented with 1.5 mg L^{-1} (6.6 µM) BA and 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA). The BA concentration was significantly higher with *C. paniculatus* but PGR response often differs among species. It seems from data analysis that *E. bungeanus* is more sensitive to BA than *E. turkestanicus*. As the PGR increased to 4 µM of BA, depending upon nutrient salt, the propagation number decreased. (Table 1), (Graph 1 and 2).

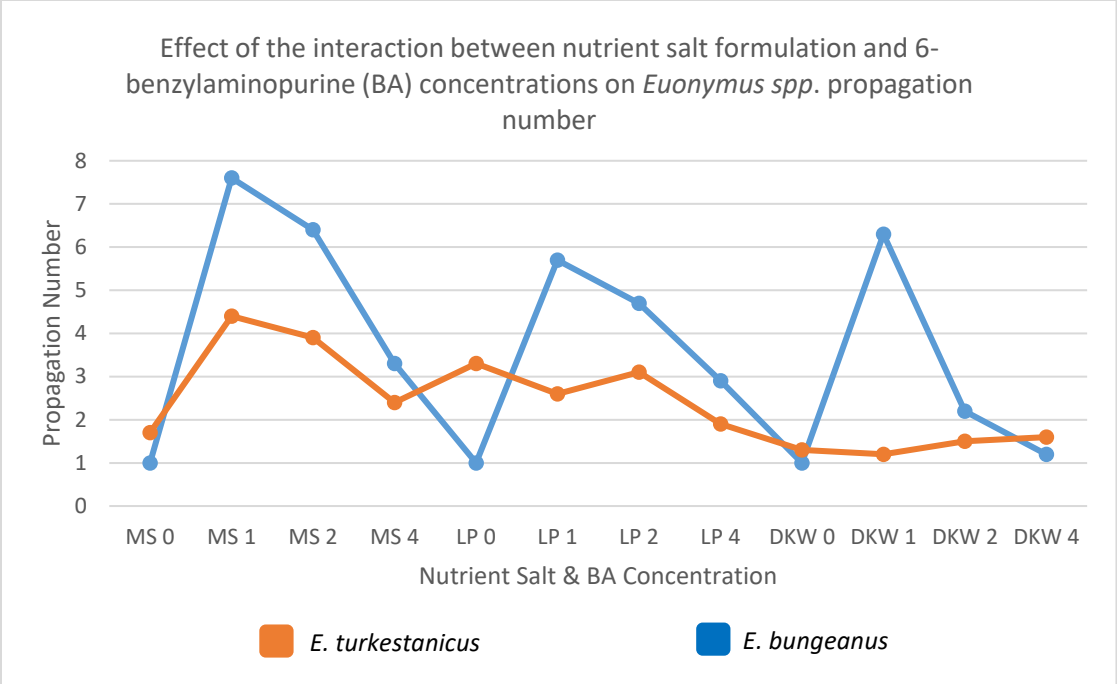
Table 1. Effect of the interaction between nutrient salt formulations and 6-Benzylaminopurine (BA) concentrations on *Euonymus spp*. shoot production and propagation number.

Nutrient Salt	Treatment (µM BA)	Shoot production		Propagation Number	
		E. turkestanicus[1]	*E. bungeanus*[1]	*E. turkestanicus*[2]	*E. bungeanus*[1]
MS	0	1.1d	1.1a	1.0c	1.7b
MS	1	5.8a	1.6a	7.6a	4.4a
MS	2	3.8b	1.7a	6.4ab	3.9ab
MS	4	2.8bc	1.5a	3.3bc	2.4b
LP	0	1.0d	1.0a	1.0c	3.3ab
LP	1	2.2c	1.0a	5.7ab	2.6b
LP	2	1.9c	1.1a	4.7b	3.1ab
LP	4	1.6c	1.1a	2.9b	1.9b
DKW	0	1.0d	1.0a	1.0c	1.3b
DKW	1	2.2c	1.0a	6.3ab	1.2b
DKW	2	1.4cd	1.3a	2.2c	1.5b
DKW	4	1.3cd	1.2a	1.2c	1.6b

Note. [1] Means with the same letter within column are not significantly different at $\alpha<0.01$.
Note. [2] Means with the same letter within column are not significantly different at $\alpha<0.05$.



Graph 1. Effect of the interaction between nutrient salt formulations and 6-Benzylaminopurine (BA) concentrations on *Euonymus spp*. shoot production



Graph 2. Effect of the interaction between nutrient salt formulations and 6-Benzylaminopurine (BA) concentrations on *Euonymus spp*. propagation number

For *E. bungeanus* as compared to *E. turkestanicus*, there was only a 1-way interaction with respect to nutrient salts and PGR (Appendix Tables A-1, A-2, A-3, A-4, Table 2, Table 3). For *E. bungeanus*, MS outperformed the other nutrient salt formulations evaluated with respect to axillary shoot (microshoot) production (Table 2). The presence of BA had an influence on axillary shoot production as compared to the control with concentration not having an influence on production (Table 2, Table 3). As compared to *C. paniculatus*, it is possible that with *E. bungeanus*, the BA concentration was too low to stimulate significant axillary shoot production. Otherwise, the reason can be attributed to the absence of naphthalene acetic acid (NAA) as indicated by Senapati et al. 2013 that a highly efficient protocol for in vitro regeneration of an indigenous, endangered medicinal plant *C. paniculatus* was achieved using nodal explants. (MS) basal medium supplemented with 0.5 g L^{-1} BA and 0.1 g L^{-1} NAA showed maximum percentage of shoot multiplication (83.4%) with 8.2 shoots/explants. Also, in a study on the production of

triploid *E. alatus* using endosperm tissues as explants, (≈50%) of immature endosperm explants and (14%) of mature endosperm explants formed compact, green calli after culture in the dark for 8 weeks and then under light for 4 weeks on Murashige and Skoog (MS) medium supplemented with 2.2 μM BA and 2.7 μM α-naphthaleneacetic acid (NAA) (Thammina et al., 2011). Approximately (5.6%) of the immature endosperm-derived calli and (13.4%) of mature endosperm-derived calli initiated shoots within 8 weeks after they were cultured on MS medium with 4.4 μM (BA) and 0.5 μM indole-3-butyric acid (IBA) (Thammina et al., 2011). On the other hand, results from a study on the effect of BA on in vitro shoot multiplication of *Musa acuminata* (banana) cv. Berangan indicated that BA at 2.2 μM and 2.7 μM induced the highest number of normal and elongated shoots, although more shoots proliferated on initiation medium supplemented with BA at 33 μM. At 11 and 6.5 μM BA, the lowest number of elongated shoots was obtained. With the addition of indole acetic acid (IAA) to BA supplemented media, shoot proliferation and elongation were generally enhanced with an optimal concentration of 22 μM BA and 2 μM IAA. Also, MS media supplemented with BA showed that the number of bud formation in shoot cultures of *M. acuminata* cv. Berangan during the initiation stage increased proportionately with the concentrations used (11, 22 and 33 μM). However, the highest concentration of BA (33 μM) simultaneously increased the formation of abnormal shoots. After the first apical bud appeared, explants were transferred to MS medium with lower concentrations of BA either with or without IAA. Proliferation media supplemented with IAA showed enhanced shoot multiplication and elongation but did not help to reduce the abnormality index that occurred (Jafari et al., 2011). Further research will need to be conducted with increased BA concentration and possibly with the addition of NAA, IBA and/or IAA.

Table 2. Effect of three nutrient salts on *Euonymus bungeanus* microshoot production.

Nutrient Salt	Average Microshoot Production[1]
MS	1.5a
LP	1.0b
DKW	1.1b

Note. [1] Means with the same letter are not significantly different at $\alpha<0.01$.

Table 3. Effect of four different concentrations of 6-Benzylaminopurine (BA) on *Euonymus bungeanus* microshoot production.

Treatment (µM BA)	Average Microshoot Production[1]
0	1.0a
1	1.2b
2	1.4b
4	1.3b

Note. [1] Means with the same letter are not significantly different at $\alpha<0.01$.

Morphologically, MS with 1 µM BA displayed the longest growth of axillary shoots for *E. turkestanicus* in run 1 and run 2 (Fig. 1), while LP with 1 µM BA was more effective on *E. bungeanus* in both runs than the other nutrient salts and the PGR concentrations (Fig. 2). The observed decrease in length of shoots at high concentrations of BA (0.4–1.0 mg L^{-1}) might be due to the inhibition of organogenesis and induction of callusogenesis (Dimitrova et al., 2011). DKW performed similarly on both species and in both runs (Fig. 3).

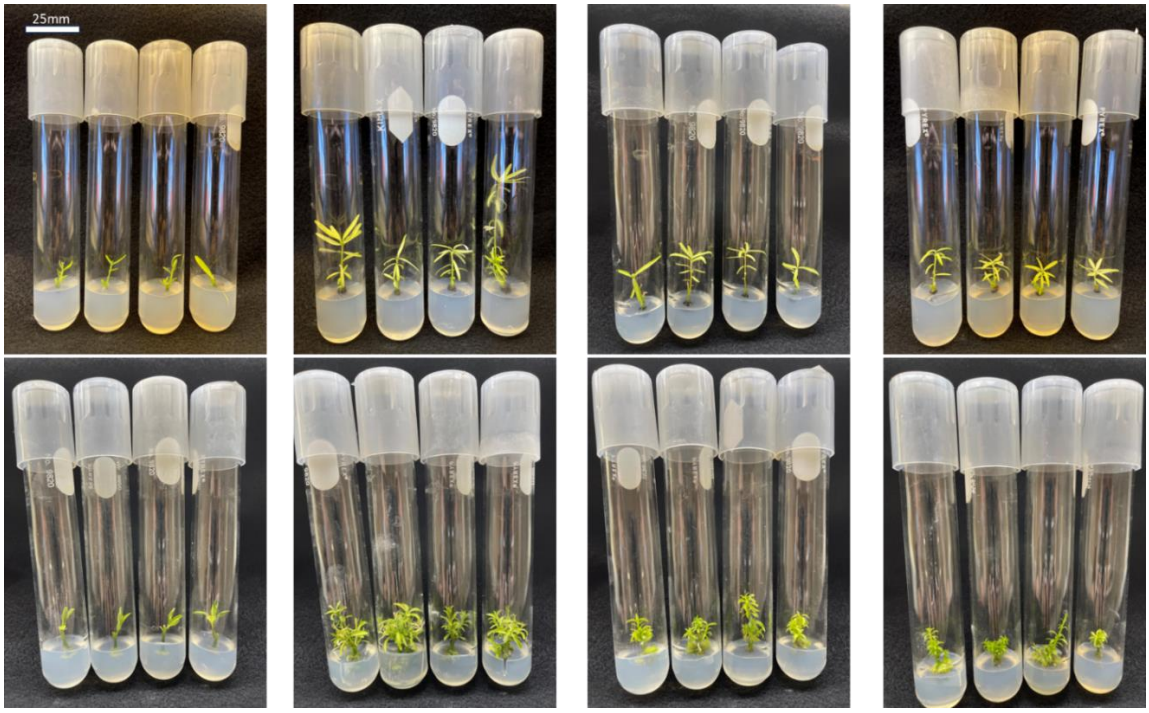


Figure 1. Nutrient salt (MS) and PGR (BA) interaction effect on *Euonymus spp*. (From left to right: Control with 0µM BA, 1µM BA, 2µM BA, 4µM BA). (*E. turkestanicus* on top, *E. bungeanus* below).

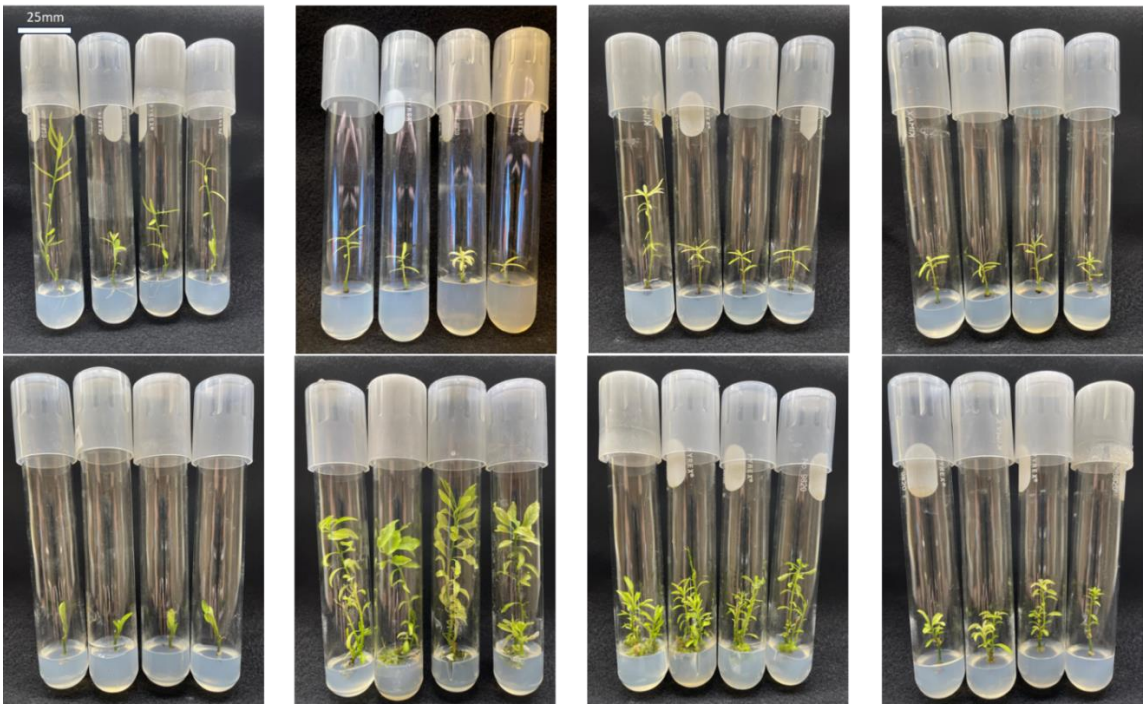


Figure 2. Nutrient salt (LP) and PGR (BA) interaction effect on *Euonymus spp*. (From left to right: Control with 0µM BA, 1µM BA, 2µM BA, 4µM BA). (*E. turkestanicus* on top, *E. bungeanus* below).

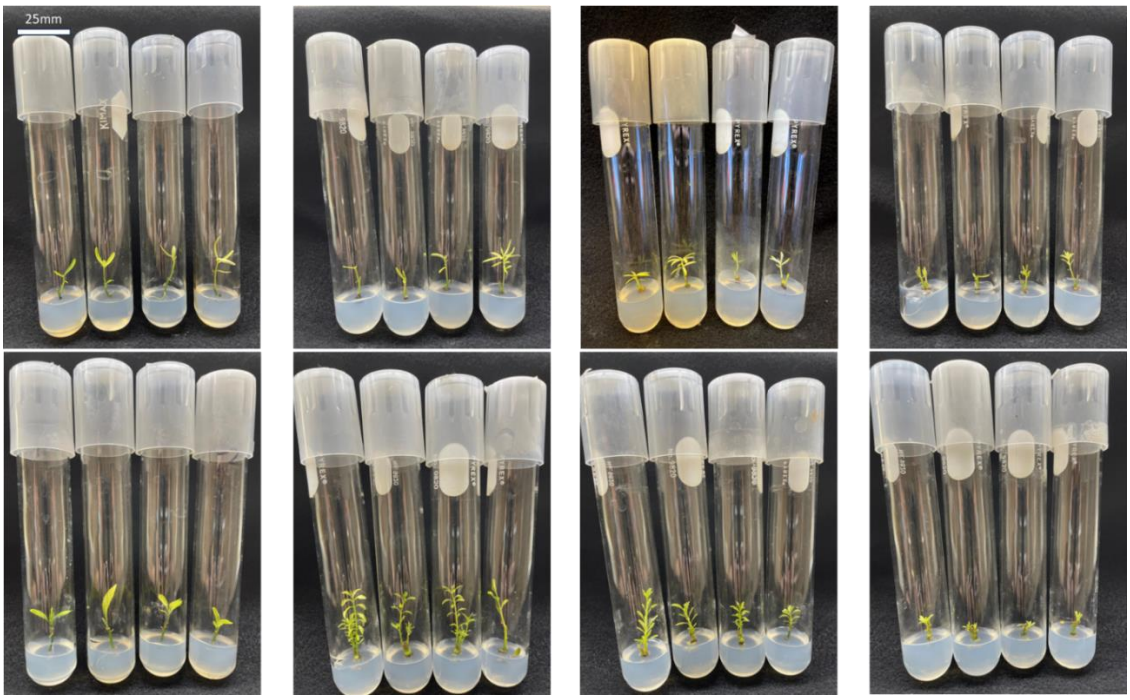


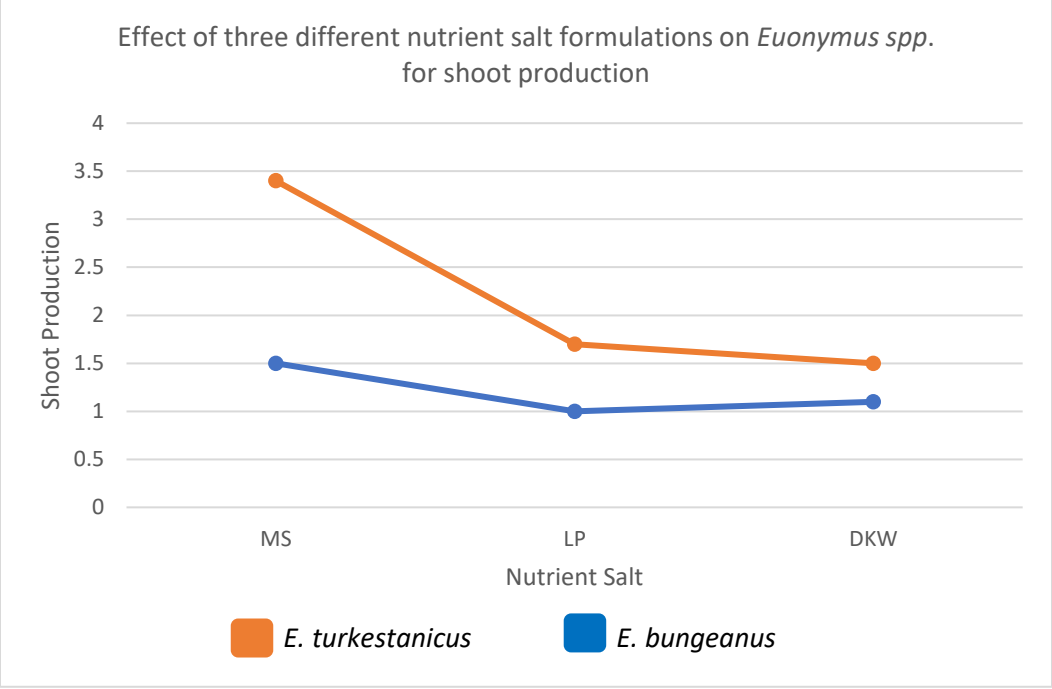
Figure 3. Nutrient salt (DKW) and PGR (BA) interaction effect on *Euonymus spp*. (From left to right: Control with 0µM BA, 1µM BA, 2µM BA, 4µM BA). (*E. turkestanicus* on top, *E. bungeanus* below).

As a single effect of nutrient salt formulations and BA concentrations, there was a significant 1-way interaction between the nutrient salt formulations (Appendix Table A-1, A-2, A-3). MS was significantly greater than the other nutrient salt formulations evaluated for *E. turkestanicus* with respect to shoot production, whereas there was no difference in effect for *E. bungeanus*. For propagation number, MS performed better than other nutrient salt formulations for *E. turkestanicus*. A possible reason for this may be the different macronutrients and/or micronutrients included in MS medium. However, MS and LP outperformed DKW, whilst were not different from each other. (Appendix Tables A-1, A-2, A-3, Table 4, Graphs 3, 4).

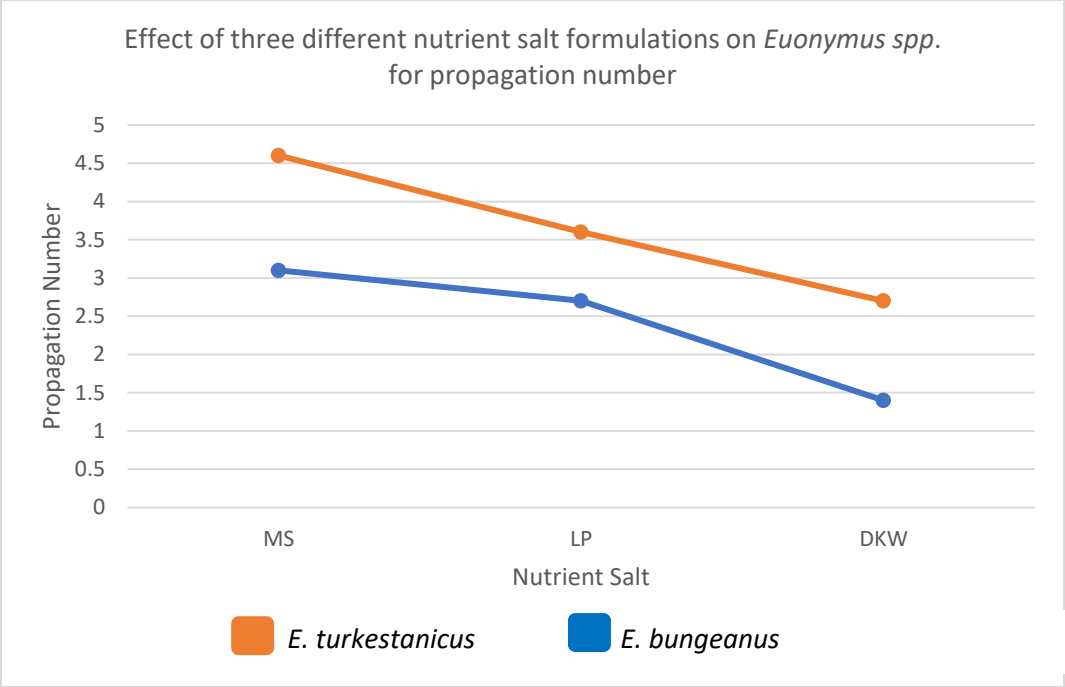
Table 4. Effect of nutrient salt formulations on *Euonymus spp*. shoot production and propagation number.

Nutrient Salt	Shoot production		Propagation Number[1]	
	E. turkestanicus	*E. bungeanus*	*E. turkestanicus*	*E. bungeanus*
MS	3.4a	1.5a	4.6a	3.1a
LP	1.7b	1.0a	3.6b	2.7a
DKW	1.5b	1.1a	2.7c	1.4b

Note. [1] Means with the same letter within column are not significantly different at $\alpha<0.0001$.



Graph 3. Effect of nutrient salt formulations on *Euonymus spp*. shoot production.



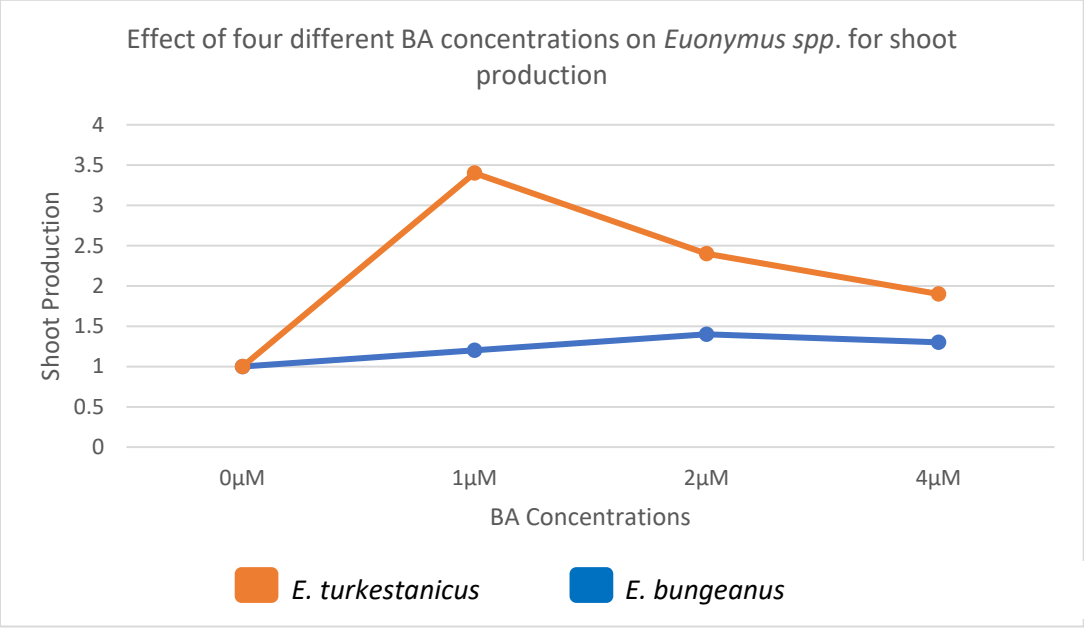
Graph 4. Effect of nutrient salt formulations on *Euonymus spp*. propagation number.

BA had an effect on shoot and propagation number. BA concentration of 1 µM outperformed the other treatments for *E. turkestanicus*, while had no significant effect on *E. bungeanus*. For shoot production, BA had an impact on propagation number with 1 µM BA being the best for *E. turkestanicus* while had no significant effect on *E. bungeanus*. (Appendix Tables A-1, A-2, A-3, Table 5, Graphs 5, 6).

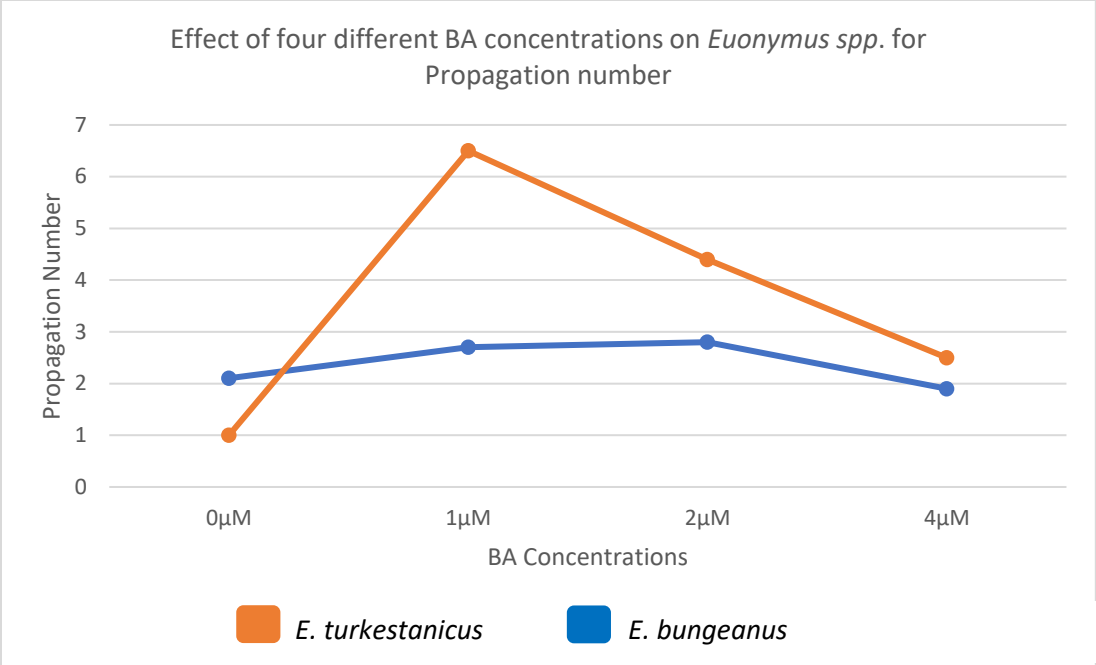
Table 5. Effect of BA concentrations on *Euonymus spp*. shoot production and propagation number.

Treatment (µM BA)	Shoot production[1]		Propagation Number[1]	
	E. turkestanicus	*E. bungeanu*[1]	*E. turkestanicus*	*E. bungeanus*
0	1.0a	1.0a	1.0a	2.1a
1	3.4b	1.2a	6.5b	2.7a
2	2.4c	1.4a	4.4c	2.8a
4	1.9c	1.3a	2.5d	1.9a

Note. [1] Means with the same letter are not significantly different at $\alpha<0.0001$.



Graph 5. Effect of BA concentrations on *Euonymus spp*. shoot production.



Graph 6. Effect of BA concentrations on *Euonymus spp*. propagation number.

3.2 Evaluating IBA Concentrations for Rooting

Data analysis showed that the concentrations of the PGR were significantly different for *E. bungeanus* but not *E. turkestanicus* for root production (Appendix Tables A-5, A-6). For *E. turkestanicus*, the presence of IBA and concentration did not have a significant effect on root production which suggests that an exogenous application of a rooting hormone may not be needed for root initiation and development as shown with the negative affect of the presence of IBA in the medium and that some control plants produced roots, which led to elongated shoots. (Table 6). For *E. bungeanus*, the presence of IBA had a significant negative effect on rooting with the best rooting in the absence of IBA and decreased rooting as IBA increased (Table 6). This suggests that IBA is likely not a good PGR for Euonymus rooting and other rooting PGRs should be evaluated for future consistent rooting. Roots were produced (Fig. 4) but were not consistently produced with the different IBA concentrations. Also, the control performed better than the concentrations of IBA in *E. bungeanus* (Table 6) and (Graph 7). In a study on eight

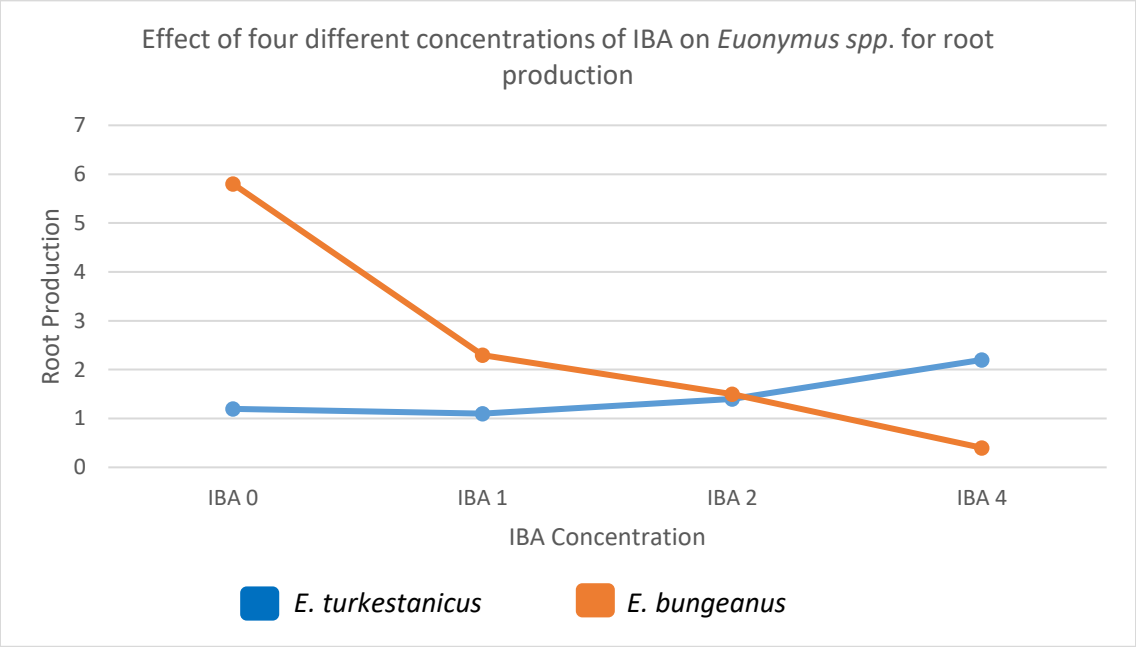
Prunus genotypes of the effect of IBA on in-vitro rooting, it has been stated that Shoots rooted with 15 µM IBA were smaller and lower quality in most genotypes. DKW maximized size and quality in six genotypes. Better shoots and larger root systems during in vitro rooting produced better plants in the greenhouse with no detrimental effect of callus growth (Lawson et al. 2023).

This finding is consistent with in vitro observations where plants grown on 15 µM IBA medium had lower quality shoots relative to other IBA treatments. This is potentially linked to reports of prolonged auxin exposure inhibiting root elongation and hindering surface area for nutrient and water exchange compared to ex-vitro rooting techniques (Lawson et al., 2023). While in an in vitro micro propagation protocol study of *C. paniculatus*, the highest rooting percentage (73.3%) was obtained in MS medium with 5.6 µM IAA and 9.6 µM IBA (Man De Silva 2010) and this indicates that high concentrations of IBA my results in high root production and for it to be effective, IBA needs to be combined with IAA. Moreover, in vitro rooting of shoots of in *C. paniculatus* (80%) was obtained when their bases were dipped in pre-autoclaved IBA solution (2.45 µM) for 10 min followed by their implantation on medium containing 1/4 MS salts (Rao and Purohit, 2006). On the contrary, in a study on the production of triploid *E. alatus* using endosperm tissues as explants, (85%) of shoots rooted after culture on woody plant medium (WPM) containing 4.9 µM IBA for 2 weeks and then on hormone-free WPM medium containing 2.0 g·L−1 activated charcoal for 4 weeks (Thammina et al., 2011). Further research will need to be conducted with increased IBA concentration and possibly with the addition of IAA and cultured on woody plant medium (WPM).

Table 6. Effect of four different concentrations of IBA on *Euonymus spp*. for root production.

Treatment (µM IBA)	Root Number[1]	
	E. turkestanicus	*E. bungeanus*
0	1.2a	5.8a
1	1.1a	2.3b
2	1.4a	1.5c
4	2.2a	0.4d

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



Graph 7. Effect of four different concentrations of IBA on *Euonymus spp*. for root production.

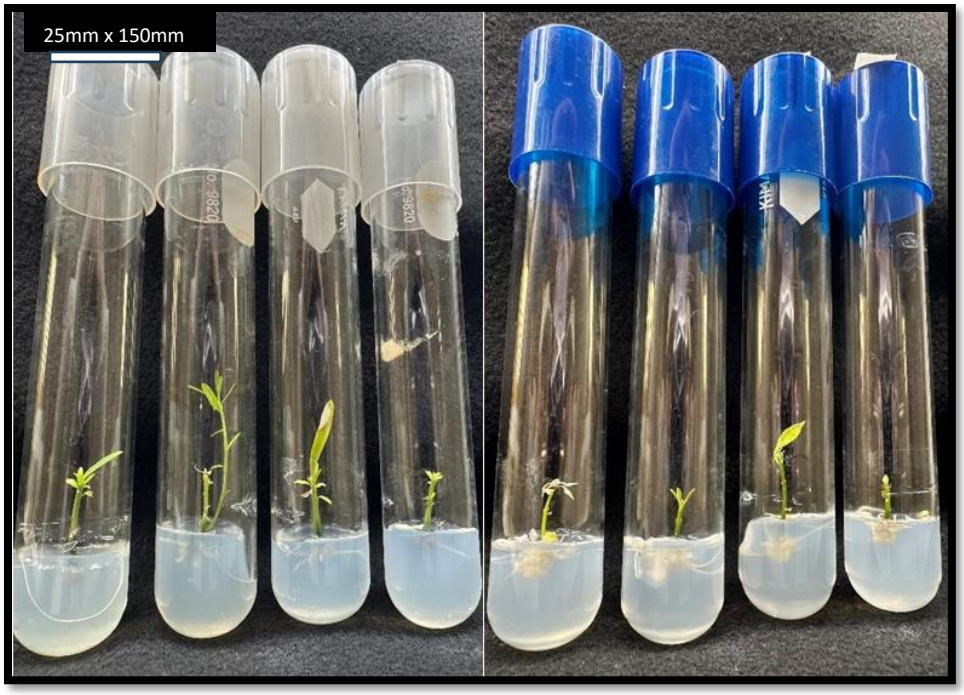


Figure 4. Effect of IBA on *Euonymus bungeanus* for root production (Control 0 µM IBA on left, 4 µM IBA on right).

3.3 Measuring Ploidy via Flow Cytometry

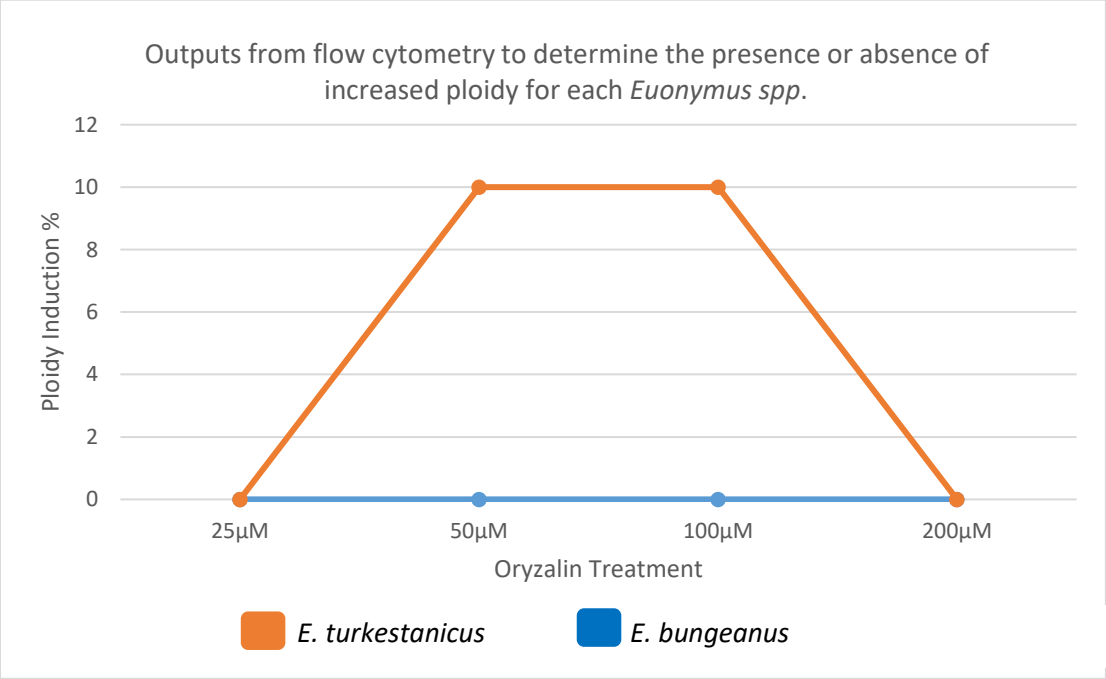
Histograms produced using flow cytometry were used to determine the presence or absence of increased ploidy for each Euonymus species. As a result of genotypic differences between the two euonymus species tested, flow cytometry histograms differed slightly between species. Euonymus species were treated with four concentrations of oryzalin (25, 50, 100 and 200 µM) in aseptic in invitro conditions. Ten samples for each concentration of oryzalin were tested for each euonymus species. There was a 10% recovery of polyploids in *E. turkestanicus* based off replicates number per treatment (Table 5) and (Graph 4). All *E. bungeanus* samples tested resulted in similar histograms (Fig. 4). Control samples of this plant produced one definitive peak of diploid nuclei (2x) at approximately mean reflective fluorescence at ~17K. While samples of plants treated with oryzalin appeared virtually identical to the controls. This

suggests no evidence of increased ploidy on treated samples tested because there are no subsequent peaks at the histograms to the treated plants.

Table 7. Outputs from Flow Cytometry to determine the presence or absence of increased ploidy for each *Euonymus spp*.

Species	No. of Replicates	Oryzalin Concentration (µM)	Ploidy Induction %1
E. turkestanicus	10	25	0a
	10	50	10b
	10	100	10b
	10	200	0a
E. bungeanus	10	25	0a
	10	50	0a
	10	100	0a
	10	200	0a

Note. [1] Means with the same letter within column are not significantly different.



Graph 8. Outputs from Flow Cytometry to determine the presence or absence of increased ploidy for each *Euonymus spp*.

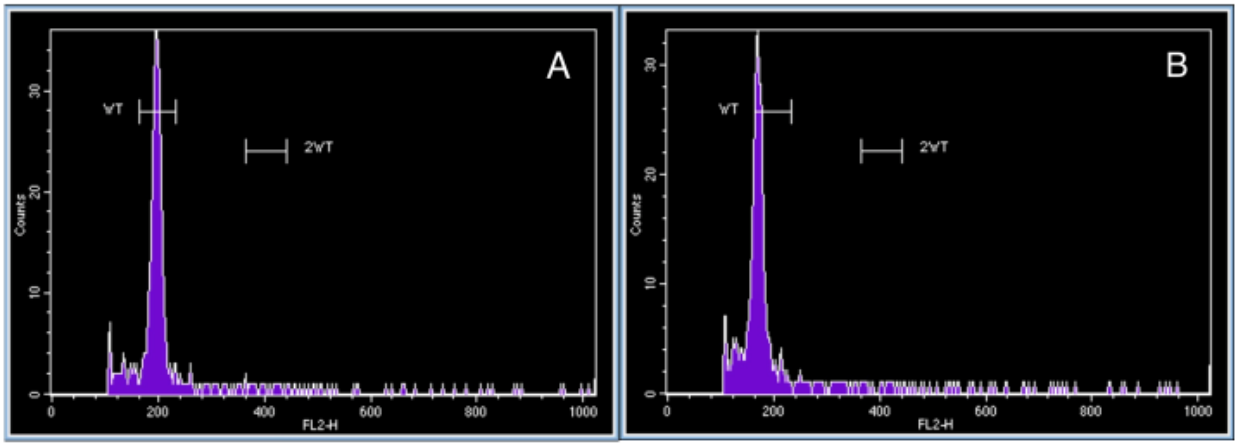


Figure 5. Flow Cytometry Histograms for *Euonymus bungeanus* (Control on left, control plus oryzalin treated on right)

Flow cytometry histograms for samples of *E. turkestanicus* were more promising in potential polyploid conversion. Samples of oryzalin-treated plant material which produced multiple DNA peaks in their histograms compared to control samples were counted as polyploid for data analysis. Placement of the second peak of DNA suggests double the DNA profile of the control sample, implying tetraploid conversion. There were only 2 samples from all the treatments that showed the presence of polyploids, sample No. 1238 treated with 50 µM of oryzalin (Fig. 5) and sample No. 1313 treated with 100 µM of oryzalin (Fig. 6). This suggests that euonymus species may not be highly sensitive to oryzalin but polyploid conversion is still possible. Sample No. 1238, displayed two peaks, one diploid (control) and one polyploid from the oryzalin treatment. These peaks were estimated at 179 and 366 (Fig. 7). Sample No. 1313 also displayed two peaks, one diploid (control) and one polyploid from the oryzalin treatment 7. These peaks were estimated at 170 and 380 (Fig. 8). No data analysis was conducted because of the low induction rates across the oryzalin treatments evaluated.

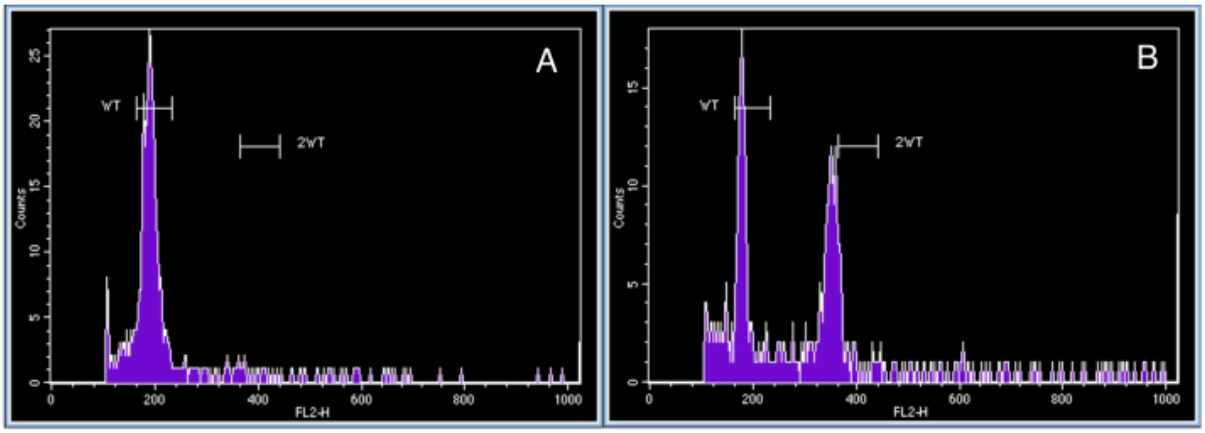


Figure 6. Flow Cytometry Graphs for *Euonymus turkestanicus* (Control 0 µM of Oryzalin on left, control plus 50 µM of Oryzalin treated polyploid on right. Sample No. 1238)

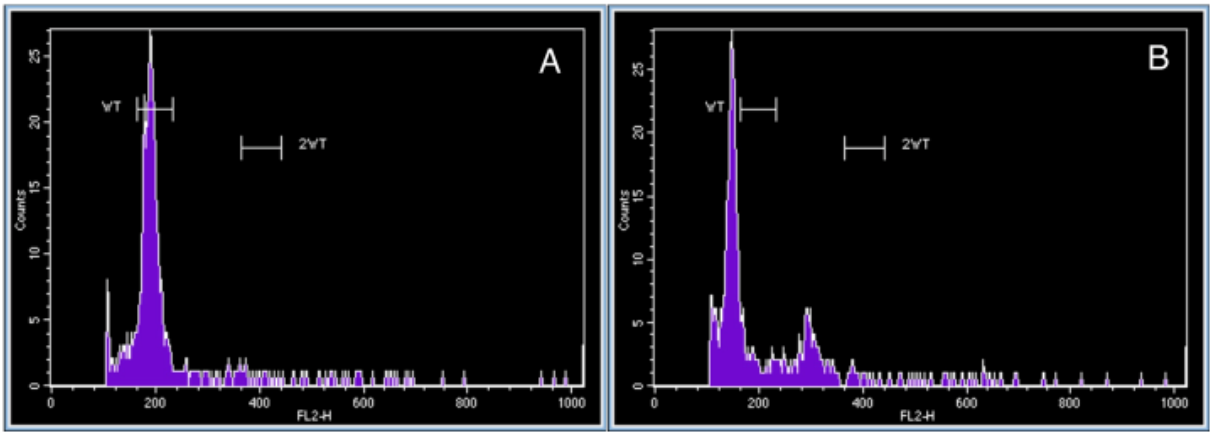
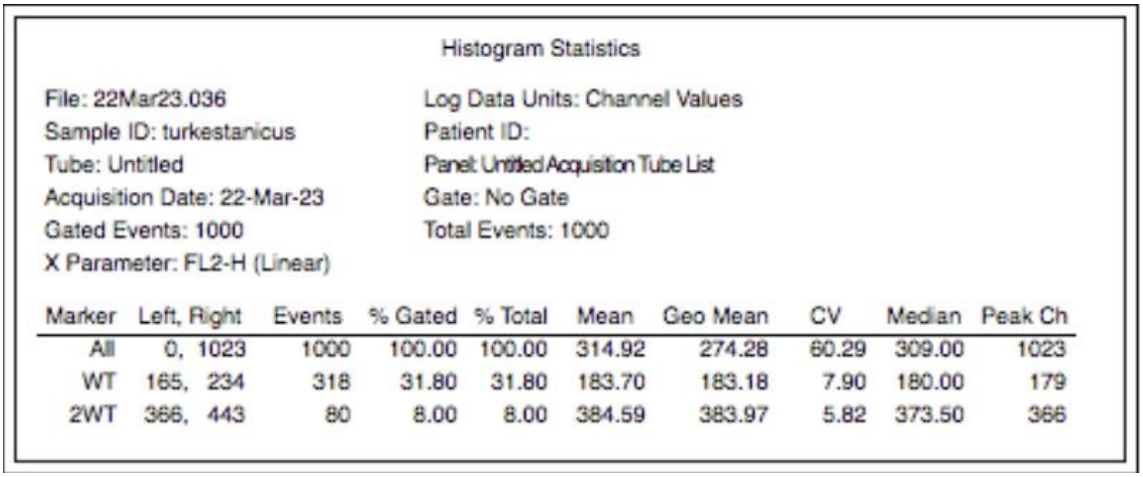


Figure 7. Flow Cytometry Graphs for *Euonymus turkestanicus* (Control 0 µM of oryzalin on left, control plus 100 µM of oryzalin treated polyploid on right. Sample No. 1313)



Histogram Statistics

File: 22Mar23.036
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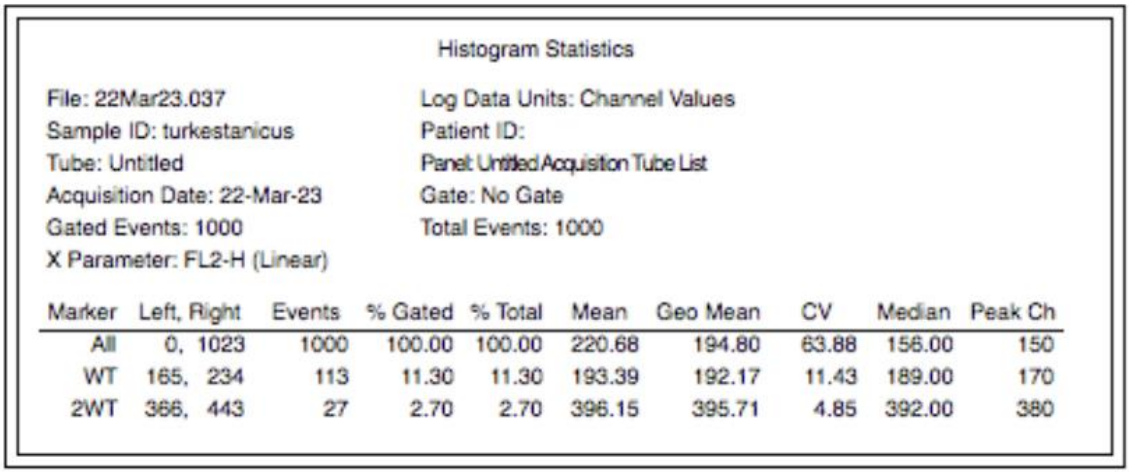
Tube: Untitled
Acquisition Date: 22-Mar-23
Gated Events: 1000
X Parameter: FL2-H (Linear)

Log Data Units: Channel Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 1000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	1000	100.00	100.00	314.92	274.28	60.29	309.00	1023
WT	165, 234	318	31.80	31.80	183.70	183.18	7.90	180.00	179
2WT	366, 443	80	8.00	8.00	384.59	383.97	5.82	373.50	366

Figure 8. Histogram statistics for *Euonymus turkestanicus* showing two peaks of WT and 2WT



Histogram Statistics

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Tube: Untitled
Acquisition Date: 22-Mar-23
Gated Events: 1000
X Parameter: FL2-H (Linear)

Log Data Units: Channel Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 1000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	1000	100.00	100.00	220.68	194.80	63.88	156.00	150
WT	165, 234	113	11.30	11.30	193.39	192.17	11.43	189.00	170
2WT	366, 443	27	2.70	2.70	396.15	395.71	4.85	392.00	380

Figure 9. Histogram statistics for *Euonymus turkestanicus* showing two peaks of WT and 2WT

Although polyploidy was confirmed in two of the four oryzalin concentrations between the two different species, the current evaluated method of oryzalin application via aseptic conditions (tissue culture) over a 3-day period may lack practical application in euonymus tetraploid production. A more successful strategy may be to increase the time of exposure to oryzalin and/or treat in vitro cultures with different concentrations of oryzalin.

3.4 Conclusions

Euonymus species: *Euonymus bungeanus* and *Euonymus turkestanicus* and can be successfully propagated in vitro from axillary nodal explants. The addition of 1 µM of BA to the solid MS culture medium can result in the best shoot production for *E. turkestanicus* as compared to the other treatments in this study. The highest propagation number can be achieved with one of the treatments; MS with 1 or 2 µM BA for both species. This data suggests that micropropagation is possible allowing for in vitro polyploid induction to be possible with plant regeneration. Polyploids can be induced in *E. turkestanicus* using 50 or 100 µM of oryzalin. More research will need to be conducted to further evaluate polyploid induction for *E. bungeanus* and to increase efficiency of oryzalin induced polyploid induction. Further research is also necessary to examine different PGRs for euonymus root production. This study suggests that micropropagation and polyploid induction is possible and will assist in the potential breeding and production of sterile cultivars of euonymus.

REFERENCES

Abeyaratne, W., & Lathiff, M. (2001). In-Vitro Propagation of Rathambala (Musa AAA) And The Occurrence Of Phenotypic Variations In The Pseudostem. *Annals of the Sri Lanka Department of Agriculture*, 4, 191–197.

Ackerman, W. L., & Dermen, H. (1972). A Fertile Colchiploid from A Sterile Interspecific Camellia Hybrid. *Journal of Heredity*, 63(2), 55–59. https://doi.org/10.1093/oxfordjournals.jhered.a108227

Ascough, G., Staden, V. J., & Erwin, J. E. (2008). Effectiveness of Colchicine and Oryzalin at Inducing Polyploidy in *Watsonia lepida* N.E. Brown. *Hortscience*, 43(7), 2248–2251. https://doi.org/10.21273/hortsci.43.7.2248

Bartels, P. G., & Hilton, J. L. (1973). Comparison Of Trifluralin, Oryzalin, Pronamide, Propham, And Colchicine Treatments on Microtubules. *Pesticide Biochemistry and Physiology*, 3(4), 462–472. https://doi.org/10.1016/0048-3575(73)90072-2

Bouman, H., & Tiekstra, A. (2005). Adaptions Of the Mineral Composition of Tissue Culture Media on The Basis of Plant Elemental Analysis and Composition of Hydroponic Substrates. *In Springer eBooks* (pp. 493–505). https://doi.org/10.1007/1-4020-3200-5_37

Bouvier, F., Hugueney, P., D'Harlingue, A., Kuntz, M., & Camara, B. (1994). Xanthophyll Biosynthesis in Chromoplasts: Isolation and Molecular Cloning of An Enzyme Catalyzing the Conversion Of 5,6-Epoxycarotenoid into Ketocarotenoid. *The Plant Journal*, 6(1), 45–54. https://doi.org/10.1046/j.1365-313x.1994.6010045.x

Buah, J. N., Van Der Puije, G. C., Bediako, E. A., Abole, E. A., & Showemimo, F. (2010). The Growth and Yield Performance of Oyster Mushroom (*Pleurotus ostreatus*) On Different Substrates. *Biotechnology*, 9(3), 338–342. https://doi.org/10.3923/biotech.2010.338.342

Cai, H., Gu, X., Li, Y., Ren, Y., Shufang, Y., & Yang, M. (2022). Cold Resistance of *Euonymus Japonicus* Beihaidao Leaves and Its Chloroplast Genome Structure and Comparison with Celastraceae Species. *Plants*, 11(19), 2449. https://doi.org/10.3390/plants11192449

Chauhan, R., Hailemariam, T., Ugo, Y., Shibru, S., Anjulo, A., Debella, A., Walche, A., & Misgana, D. (2021). Growth And Carbon Sequestration Potential of Moringa (*Moringa stenopetala*) Along an Altitudinal Gradient in Southern Ethiopia. *Tropical and Subtropical Agroecosystems*, 24(3). https://doi.org/10.56369/tsaes.3576

Chen, Y., Lu, L., Deng, W., Yang, X., McAvoy, R., Zhao, D., Pei, Y., Luo, K., Duan, H., Smith, W., Thammina, C., Zheng, X., Ellis, D., & Li, Y. (2006). In Vitro Regeneration and Agrobacterium-Mediated Genetic Transformation of *Euonymus alatus*. *Plant Cell Reports*, 25(10), 1043–1051. https://doi.org/10.1007/s00299-006-0168-8

Davies FT, Geneve RL, Wilson SB. 2018. Hartmann And Kester's Plant Propagation: Principles and Practices. 9th Ed. *Pearson Education*, New York, NY.

Dimitrova, M., Yordanova, Z., & Kapchina-Toteva, V. (2012). Influence Of 6-Benzylaminopurine and Indole-3-Butyric Acid On In Vitro Propagation and Secondary Metabolites Accumulation in Lamium Album from Lozen Mountain. *Acta Horticulturae*, 955, 315–320. https://doi.org/10.17660/actahortic.2012.955.47

Ding, J., Reardon, R. C., Wu, Y., Zheng, H., & Fu, W. (2006). Biological Control of Invasive Plants Through Collaboration Between China and The United States of America: A Perspective. *Biological Invasions*, 8(7), 1439–1450. https://doi.org/10.1007/s10530-005-5833-2

Dirr, M. A. (2009). Manual of Woody Landscape Plants. Their Identification, Ornamental Characteristics, Culture, Propagation and Uses. *Stipes Publishing*, Champaign, IL.

Dirr, M. A., & Heuser, C. W. (2006). The Reference Manual of Woody Plant Propagation from Seed to Tissue Culture. 2nd Edition. *Timber Press*, Portland, OR.

Driver, J. A., Suttle, G. R. L. (1987). Nursery Handling of Paradox Walnut Rootstock. *HortScience*, 19:507-509.

Ebinger, J. E. (1996). *Euonymus alatus*. Winged Euonymus, Burning Bush. P. 55 In J. M. Randall and J. Marinelli, Eds. Invasive Plants. Weeds of the Global Garden. Handbook 147. *Brooklyn Botanic Garden*, Brooklyn, NY.

Eisel, L. M., & Gaudette, J. J. (1974). Comments On 'An Environmental Evaluation System for Water Resource Planning' by Norbert Dee et al. *Water Resources Research*, 10(2), 379–380. https://doi.org/10.1029/wr010i002p00379

Fan, L., Zhang, C., Ai, L., Wang, L., Li, L., Fan, W., Li, R., He, L., Wu, C., & Huang, Y. (2020). Traditional Uses, Botany, Phytochemistry, Pharmacology, Separation, And Analysis Technologies of *Euonymus Alatus* (Thunb.) Siebold: A Comprehensive Review. *Journal of Ethnopharmacology*, 259, 112942. https://doi.org/10.1016/j.jep.2020.112942

Galbraith, D. W., Harkins, K. R., Maddox, J. M., Ayres, N. M., Sharma, D. P., & Firoozabady, E. (1983). Rapid Flow Cytometric Analysis of The Cell Cycle in Intact Plant Tissues. *Science*, 220(4601), 1049–1051. https://doi.org/10.1126/science.220.4601.1049

Galbraith, D. W., Loureiro, J., Antoniadi, I., Bainard, J. D., Bureš, P., Cápal, P., Castro, M., Castro, S., Čertner, M., Čertnerová, D., Chumová, Z., Doležel, J., Giorgi, D., Husband, B. C., Kolář, F., Koutecký, P., Kron, P., Leitch, I. J., Ljung, K., Lopes, S., Lučanová, M., Lucretti, S., Ma, W., Melzer, S., Molná,r I., Novák, O., Poulton, N., Skalický, V., Sliwinska, E., Šmarda, P., Smith, T. W., Sun, G., Talhinhas, P., Tárnok, A., Temsch,

EM., Trávníček, P., & Urfus, T. (2021). Best Practices in Plant Cytometry. *Cytometry. Part A*, 99(4), 311–317. https://doi.org/10.1002/cyto.a.24295

Gamborg, O. L., Murashige, T., Thorpe, T. A., & Vasil, I. K. (1976). Plant Tissue Culture Media. *In Vitro*, 12(7), 473–478. https://doi.org/10.1007/bf02796489

Gamborg, O. L., & Phillips, G. C. (1995). Plant Cell, Tissue and Organ Culture. *In Springer eBooks*. https://doi.org/10.1007/978-3-642-79048-5

Gaspar, T., Kevers, C., Penel, C., Greppin, H., Reid, D. M., & Thorpe, T. A. (1996). Plant Hormones and Plant Growth Regulators in Plant Tissue Culture. *In Vitro Cellular & Developmental Biology. Plant*, 32(4), 272–289. https://doi.org/10.1007/bf02822700

Martin, G., Geetha, S., Raja, S. S., Raghu, A. V., Balachandran, I., & Ravindran, P. N. (2006). An Efficient Micropropagation System for *Celastrus paniculatus* Willd.: A Vulnerable Medicinal Plant. *Journal of Forest Research*, 11(6), 461–465. https://doi.org/10.1007/s10310-006-0237-4

George, E. F., Hall, M. A., & Klerk, G. D. (2007). Plant Propagation by Tissue Culture 3rd Edition. *Springer*. Edington, GB

Gonçalves, T. T., DeSouza, O., Junior, R. R., & Pontes, R. (2005). Effect Of Tree Size and Growth Form on The Presence and Activity of Arboreal Termites (Insecta: Isoptera) In The Atlantic Rain Forest. *Sociobiology*, 59:903-910

Harbard, J. L., Griffin, A. R., Foster, S. A., Brooker, C., Kha, L. D., & Koutoulis, A. (2012). Production Of Colchicine-Induced Autotetraploids as A Basis for Sterility Breeding in *Acacia mangium* Willd. *Forestry*, 85(3), 427–436. https://doi.org/10.1093/forestry/cps041

Harrington, R. A., Kujawski, R., & Ryan, H. D. P. (2003). Invasive Plants and The Green Industry. *Arboriculture & Urban Forestry*, 29(1), 42–48. https://doi.org/10.48044/jauf.2003.006

Jafari, N., Othman, R. Y., & Khalid, N. (2011). Effect of Benzylaminopurine (BAP) Pulsing On In Vitro Shoot Multiplication of *Musa acuminata* (banana) cv. *Berangan*. https://api.semanticscholar.org/CorpusID:32055699

Kehie, M., Kumaria, S., & Tandon, P. (2011). In Vitro Plantlet Regeneration from Nodal Segments and Shoot Tips of Capsicum Chinense Jacq. Cv. Naga King Chili. *3 Biotech*, 2(1), 31–35. https://doi.org/10.1007/s13205-011-0025-5

Kermani, M. J., Sarasan, V., Roberts, A., Yokoya, K., Wentworth, J., & Sieber, V. (2003). Oryzalin-Induced Chromosome Doubling in Rosa and Its Effect on Plant Morphology and Pollen Viability. *Theoretical and Applied Genetics*, 107(7), 1195–1200. https://doi.org/10.1007/s00122-003-1374-1

Lawson, J. D., Bridges, W. C., & Adelberg, J. (2023). IBA Delivery Technique and Media Salts Affected In Vitro Rooting and Acclimatization of Eight Prunus Genotypes. *Plants*, 12(2), 289. https://doi.org/10.3390/plants12020289

Li, Z., & Ruter, J. M. (2017). Development and Evaluation of Diploid and Polyploid *Hibiscus moscheutos*. *HortScience*, 52(5), 676–681. https://doi.org/10.21273/hortsci11630-16

Lin, L., Pan, M., Hong, G., Tian, C., & Fan, X. (2023). The Potential Fungal Pathogens of *Euonymus japonicus* in Beijing, China. *Journal of Fungi (Basel)*, 9(2), 271. https://doi.org/10.3390/jof9020271

Lloyd, G., & Mccown, B. (1980). Commercially-Feasible Micropropagation Of Mountain Laurel, Kalmia Latifolia, By Use Of Shoot-Tip Culture. *Environmental Science, Biology, Agricultural and Food Sciences*, https://api.semanticscholar.org/CorpusID:83728629

Madhulatha, P., Anbalagan, M., Sadaksharam, J., & Sakthivel, N. (2004). Influence Of Liquid Pulse Treatment with Growth Regulators On In Vitro Propagation of Banana (Musa Spp. AAA*). Plant Cell, Tissue and Organ Culture (Print)*, 76(2), 189–192. https://doi.org/10.1023/b:ticu.0000007291.31439.6c

Mattingly, K. Z., McEwan, R. W., Paratley, R. D., Bray, S. R., Lempke, J. R., & Arthur, M. A. (2016). Recovery Of Forest Floor Diversity After Removal of The Nonnative, Invasive Plant *Euonymus fortune*. *The Journal of the Torrey Botanical Society*, 143(2), 103–116. https://doi.org/10.3159/torrey-d-14-00051

Mir, M. Y., Kamili, A. N., Hassan, Q. P., Rafi, S., Parray, J. A., & Jan, S. (2018). In Vitro Regeneration and Free Radical Scavenging Assay of *Hypericum perforatum* L. National Academy Science Letters, 42(2), 161–167. https://doi.org/10.1007/s40009-018-0699-x

Morel, G. M. (1960). Producing Virus-Free Cymbidium. *American Orchid Society Bulletin*, 29:495-497

Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiologia Plantarum*, 15:473-497.

Nas, M. N., & Read, P. E. (2004). A Hypothesis for The Development of a Defined Tissue Culture Medium of Higher Plants and Micropropagation of Hazelnuts. *Scientia Horticulturae*, 101(1–2), 189–200. https://doi.org/10.1016/j.scienta.2003.10.004

Németh, G. (1986). Induction Of Rooting. *In Biotechnology in agriculture and forestry* (pp. 49–64). https://doi.org/10.1007/978-3-642-70576-2_4

Niemiera, A. X., & Von Holle, B. (2008). Invasive Plant Species and The Ornamental Horticulture Industry. *In Springer eBooks* (pp. 167–187). https://doi.org/10.1007/978-1-4020-9202-2_9

Olsen, J. V., Blagoev, B., Gnad, F., Maček, B., Kumar, C., Mortensen, P. E., & Mann, M. (2006). Global, In Vivo, And Site-Specific Phosphorylation Dynamics in Signaling Networks. *Cell*, 127(3), 635–648. https://doi.org/10.1016/j.cell.2006.09.026

Park, S., Wakana, A., Kim, J., & Jeong, C. (2002). Male And Female Fertility in Triploid Grapes (*Vitis* Complex) With Special Reference to The Production of Aneuploid Plants. *EurekaMag*. https://eurekamag.com/research/003/838/003838823.php

Preece, J. E. (1995). Can Nutrient Salts Partially Substitute for Plant Regulators? *Plant Tissue Culture and Biotechnology* 1(1): 26-27

Pryor, R. L., & Frazier, L. C. (1968). Colchicine-Induced Tetraploid Azaleas. *Journal of the American Society for Horticultural Science*, 3(4), 283–286. https://doi.org/10.21273/jashs.3.4.283

Pullman, G. S., Zhang, Y., & Phan, B. H. (2003). Brassinolide Improves Embryogenic Tissue Initiation in Conifers and Rice. *Plant Cell Reports*, 22(2), 96–104. https://doi.org/10.1007/s00299-003-0674-x

Ramage, C., & Williams, R. R. (2002). Mineral Nutrition and Plant Morphogenesis. *In Vitro Cellular & Developmental Biology. Plant*, 38(2), 116–124. https://doi.org/10.1079/ivp2001269

Rao, M. S., & Purohit, S. D. (2006). In Vitro Shoot Bud Differentiation and Plantlet Regeneration in *Celastrus paniculatus* Willd. *Biologia Plantarum*, 50(4), 501–506. https://doi.org/10.1007/s10535-006-0079-0

Reichard, S. H., & White, P. (2001). Horticulture As a Pathway of Invasive Plant Introductions in The United States: Most Invasive Plants Have Been Introduced For Horticultural Use By Nurseries, Botanical Gardens, And Individuals. *OUP Academic*, https://doi.org/10.1641/0006-3568(2001)051

Rugini, E. (1984). In Vitro Propagation of Some Olive (*Olea Europaea Sativa L.*) Cultivars with Different Root-Ability, And Medium Development Using Analytical Data from Developing Shoots and Embryos. *Scientia Horticulturae*, 24(2), 123–134. https://doi.org/10.1016/0304-4238(84)90143-2

Searcy, K. B. (2000). Flora Of the Northeast: A Manual of The Vascular Flora of New England and Adjacent New York. Dennis W. Magee, Harry E. Ahles. *The Quarterly Review of Biology*, 75(3), 321–322. https://doi.org/10.1086/393538

Senapati, S. K., Aparajita, S., & Rout, G. R. (2013). Micropropagation And Assessment of Genetic Stability in *Celastrus Paniculatus*: An Endangered Medicinal Plant. *Biologia (Bratislava)*, 68(4), 627–632. https://doi.org/10.2478/s11756-013-0187-1

Šípošová, K., Kollárová, K., Lišková, D., & Vivodová, Z. (2019). The Effects of IBA On The Composition Of Maize Root Cell Walls. *Journal of Plant Physiology*, 239, 10–17. https://doi.org/10.1016/j.jplph.2019.04.004

Skirvin, R. M., Chu, M., Mann, M. L., Young, H., Sullivan, J. G., & Fermanian, T. W. (1986). Stability Of Tissue Culture Medium Ph as A Function of Autoclaving, Time, And Cultured Plant Material. *Plant Cell Reports*, 5(4), 292–294. https://doi.org/10.1007/bf00269825

Thammina, C., He, M., Lu, L., Cao, K., Yu, H., Chen, Y., Tian, L., Chen, J., McAvoy, R., Ellis, D., Zhao, D., Wang, Y., Zhang, X., & Li, Y. (2011). In Vitro Regeneration of Triploid

Plants of *Euonymus alatus* ‘Compactus’ (Burning Bush) from Endosperm Tissues. *Hortscience*, 46(8), 1141–1147. https://doi.org/10.21273/hortsci.46.8.1141

TLC Garden Centers. (2023). Dwarf Turkestan Burning Bush *Euonymus turkestanicus.* TLC Garden Centers. https://tlcgarden.com/shrubs/dwarf-shrubs-more-beauty-less-effort/

Trigiano, R. N., & Gray, D. J. (2018). Plant Tissue Culture Concepts and Laboratory Exercises, 2nd Edition. *In Routledge eBooks*. https://doi.org/10.1201/9780203743133

Väinölä, A. (2000). Polyploidization And Early Screening of Rhododendron Hybrids. *Euphytica*, 112(3), 239–244. https://doi.org/10.1023/a:1003994800440

Woo, Y., Lim, J. S., Oh, J., Lee, J. S., & Kim, J. (2020). Neuroprotective Effects of *Euonymus Alatus* Extract on Scopolamine-Induced Memory Deficits in Mice. *Antioxidants*, 9(5), 449. https://doi.org/10.3390/antiox9050449

Yemets, A. I., & Blume, Y. B. (2008). Progress In Plant Polyploidization Based on Antimicrotubular Drugs. *The Open Horticulture Journal*, 1(1), 15–20. https://doi.org/10.2174/1874840600801010015

Yıldız, M. (2013). Plant Responses at Different Ploidy Levels. *In InTech eBooks*. https://doi.org/10.5772/55785

APPENDIX

Table A1. SAS GLM Output Using All Nutrient Salts, PGRs, Nutrient Salts*PGRs. Dependent Variable: Propagation Number of *Euonymus turkestanicus*

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
RUN	1	74.816667	74.816667	10.51	0.0014
REP	9	67.850000	7.538889	1.06	0.3946
N SALT	2	150.325000	75.162500	10.56	<.0001
PGR	3	1015.333333	338.444444	47.53	<.0001
N SALT*PGR	6	117.041667	19.5069444	2.74	0.0138

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
RUN	1	74.816667	74.816667	10.51	0.0014
REP	9	67.850000	7.538889	1.06	0.3946
N SALT	2	150.325000	75.162500	10.56	<.0001
PGR	3	1015.333333	338.444444	47.53	<.0001
N SALT*PGR	6	117.041667	19.5069444	2.74	0.0138

Table A2. SAS GLM Output Using All Nutrient Salts, PGRs, Nutrient Salts*PGRs. Dependent Variable: Shoot Production of *Euonymus turkestanicus*

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
RUN	1	3.1510417	3.1510417	1.72	0.1909
REP	9	17.1343750	1.9038194	1.04	0.4090
N SALT	2	170.8187500	85.4093750	46.66	<.0001
PGR	3	175.3197917	58.4399306	31.93	<.0001
N SALT*PGR	6	86.9895833	14.4982639	7.92	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
RUN	1	3.1510417	3.1510417	1.72	0.1909
REP	9	17.1343750	1.9038194	1.04	0.4090
N SALT	2	170.8187500	85.4093750	46.66	<.0001
PGR	3	175.3197917	58.4399306	31.93	<.0001
N SALT*PGR	6	86.9895833	14.4982639	7.92	<.0001

Table A3. SAS GLM Output Using All Nutrient Salts, PGRs, Nutrient Salts*PGRs. Dependent Variable: Propagation Number of *Euonymus bungeanus*

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
RUN	1	1.5041667	1.5041667	0.78	0.3777
REP	9	9.9208333	1.1023148	0.57	0.8189
N SALT	2	122.2750000	61.1375000	31.75	<.0001
PGR	3	33.9458333	11.3152778	5.88	0.0007
N SALT*PGR	6	87.5916667	14.5986111	7.57	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
RUN	1	1.5041667	1.5041667	0.78	0.3777
REP	9	9.9208333	1.1023148	0.57	0.8189
N SALT	2	122.2750000	61.1375000	31.75	<.0001
PGR	3	33.9458333	11.3152778	5.88	0.0007
N SALT*PGR	6	87.5916667	14.5986111	7.57	<.0001

Table A4. SAS GLM Output Using All Nutrient Salts, PGRs, Nutrient Salts*PGRs. Dependent Variable: Shoot Production of *Euonymus bungeanus*

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
RUN	1	0.33750000	0.33750000	1.50	0.2214
REP	9	0.78750000	0.08750000	0.39	0.9392
N SALT	2	7.55833333	3.77916667	16.84	<.0001
PGR	3	3.17916667	1.05972222	4.72	0.0033
N SALT*PGR	6	2.20833333	0.36805556	1.64	0.1373

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
RUN	1	0.33750000	0.33750000	1.50	0.2214
REP	9	0.78750000	0.08750000	0.39	0.9392
N SALT	2	7.55833333	3.77916667	16.84	<.0001
PGR	3	3.17916667	1.05972222	4.72	0.0033
N SALT*PGR	6	2.20833333	0.36805556	1.64	0.1373

Table A5. SAS GLM Output Using Four PGR Concentrations (0, 1, 2, 4 µM IBA). Dependent Variable: Root Production of *Euonymus turkestanicus*

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
RUN	1	0.75000000	0.75000000	0.14	0.7088
REP	5	23.16666667	4.63333333	0.87	0.5071
PGR	3	8.75000000	2.91666667	0.55	0.6508

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
RUN	1	0.75000000	0.75000000	0.14	0.7088
REP	5	23.16666667	4.63333333	0.87	0.5071
PGR	3	8.75000000	2.91666667	0.55	0.6508

Table A6. SAS GLM Output Using Four PGR Concentrations (0, 1, 2, 4 µM IBA). Dependent Variable: Root Production of *Euonymus bungeanus*

Source of Variation	df	Type I SS	Mean Square	F Value	Pr > F
RUN	1	4.0833333	4.0833333	1.27	0.2675
REP	5	37.2500000	7.4500000	2.31	0.0629
PGR	3	198.1666667	66.0555556	20.49	<.0001

Source of Variation	df	Type III SS	Mean Square	F Value	Pr > F
RUN	1	4.0833333	4.0833333	1.27	0.2675
REP	5	37.2500000	7.4500000	2.31	0.0629
PGR	3	198.1666667	66.0555556	20.49	<.0001