

MICROPROPAGATION AND *IN VITRO* MUTAGENESIS
OF *VITEX AGNUS-CASTUS*

A Thesis

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by

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ABSTRACT

Vitex agnus-castus (Linnaeus, C., 1799) has many ornamental properties that make it a popular ornamental plant. Currently however, the shrub is overlooked and underutilized because of a few weaknesses. These weaknesses could be eliminated by creating polyploid versions of the plant using *in vitro* chemical mutagenesis. To achieve this, the micropropagation protocol for *V. agnus-castus* has been outlined and the effects of oryzalin exposure to the plant *in vitro* recorded. For micropropagation, cuttings were surfaced sterilized using detergent and bleach solution and were successfully introduced into sterile medium with 94% success rate. To multiply the cuttings, two cytokinins were tested at various concentrations. It was observed that hormone 6-benzylaminopurine (BA) at concentration of 4.44 μM is the most effective at inducing shoot formation without compromising the length of shoots produced as compared to hormone 6-(γ,γ -dimethylallylamino)purine (2iP). To induce root formation, two auxins were tested at various concentrations and the hormone indole-3-butyric acid (IBA) at concentration 5 μM was observed to be the most effective when compared to 1-naphthaleneacetic acid (NAA) after eight weeks of growth. Lastly, rooted tissue culture plants were successfully acclimated into the greenhouse with 85% survival during the acclimatization protocol. The application of oryzalin to *in vitro* plants produced phenotypic changes and the percent survival of *V. agnus-castus* after exposure to oryzalin was recorded at different concentrations and exposure times. After 16 weeks of observation, the lethal dose with 50% survival rate to oryzalin was determined to be at a concentration of 0.3 millimolar for 60 minutes exposure time.

BIOGRAPHICAL SKETCH

Nor Kamal Ariff Nor Hisham Shah, known as Nor by his friends, was born and raised in Malaysia. Growing up in a large city did not offer Nor the opportunity to grow plants and learn about them. However, he was able to visit his grandfather's orchards during school holidays and learned to appreciate plants. After finishing high school, Nor was selected to receive a scholarship to study abroad and was enrolled in Pennsylvania State University to study biotechnology. In August 2008, at the age of 18, he moved to the United States in pursuit of science and discovery.

Starting college is challenging for any 18-year-old, but the new learning environment and culture in the United States meant additional lessons other than just learning about science in lecture halls. As he progressed in his degree program, Nor began to learn about the limitless potentials of molecular biology and biotechnology. However, Nor was feeling unfulfilled as he wanted to observe how the theories learned in classes applied to a research environment. During his third year at Penn State, Nor was fortunate to be accepted in a plant science laboratory and learned under the guidance of Dr. Michael Robbins who was working on epigenetic effects on stress-induced maize plants. For two semesters, his spare time and holidays were spent in enhancing his laboratory skills and working in the greenhouse. It is during this time that the passion to pursue molecular biology and plants was born.

Upon returning home to Malaysia in 2012, after receiving his B.S. degree, his qualifications secured Nor a position as a molecular biologist at the Malaysian Genomics Resource Center

(MGRC), a commercial sequencing laboratory where he worked for seven months. While working at MGRC, Nor felt that more learning and training was required to have a successful career in this field of science. Nor applied for a Master's degree program at Cornell University and secured a scholarship to fund his study abroad.

In 2015, Nor returned to the United States for the second time to work on his graduate degree. For his Master's degree, he wanted to combine the two passions of biotechnology and plants into his project. Knowing this, his advisor, Dr. Mark Bridgen, suggested a project that involved tissue culture and an ornamental shrub called *Vitex* for his thesis.

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The horticulture industry requires a constant supply of new plants. The introduction of new plants ensures a supply of new products for growers to sell and make revenue for their establishments. Additionally, the introduction of new plant products will ensure a wide selection of products available for growers to cater to the different needs of their customers. Besides from ensuring a wide selection of products for growers and customers, new plants are needed to supplant the ones that are removed and banned from circulation. Because of these reasons, there is a constant demand from the industry for new plants in order for it to flourish and be profitable.

Based on the need of the industry, the long-term goal with *Vitex agnus-castus* is to introduce new cultivars into the industry. *V.agnus-castus* possesses many great ornamental qualities that could make it a profitable ornamental plant but it has several weaknesses that need to be addressed (Dirr, 2015) . *Vitex agnus-castus* has an aggressive growth habit, it is not cold tolerant, and it has limited flower colors. If these weaknesses are eliminated, coupled with the existing qualities that *Vitex* already possess, it has the potential to be a great plant.

The issue that was to be addressed first, was the aggressive growth habit of *Vitex*. To do this, it was decided that a suitable method to be used would be *in vitro* chemical mutagenesis using tubulin inhibitors. Chemical mutagenesis allows the production of polyploids of *Vitex* and those would have the potential to mollify its growth habit. If polyploids could be successfully created from the mutagenesis treatment, then plants could be produced with new phenotypes from the extra sets of chromosomes. Thus, the treatment with tubulin inhibitors will not only solve the issue with the growth habit, but also has the potential to produce novel phenotypes of *Vitex*.

While it is common for chemical mutagenesis to be performed *ex vivo*, performing the procedure *in vitro* offer several advantages. The tissue culture environment provides an optimum location for the plants to grow. The sterile location is fully controlled thus removing any external factors that might negatively influence the growth of the plants especially when they have been treated with mutagens. In addition, using tissue culture allows more individual plants to be treated as compared to doing the experiment in the greenhouse. Tissue cultured plants are small and do not require a lot of space as compared to plants grown in the greenhouse. More individuals can be grown in the laboratory and experimented on thus increasing the chance of finding more mutants from the mutagenesis treatment. Lastly performing the experiments *in vitro* confine the mutagens only in the laboratory. This ensures that no mutagen residue escapes into the greenhouse because treated plants are grown in culture vessels in the laboratory.

To perform *in vitro* chemical mutagenesis and to produce polyploid plants, a large number of plants *in vitro* is required due to the mortality that is experienced when plants are exposed to mutagens. Thus this research was designed to outline the micropropagation protocol for *V.agnus-castus*, to propagate the plants *in vitro*, and to increase the numbers in order to prepare for mutagenesis treatments.

1.2 *Vitex agnus-castus*

Vitex agnus-castus is known by many common names such as Abraham's balm, monk's pepper, chasteberry tree or the chaste tree and it is an important medicinal plant. It is native to the Mediterranean region and it was introduced and naturalized in the United States.

Besides being an important herb, *Vitex agnus-castus* also has great ornamental features (Gilman and Watson, 1994). The deciduous shrub has fragrant, palmately compound leaves that have five to seven lance-shaped leaflets with entire margins. The flowers are produced in mid to late summer, grow on a loose panicle, and are showy, fragrant, and attract pollinators. The *Vitex* plant is easy to grow in many types of soil as long as it has good drainage. In addition, the plant does not have any major pests or diseases; nor does the plant attract deer. *Vitex* plants can be grown in USDA winter hardiness Zones 9 to 5.

This underutilized shrub belongs to the family *Lamiaceae*, but it was initially classified under *Verbenaceae*, thus some sources would label it as its alternate family (USDA, n.d.). The genus has 250 species (Harley *et al.*, 2004) and because of the wide dispersion of the species in the Mediterranean and Asia region, growing in different climates of sub-tropical and tropical regions, it has been difficult to classify *Vitex* despite the use of sequencing technology (Bramley *et al.*, 2009).

There are several species of *Vitex* that are in cultivation for different uses and purposes but most are for ornamental purposes. One example is *Vitex negundo* commonly known as the Chinese Chastetree or Horseshoe *Vitex* that is native to Asia and Southeast Asia. The botanical features of *V. negundo* is very similar to *V. agnus-castus* except for its longer lanceolate lance shape leaves. The features are less ornamental than *V. agnus-castus* but it is suggested in that it might be more cold tolerant than *V. agnus-castus* (Missouri Botanical Garden, n.d.).

Another example of well-known *Vitex* is *Vitex trifolia*, also known as Arabian Lilac or Simpleleaf Chastetree. The plant is an ornamental plant that has three leaflets as compared to *V. agnus-castus* with five to seven leaflets, able to grow to five meters tall with the same aggressive growth habit. There are cultivars reported to *V. trifolia purpurea* that has purple underside leaves and another cultivar that is variegated (Top Tropicals, n.d.).

Another species is *Vitex rotundifolia* or the Beach *Vitex*; this plant is native to the seashores of the Pacific region. It was first introduced to be used to control erosion for beaches in the United States (Olsen and Bell, 2005). The shrub does not grow as tall as *V. agnus-castus*, growing only to about 1 to 1.5 meter in length. It has simple to obovate leaves but the growth habit is classified as aggressive because of the runners that it produces. Because of this, several studies and reports have been published and it is now classified as an invasive species in the United States (Cousins *et al.*, 2010).

1.3 Tissue culture

Plant tissue culture can be defined as the culture of protoplasts, cells, tissues, organs, embryos, or seed on nutrient medium *in vitro* under controlled environmental conditions (Pierik, 1987). Tissue culture is synonymous with many names such as cell, organ or *in vitro* culture. Regardless of the names used, tissue culture is a simple yet powerful technique that allows researchers to grow and manipulate plants in a sterile environment.

Tissue culture relies heavily on several scientific concepts and yet it can be performed at home with basic kitchen appliances and household chemicals. (Kyte *et al.*, 2013). This widely known

laboratory technique resulted from the hypothesis proposed by Gottlieb Haberlandt in 1902 (Haberlandt, 1902) that suggested plant cells are totipotent, thus were able to regenerate into new plants. The hypothesis of plant totipotency was tested and the components of tissue culture were investigated. The first development in tissue culture was shown by Knudson in 1922 (Knudson, 1922) when he successfully germinated and grew orchid seeds in defined medium. The medium developed was later known as Knudson C medium and was routinely used to germinate orchid seeds. The second development that further pushed the discovery of tissue culture was the discovery of root-forming substance in 1929 by Went (Went, 1929). Despite the discoveries made, none have yet able to multiply plants *in vitro* until the discovery in 1948 by Skoog and Tsui. Both researchers discovered kinetin which allowed shoot induction, thus enabling multiplication of plants in tissue culture (Skoog & Tsui, 1948). Skoog later collaborated with Miller and showed the importance of the ratio of auxin to cytokinin and the effects of the ratio on shoot induction, root and callus formation (Skoog & Miller, 1957). All of the advancement in tissue culture were analyzed by Murashige which gave birth to micropropagation in 1974 and the stages of micropropagation (Murashige, 1974).

1.4 Tissue culture media

Tissue cultured plants are grown on an artificial medium, which supplies all the necessary nutrients for healthy, vigorous growth. The medium provides all essential elements consisting of both macro- and micro-plant nutrients that make up the mineral composition of the growth medium.

Plant growth regulators, or growth substances, such as cytokinins, auxins, gibberellins, abscisic acid and their analogues and inhibitors are added to tissue culture media to regulate and manipulate the growth of tissue culture plants or organs.

Sucrose is most commonly added to the medium as a carbon source to replace the carbon that the plants usually fix via photosynthesis. Other organic compounds that are added include vitamins and sometimes amino acids. Coconut water (CW) is sometimes added to provide nutrients and plant growth substances (George *et al.*, 2008). The use of CW is limited in research because the precise composition is unknown; CW composition will vary largely and can therefore have an effect on the repeatability of experiments.

The most commonly used medium in tissue culture is the formulation outlined by Murashige and Skoog (Murashige and Skoog, 1962). Commonly referred to as MS medium, it was originally developed for optimal growth of tobacco callus. The elementary composition of plants varies, thus MS medium may not be the optimum medium for other plants. However, the salts in MS medium have been successfully used to micropropagate many plant species. MS can be adapted for other plants and can result in improved growth.

Plant tissue cultures can grow in either liquid media or semi-solid media with a solidifying agent. Agar and gellan gum are the most commonly used gelling agents (George *et al.*, 2008). There are, however, various alternatives to agar such as, synthetic polymers, alginate, and gelrite (Kyte *et al.*, 2013).

1.5 Micropropagation

When plants are multiplied vegetatively or asexually, all the offspring from a single plant can be classified as clones. This means that the genetic make-up of each offspring is identical to that of

all the other offspring and to that of the single parent. The term “cloning,” with respect to tissue culture, refers to the process of propagating in culture large numbers of selected plants with the same genotype as their parent plant (Kyte *et al.*, 2013). This is the process of micropropagation.

There are five stages (0-4) of micropropagation that are recognized internationally (Debergh and Maene, 1981). When micropropagation was first described, only three stages were identified and the stages were establishment, multiplication and preparation for re-establishment in soil (Murashige, 1974). The stages of micropropagation was later redefined by Anderson in 1980 (Anderson, 1980) to include 5 stages which starts with stock plant selection, establishment, multiplication, pre-transplant/rooting and transplantation. The additional stages were proven to be more effective for micropropagation as each stage has its own goals to be accomplished.

1.5.1 Stage 0

The stages of micropropagation start with stock plant selection designated as Stage 0. There are several requirements for this stage. During Stage 0, the mother or the donor plant is carefully examined in order to ensure future success of micropropagation (Read, 1992). The mother plant must be ensured to be the correct plant in terms of species or cultivar because this will be the starting material for plant cultures. If the wrong cultivar is chosen, it will be multiplied and all the clones will not be usable. Plants should be healthy and disease-free, having a relatively clean surface and at the right physiological age. The mother plant must be healthy since cloning a diseased plant will only multiply the pathogens along with the clones produced. It is preferred to select a donor plant that have relatively clean surface to ensure establishment will not require harsh decontamination procedure. Lastly, the donor plants should be selected for their physiological age

because young or immature part of the donor plant will respond better in tissue culture as compared to the adult or mature part of the plant. Many pretreatment methods have been formulated to ensure the requirements of Stage 0 can be met even for wild collected plants.

1.5.2 Stage 1

When all of the requirements of Stage 0 are met, the plants will be used for Stage 1: the establishment stage. There are two goals for stage 1: successfully decontaminate the surface of the plant and ensure that the decontamination process is not fatal. The protocol of the decontamination process varies depending on the starting materials to be used to establish the culture. Starting materials could be seeds, cuttings, tubers, roots, anther, pollen and even leaves. After selecting the starting material, the proper sterilization protocol must be selected to decontaminate the explants successfully without damaging them. Strong or harsh sterilization treatments can be selected for explants that are considered to be tough, such as seeds, and more delicate decontamination procedures must be selected for more sensitive explants such as soft tissue cuttings. Typically, stem cuttings would be used as the starting material for Stage 1; the common surface sterilization technique is to wash under running water followed by sterilization, usually with a sodium hypochlorite bleach or bleach solution. At the end of stage 1, the explants will be free from spores and bacteria. After sterilization, the explants are cultured on tissue culture medium for a couple of days to observe for any obvious contamination. If no obvious contamination is present the explants can be moved to stage II (George *et al.*, 2008).

1.5.3 Stage 2

Stage 2 is considered the proliferation or multiplication stage. The objective of stage 2 is to produce propagules which will be capable of growing into new plants. For stage 2, the explants established will be induced to produce shoots by using high cytokinin levels (Trigiano and Gray, 2005). There are several cytokinin that can be used to induce shoot production such as 6-benzylaminopurine (BA), kinetin, zeatin, and 6-(γ,γ -Dimethylallylamino)purine (2iP). However, depending on the species of the plants, some cytokinin are more effective than others at inducing shoots for a specific species. Another consideration that need to be addressed is the effect of apical dominance (Cline, 1991). Apical dominance might reduce the effect of cytokinin treatment; thus the apical bud may need to be removed in order to maximize shoot production. Since apical buds produced auxins innately, it might affect the cytokinin to auxin ratio during stage 2. Because it has been shown that the ratio of phytohormone will affect the growth of the explants (Skoog & Miller, 1957), optimum concentration of the right cytokinin need to be determined experimentally in order to maximize shoot production in Stage 2.

1.5.4 Stage 3

Stage 3 is the pre-transplant or rooting stage; the shoots that have been multiplied will be induced to root *in vitro*. The treatment to root explants *in vitro* utilizes phytohormones called auxins. There are several auxins that could be used such as indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), and indole-3-acetic acid (IAA). It has been shown that synthetic auxin such as IBA and NAA are more effective at inducing root compared to natural auxin IAA (Zimmerman and Wilcoxon, 1935). Explants are rooted *in vitro* for several reasons. Typically, plants that are selected for micropropagation are those that are hard to be propagated by normal propagation and

usually difficult to root. Root initiation or rooting *in vitro* speeds up the rooting phase and ensure high survivability of cloned microcuttings. Additionally, rooted cuttings will be easier to use in stage 4, which is the acclimatization phase since the rooted cuttings will be able to absorb water and thus mitigate the effect of water loss. In some situations, for fast-to-root plants, Stage 3 is skipped and cuttings are moved directly to Stage 4.

1.5.5 Stage 4

The fifth and last stage of micropropagation is Stage 4 where *in vitro* grown plants are transplanted or acclimated. In stage 4, the goal is to take *in vitro* propagated explants out of tissue culture. After the plants are transplanted, they are usually kept in high humidity environment (e.g. a mist tunnel) for several days and from there they are moved to the greenhouse (George *et al.*, 2008).

There are several factors that contribute to the success of this stage (Preece and Sutter, 1991). The first factor is water loss. *In vitro* plants grew in an artificial environment with high humidity. The humidity causes the plants to lack epicuticular wax thus making them prone to desiccation when placed in a lower humidity environments (Sutter and Langhans, 1979). In order to acclimatize them, they need to develop cuticle before they are to be taken out of tissue culture. To do this, the humidity of the growing environment need to be reduced gradually to allow the plants to develop cuticle layer.

The second factor that need to be considered is the effect of sugar supplement used to grow the plants. If stage 3 plants have been grown on a medium containing agar, the agar is usually washed away from the roots before transplanting to an appropriate rooting medium. It has been shown

that growing plants *in vitro* changed the physiology of the plants that the photosynthetic machinery of tissue cultured plants are not well equipped as their normal counter parts (Desjardins *et al.*, 1995). Tissue cultured plants have less dense chlorophylls and non-functioning or slow responding stomata. The photosynthetic machinery is not optimum because the plants have been using sugar as an energy source, negating the need to photosynthesize at full capacity. In order to activate the machinery, the plants need to be grown without sugar and this will cause the plants to start photosynthesizing on their own.

The last factor that needs to be considered at Stage 4 is light exposure or intensity. Tissue cultured plants are grown under artificial lights and the intensity of the light is very low. Plants taken out of culture need to be protected from full sun exposure. Full sun exposure not only will cause light stress, but also water stress. Tissue culture plants should be placed under shade before they are gradually exposed to full sunlight.

1.6 Application of tissue culture

There are many uses for tissue culture techniques in the field of horticulture, plant breeding, pharmaceutical and biotechnology (Pasqual *et al.*, 2014). Apart from mass production of clones using micropropagation, the sterile and controlled environment of tissue culture is used for germplasm preservation, manufacture of botanical substances, and production of disease-free plants.

Additionally, tissue culture is used for new plant production. In plant breeding, tissue culture is used to create homozygous recessive plants through haploid plant production by using anther or

pollen culture. These techniques cost less and use less time as compared to using traditional backcrossing methods to create homozygous plants. Another technique of creating new plant with tissue culture is called somatic fusion; this technique uses somatic fusion to fuse together two sexually incompatible plants. Protoplasts and cell cultures have allowed this technique to succeed; it is tremendously helpful in research and new plant development. Because of the ability to regenerate plants from cells, tissue culture is also utilized in the biotechnology field to create transgenic plants. Tissue cultured plants undergo a transformation process and new genes are inserted into them. The transformed cells are then regenerated into a new plant with the newly introduced gene giving the desirable trait.

Other tissue culture techniques that are used for plant breeding and development include embryo culture, somatic embryogenesis, synthetic seed production, callus culture, and flower culture. Lastly tissue culture has allowed the creation of mutant plants through a process called mutagenesis. Through the process of mutation breeding, mutagenesis has been a great source of many new plants.

1.7 Mutagenesis/ mutation breeding

When micropropagating plants, the mutation of plants and plant cells is a concern because clones are needed. However, plant breeders encourage mutagenesis to develop new plant introductions. Mutation is a source of variation for organisms and it occurs spontaneously in nature; some species are more prone to mutation than others. Before the discovery of inducible mutation, mutants could only be identified and selected from a population by chance; this is greatly inefficient for breeding and developing cultivars.

In 1927, the work of Muller (Muller, 1927) on fruit flies showed that it was possible to induce mutations with x-ray and the same concept was proved possible in plants by Stadler in 1928 (Stadler, 1928). Later in 1947, the effect of chemical mutagen was proven in fruit flies which led to more discovery on chemical mutagens (Auerbach, 1949). Thus, the methods for mutation induction are grouped into two categories: physical and chemical.

For physical mutagenesis, ionizing radiation is used and the source is varied from x-rays, radioactive materials, UV and even space as a source to alter the genetic makeup. For chemical mutagenesis, several chemicals were discovered to be able to induce mutation from alkylating agent, nitroso compounds, base analogues, phenols, and many more with different mutagen able to induce different type of mutation (Auerbach, 1973).

After the discovery, mutation was induced to create many mutants for breeding and horticulture industry. Some of the mutants created were registered and recorded in Mutant Variety Database and up to March of 2017, 3246 mutants are listed on the website (Mutant Variety Database, n.d.). The mutants registered include but not limited to rice, wheat, cotton, carnation, strawberry and many more. From the list of mutants created, it is evident that mutagenesis is used in many different breeding programs to achieve diverse breeding goals.

One of the methods that is used to create mutants by chemical induction is to use an herbicide commonly known as Oryzalin. Oryzalin has many commercial trade names such as Dirimal, EL-

119, Rycelan, Ryzelan, Ryzelon and Surflan, with the chemical name of 3,5-dinitro-N4, N4-dipropylsulfanilamide. It is in the chemical class of dinitroaniline sulfonamide.

The effect of oryzalin on microtubules were controversial until a study done by Morejohn *et al.* (Morejohn *et al.*, 1987) that showed oryzalin is able to disrupt the polymerization of tubulin in plant cells thus could be used to alter the ploidy level of plants. Additionally, oryzalin was observed to be more effective than colchicine at disrupting the tubulin in plants. After the discovery of mutagenic effect of oryzalin, it has been used extensively to produce many mutant plants.

To perform chemical mutagenesis using oryzalin, the chemical needs to be exposed to the plant of interest while considering several factors to ensure high probability of creating mutants. The first factor to be considered is the stage of growth of the meristem cells. The meristem cells need to be actively growing and dividing and that is because oryzalin only affects actively dividing cells. The chemical has been shown to disrupt the tubulin formed during cell division when cells try to divide the chromosome between the daughter cells. By ensuring the plants used are actively growing, the chance of creating a mutant will be greater.

The other factors that need to be considered involve the combination of concentration and exposure time of oryzalin. The combination of optimum concentration and exposure time will increase the chance of producing mutants. Using a concentration that is too high will lead to excessive cell damage and plant death while using too low of a concentration will not have enough of an effect to cause mutation. As for the exposure time, the plants must be exposed to the mutagen long enough to allow the mutagen to penetrate into the plants and disrupt the cell cycle to have an effect

but not to cause excessive damage that will cause the plants to die. Because of the combination of both concentration and exposure time, researchers used LD₅₀ or the lethal dose that cause 50% death as an indicator to determine the optimum combination of the concentration and exposure time for specific mutagen and plant of interest (Heinze and Schmidt, 1995).

Based on the result of the Master's Degree, I have learned that micropropagation of *Vitex agnus-castus* is possible using standard woody plant micropropagation protocol. In this project, cuttings of *V.agnus-castus* were surfaced sterilized using bleach solution, multiplied in tissue culture using cytokinin, BA, induced to root using auxin, IBA and successfully acclimatized into the greenhouse.

And for the mutagenesis of *Vitex*, the effects of oryzalin were documented for 16 weeks for the project. With the result obtained, the Lethal Dose of 50% (LD₅₀) of oryzalin on *Vitex* was determined and because of this, now it is possible to use oryzalin to create polyploid of *Vitex*.

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CHAPTER 2: MICROPROPAGATION OF *VITEX AGNUS-CASTUS*

2.1 Abstract

The objective of this study was to develop a micropropagation procedure for *Vitex agnus-castus*. Micropropagation is a tissue culture technique that allows the mass production of clones. This technique will allow the production of clones of *Vitex agnus-castus in vitro*. The long-term goal is to use this micropropagation technique to clone mutant plants that are produced by chemical mutagenesis studies in the future. To clone *Vitex* efficiently, the protocol for each of the micropropagation stages in this research was outlined, except for stage 0. For stage 1, cuttings of *Vitex* were successfully introduced into tissue culture with a 94% success rate using a bleach solution wash. The cuttings were then grown in two cytokinin media with several concentrations for four and eight weeks to promote shoot multiplication. It was observed that 6-benzylaminopurine (BA) is more effective at inducing shoots when compared to 6-(γ,γ -dimethylallylamino)purine (2iP) and the optimum concentration of BA to use is 4.44 μM . For stage 3, to induce rooting, two auxins were tested at several concentrations for four and eight weeks. Indole-3-butyric acid (IBA) produced more rooted explants when compared to explants that were grown in 1-naphthaleneacetic acid (NAA) after eight weeks of growth. The optimum concentration of IBA to use is 5 μM for eight weeks. Lastly, the procedure for stage 4 was outlined and rooted tissue culture plants were successfully acclimatized into the greenhouse with 84% explants surviving the acclimatization procedure.

2.2 Introduction

Vitex agnus-castus or the chaste tree is a large shrub that is important both as a medicinal and an ornamental plant. It is well known for its medicinal properties in hormone regulations (Snow, 1996); research is actively being conducted to prove the medicinal properties of the shrub (Van

die *et al.*, 2012). In addition to being an important herbal medicine, the shrub also possesses ornamental properties with promising marketable value (Dirr, 2015). The shrub has fragrant, palmately compound leaves and produces spikes of lavender flowers in the late summer that attract pollinators (Gilman and Watson, 1994). It can grow in many types of soil with good drainage and the shrub is not bothered by deer grazing. Lastly, *Vitex* is not known to be affected by any major pest or diseases.

Vitex has a few weaknesses (Dirr, 2015); the shrub has an aggressive growth habit and can grow up to 20 feet in length and width. This quality requires pruning to maintain the plant in a manageable shape and likely deters home owners and landscape architects to use *Vitex* in a landscape. Another weakness of *Vitex agnus-castus* is that it has limited flower colors. Currently the flower colors that are available commercially are limited to two: purple-blue and pink. Lastly, the shrub is not cold tolerant or hardy in zones colder than zone 7 (USDA, n.d.). This limits the landscape or area that the plant can be used.

Considering the economic potential of *Vitex*, a breeding program will be able to improve *Vitex* as an ornamental plant by mitigating or breeding out the weaknesses identified in the plant. With this in mind, a long-term plan to improve *Vitex* by using *in vitro* chemical mutagenesis to alter the ploidy level of its chromosomes in order to obtain a new variety with novel phenotypes requires an *in vitro* micropropagation protocol. A successful micropropagation protocol for *Vitex* would allow the production of an ample supply of the plant *in vitro* for mutation purposes. Subsequently a micropropagation procedure would also allow new cultivars of *Vitex* to be clonally propagated if a new mutant is identified from the *in vitro* chemical mutagenesis treatments.

Micropropagation is one of several plant tissue culture techniques that allows the production of clones in an optimum artificial environment. The idea of micropropagation of plants was first proposed by Gottlieb Haberlandt in 1902 who suggested plant cells are totipotent and thus were able to regenerate into new plants (Haberlandt, 1902). The idea prompted research into growing plants in sterile environments. The first discovery that suggested it is plausible to grow plants *in vitro* was when Knudson successfully germinated orchid seed in a defined medium (Knudson, 1922). When it was proven possible to grow plants *in vitro*, it led to more research into plant regeneration and thus led to the discovery of root forming substance by Went in 1929 (Went, 1929) and shoot multiplication with kinetin in 1948 by Skoog and Tsui (Skoog and Tsui, 1948). These fundamental discoveries of growing plants *in vitro*, and the ability to induce shoot and root formation, gave birth to micropropagation in 1974 by Murashige when he outlined the stages of micropropagation (Murashige, 1974).

Toshio Murashige identified three main stages of micropropagation: establishment, multiplication and preparation for re-establishment in soil. These three stages eventually evolved into five stages that are internationally recognized (Kyte *et al.*, 2013). The five stages of micropropagation are now stock plant selection, establishment, multiplication, pre-transplant/rooting and transplantation. The additional stages that were added were proven to be more effective for micropropagation as each stage has its own goals to be accomplished.

To successfully outline the micropropagation protocol for *Vitex*, it is imperative to understand each of the stages that is involved and their goals. The first stage of micropropagation is designated as Stage 0 which is the selection stage. In Stage 0, there are several goals that need to be fulfilled to

ensure the success in the next stage. The first one is to identify the plant of interest correctly in terms of species or cultivar as this will be the starting material for plant cultures. In addition, the individual selected should be healthy and disease free, having a relatively clean surface and at the right physiological age. The mother plant must be healthy since cloning a diseased plant will only multiply the pathogens along with the clones produced. It is preferred to select a donor plant that have relatively clean surface to ensure establishment will not require harsh decontamination procedure. Lastly, the donor plants should be selected for its physiological age because young or immature part of the donor plant will respond better in tissue culture as compared to the adult or mature part of the plant. Many pretreatment methods have been formulated to ensure the requirements of Stage 0 can be met even for wild collected plants.

When all the requirements of Stage 0 are met, the plants will be used for Stage 1: the establishment stage. There are two goals for stage 1: to successfully decontaminate the surface of explant and to ensure that the decontamination process is not fatal. The protocol of the decontamination process varies depending on the starting materials that are to be used to establish the culture. Starting materials can be seeds, cuttings, tubers, roots, anther, pollen and leaves. After selecting the starting material, the proper sterilization protocol must be selected to decontaminate the explants successfully without damaging them. Strong or harsh sterilization treatments can be selected for explants that are tough, such as seeds, and more delicate decontamination procedure must be selected for a more sensitive explant such as soft tissue cuttings. Typically, stem cuttings will be used as the starting material and the common surface sterilization is to wash under running water followed by sterilization with a sodium hypochlorite or bleach solution. At the end of stage 1, if successful, the explants will be free from spores and bacteria. After sterilization and a rinse in

sterile water, the explants are cultured on tissue culture medium for a few days to observe for any obvious contamination. If no obvious contamination is present, the explants can be moved to stage II (George *et al.*, 2008).

Stage 2 is considered the proliferation or multiplication stage. The objective of Stage 2 is to produce propagules which will be capable of growing into new plants. For stage 2, the explants that are established will be induced to produce shoots by using high cytokinin levels (Trigiano and Gray, 2005). There are several cytokinins that can be used to induce shoot production such as benzyl aminopurine (BA), kinetin, zeatin, and 6-(γ,γ -dimethylallylamino)purine (2iP). Depending on the species of the plants, some cytokinins are more effective than others at inducing shoots for a specific species. Another consideration that needs to be addressed is the effect of apical dominance. Apical dominance might reduce the effect of cytokinin treatments, thus apical buds need to be removed to maximize shoot production. Since apical buds produce auxins innately, they might affect the cytokinin to auxin ratio during Stage 2. Because it has been shown that the ratio of phytohormones will affect the growth of the explants, optimum concentrations of the best cytokinin needs to be determined experimentally to maximize shoot production in Stage 2.

Stage 3 is the pre-transplant or rooting stage; here, the shoots that were multiplied during Stage 2 will be induced to root *in vitro*. The treatment to root explants *in vitro* utilizes phytohormones called auxins. There are several auxins that can be used such as indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), and indole-3-acetic acid (IAA). It has been shown that synthetic auxin such as IBA and NAA are more effective at inducing root compared to natural auxin IAA (Kyte *et al.*, 2013). Explants are rooted *in vitro* for several reasons. Typically, plants that are

selected for Stage 3 rooting are those that are difficult to be rooted by normal propagation or plants of high economic value. Root initiation or rooting *in vitro* speeds up the rooting phase and ensure high survivability of cloned microcuttings. Additionally, rooted cuttings will be easier to use in Stage 4, which is the acclimatization phase, since the rooted cuttings will be able to absorb water and mitigate the effect of water loss. In some situations, for east-to-root plants, Stage 3 is skipped and cuttings are moved directly to Stage 4.

The fifth and last stage of micropropagation is Stage 4, where *in vitro* grown plants are acclimated; this is called the transplanted or acclimated stage. In Stage 4, the goal is to take *in vitro* propagated explants out of tissue culture. After the plants are transplanted, they are usually kept in high humidity environment (e.g. a mist tunnel) for several days and from there they are moved to the greenhouse (George *et al.*, 2008).

There are several factors that contribute to the success of Stage 4. The first factor that is considered is water loss. *In vitro* plants grow in an artificial environment with high humidity. The humidity causes the plants to lack epicuticular wax thus making them prone to desiccation when placed in a lower humidity environments (Sutter and Langhans, 1979). In order to acclimatize them, they need to develop cuticle before they are to be taken out of tissue culture. To do this, the humidity of the growing environment need to be reduced gradually in order to allow the plants to develop a cuticle layer.

The second factor that needs to be considered is the effect of the sugar supplement that is used to grow the plants. The photosynthetic machinery of tissue cultured plants is not as functional as their

normal counter parts because they were raised on sugar. Tissue cultured plants have less dense chlorophylls, less stomata and many other morphological differences. The photosynthetic machinery is not optimum because the plants have been using sugar as an energy source, negating the need to photosynthesize at full capacity. In order to activate the machinery, the plants need to be grown without sugar and this will cause the plants to start photosynthesizing on their own. If Stage 3 plants have been grown on a medium containing agar, the agar is usually washed away from the roots before transplanting to an appropriate rooting medium.

The last factor that needs to be considered is light exposure or intensity. Tissue cultured plants are grown under artificial lights and the intensity of the light is very low. Plants taken out of culture need to be protected from immediate full sun exposure. Full sun exposure not only will cause light stress, but also water stress. Tissue culture plants should be placed under shade before they are gradually exposed to full sunlight.

2.3 Materials and Methods.

Seeds of *Vitex agnus-castus* were used as the original source of plant material for this research. Fresh seeds were harvested from several plants growing on Long Island, New York. The seeds were germinated and grown in the greenhouse for approximately three to five months before stem cuttings were taken to be used as explants for tissue culture initiation.

2.3.1 Surface sterilization – Stage 1

Stem cuttings were taken from plants, approximately 3-5 months old, which were actively growing in the Cornell University greenhouses. Cuttings were wrapped in wet paper towels and transported

to the laboratory in a closed container. The apical meristem and the leaves were removed and the cuttings were cut to 7-10 cm. The cuttings were then individually washed under warm running water for 30-60 seconds before they were stirred in soapy solution for 30 seconds. The cuttings were washed under running water for another 30-60 seconds to remove the soapy solution. After washing, the cuttings were placed in Magenta™ boxes containing 200 ml of sterile water and placed under the laminar air flow hood before they were soaked in 10% bleach (8.25% NaOCl) solution for 15 minutes with occasional stirring every 5 minutes. After 15 minutes, the cuttings were washed three times with sterile water to remove any bleach solution. Then individual nodes, with internodes removed, were cultured into test tubes containing 10 ml half strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) nutrient medium with vitamins, 30 g/l sucrose, and 6 g/l agar micropropagation grade (Phytotech Laboratories®) with pH calibrated to 5.70- 5.75. The explants were then placed under cool white florescent lights and allowed to grow for four weeks. Contamination and survivability of explants were recorded after four weeks.

2.3.2 Shoot multiplication – Stage 2

For shoot multiplication media, full strength MS media was prepared with 30 g/l sucrose, 6 g/l agar and the pH calibrated to 5.70-5.75 after cytokinin was added, and then autoclaved. The media was poured into Magenta™ boxes with 40 ml per box and five explants were cultured into each box.

The explants used for the experiment were maintained in full strength MS media. The explants were cut into approximately 2.5 cm in length with the apical meristem removed to remove apical dominance. Twenty explants were used for each treatment with a total of 9 treatments including

the control. The treatments were control (no growth regulator), 0.44 μM BA, 2.22 μM BA, 4.44 μM BA, 22.2 μM BA, 0.5 μM 2iP, 2.5 μM 2iP, 5 μM 2iP, and 24.6 μM 2iP. The explants were allowed to grow for four or eight weeks before the number of shoots and the length of shoots produced were recorded.

2.3.3 Rooting – Stage 3

For rooting media, full strength MS medium was prepared with 30 g/l sucrose, 6 g/l agar and the pH was calibrated to 5.70-5.75 after auxins were added, and then autoclaved. Twenty explants were used for each concentration tested and the explants were taken from stock plants that were maintained in full strength MS media supplemented with 4.44 μM BA. The explants were cut to 2.5 cm in length, each with the apical meristem included on the explant. The explants were cultured in media supplemented with either IBA or NAA at concentrations of 0.5 μM IBA, 2.5 μM IBA, 5 μM IBA, 24.6 μM IBA, 0.54 μM NAA, 2.7 μM NAA, 5.4 μM NAA, or 26.9 μM NAA. Control medium were also prepared with no auxin present. Five explants were cultured into one Magenta™ box and they were allowed to grow for four or eight weeks before the number of explant rooted and the length of primary roots produced were measured.

2.3.4 Acclimatization – Stage 4

For these experiments, explants were taken from plants that were grown *in vitro* and maintained on MS media supplemented with 5 μM IBA. The explants were washed under running water to remove tissue culture media before they were potted into Magenta™ boxes containing sterile 50:50 mix of perlite and peat moss. Four explants were placed inside each box and thin transparent plastic bags were used to loosely cover the top of the magenta boxes. The explants were then placed under

fluorescent light for four weeks. The explants were watered with 15 ml sterile water on the first day and every two days after or as needed. After one week, a vertical slit with 0.5 cm in length was made on the plastic cover and the length of the opening was doubled every four days. On the fourth week, after two days with the opening of the slit to be 8 cm, the explants were taken to the greenhouse and potted into 10 cm pots and were placed under shade cloth. The survival of the explants was recorded at the end of the 5th week.

2.3.5 Result analysis

The data obtained for shoot multiplication and rooting experiments were analyzed using JMP PRO version 12 software produced by SAS Institute Inc. (JMP®, Version 12. SAS Institute Inc). Each treatment in the experiments were given a treatment number in order to perform statistical analysis.

2.4 Results.

2.4.1 Surface sterilization – Stage 1

Table 1 summarizes the results of the surface sterilization method. Cuttings that were taken from the greenhouse were sterilized before they were cultured individually in test tubes. Fifty nodes were obtained from the sterilized cuttings and after four weeks in culture, three test tubes were observed to be contaminated. The success of the procedure was calculated to be 94%.

Table 1. Results of surface sterilization method on *Vitex agnus-castus* plants. Cuttings were successfully introduced into a sterile environment with a 94% success rate.

Number of explants/nodes	Number of contaminated explants	Percent Success
50	3	94 %

2.4.2 Shoot multiplication for four weeks of growth – Stage 2

To analyze the number of shoots that were produced on the different media, a one-way ANOVA test was done with the data collected (Table 2). The test showed that the treatments done in the experiment were significant with a P-value of less than 0.05. To visualize the results, a bar graph was made from the ANOVA result (Figure 1).

Table 2. Results of ANOVA analysis after 4 weeks *in vitro* growth of *Vitex agnus-castus* plants for shoot production in different cytokinin treatments.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	58.57004	7.32125	12.5003
Error	343	200.89041	0.58569	Prob > F
C. Total	351	259.46045		<.0001*

To further analyze the results and confirm the trend observed from the graph, a Tukey test was conducted and each treatment was compared to one another to determine if the treatments had any significant effects on shoot production (Table 3).

Based on the data obtained from the Post Hoc ANOVA Tukey test, it showed that 0.44 μM BA and 0.5 μM 2iP did not affect shoot production when compared to the control treatment. When the cytokinin concentrations were increased to 2.2 μM for BA and 2.5 μM for 2iP, both BA and 2iP showed that it improved shoot production as compared to the control.

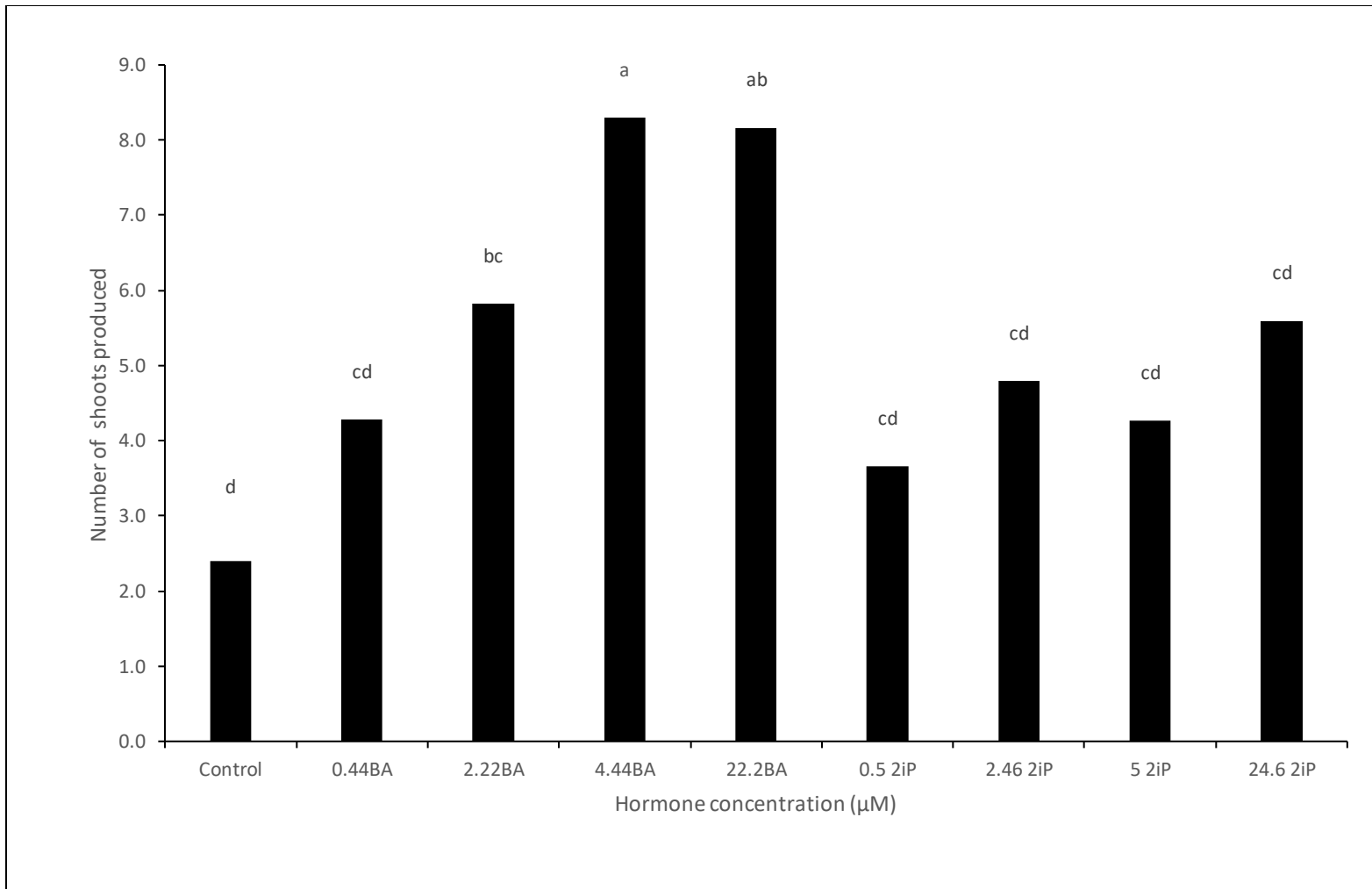


Figure 1. Graph of the number of shoots that were produced after *Vitex agnus-castus* plants were grown for four weeks in different cytokinin treatments. Treatments that do not share the same letter are significantly different. The α value was set to 0.05.

Table 3. Tukey test results of four weeks shoot production of *Vitex agnus-castus* plants grown *in vitro* in different cytokinin treatments.

Hormone	Hormone	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
4.44μM BA	Control	1.329247	0.1722201	0.791623	1.866871	<.0001
22.2μM BA	Control	1.305891	0.1711266	0.77168	1.840101	<.0001
4.44μM BA	0.5μM 2iP	0.965725	0.1722201	0.428102	1.503349	<.0001
22.2μM BA	0.5μM 2iP	0.942369	0.1711266	0.408159	1.476579	<.0001
2.22μM BA	Control	0.862376	0.1711266	0.328166	1.396587	<.0001
24.6μM 2iP	Control	0.814361	0.1771329	0.261401	1.367321	0.0002
4.44μM BA	5μM 2iP	0.813882	0.1733067	0.272866	1.354897	0.0001
4.44μM BA	0.44μM BA	0.811682	0.1722201	0.274058	1.349306	0.0001
22.2μM BA	5μM 2iP	0.790525	0.1722201	0.252901	1.328149	0.0002
22.2μM BA	0.44μM BA	0.788325	0.1711266	0.254115	1.322535	0.0002
4.44μM BA	2.5μM 2iP	0.690098	0.1733067	0.149083	1.231114	0.0027
22.2μM BA	2.5μM 2iP	0.666742	0.1722201	0.129118	1.204366	0.0041
2.5μM 2iP	Control	0.639149	0.1722201	0.101525	1.176772	0.0073
0.44μM BA	Control	0.517565	0.1711266	-0.016645	1.051776	0.0659
5μM 2iP	Control	0.515366	0.1722201	-0.022258	1.052989	0.072
4.44μM BA	24.6μM 2iP	0.514886	0.1781895	-0.041373	1.071144	0.0948
2.22μM BA	0.5μM 2iP	0.498855	0.1711266	-0.035356	1.033065	0.0887
22.2μM BA	24.6μM 2iP	0.491529	0.1771329	-0.061431	1.044489	0.1267
4.44μM BA	2.22μM BA	0.466871	0.1722201	-0.070753	1.004494	0.1478
24.6μM 2iP	0.5μM 2iP	0.45084	0.1771329	-0.102121	1.0038	0.2144
22.2μM BA	2.22μM BA	0.443514	0.1711266	-0.090696	0.977724	0.1939
0.5μM 2iP	Control	0.363522	0.1711266	-0.170688	0.897732	0.4585
2.22μM BA	5μM 2iP	0.347011	0.1722201	-0.190613	0.884635	0.534
2.22μM BA	0.44μM BA	0.344811	0.1711266	-0.189399	0.879021	0.534
24.6μM 2iP	5μM 2iP	0.298996	0.1781895	-0.257263	0.855254	0.7597
24.6μM 2iP	0.44μM BA	0.296796	0.1771329	-0.256164	0.849756	0.7611
2.5μM 2iP	0.5μM 2iP	0.275627	0.1722201	-0.261997	0.813251	0.8042
2.22μM BA	2.5μM 2iP	0.223228	0.1722201	-0.314396	0.760851	0.932
24.6μM 2iP	2.5μM 2iP	0.175213	0.1781895	-0.381046	0.731471	0.9872
0.44μM BA	0.5μM 2iP	0.154044	0.1711266	-0.380167	0.688254	0.9929
5μM 2iP	0.5μM 2iP	0.151844	0.1722201	-0.38578	0.689468	0.9938
2.5μM 2iP	5μM 2iP	0.123783	0.1733067	-0.417233	0.664799	0.9986
2.5μM 2iP	0.44μM BA	0.121583	0.1722201	-0.41604	0.659207	0.9987
2.22μM BA	24.6μM 2iP	0.048015	0.1771329	-0.504945	0.600975	1
4.44μM BA	22.2μM BA	0.023357	0.1722201	-0.514267	0.56098	1
0.44μM BA	5μM 2iP	0.0022	0.1722201	-0.535424	0.539824	1

However, there is no significant difference on shoot production between 2.2 μM BA and 2.5 μM 2iP. Shoot production for 4.4 μM BA showed that it is more significant than control and 5 μM 2iP but it did not show any significant difference than 2.2 μM BA thus there is no difference in shoot production between 2.2 μM BA and 4.4 μM BA. For the 22.2 μM BA tested, the data showed that 22.2 μM BA is more significant than the control treatment, 0.44 μM BA, 0.5 μM 2iP, 2.5 μM 2iP and 5 μM 2iP but there is no significant difference between 22.2 μM BA and 2.2 μM BA or 4.4 μM BA. Lastly treatment 24.6 μM 2iP showed that it is more significant than the control and not significant with all of the other treatments.

2.4.3 Average shoot length analysis for four weeks of growth.

The same ANOVA test was done on the average shoot length of the shoots produced after four weeks of growth (Table 4). The test showed that the treatments tested for the experiment was significant and thus the least square mean table was used to graph the result and visualize the data (Figure 2).

Table 4. ANOVA analysis of average shoot length of plants of *Vitex agnus-castus* after four weeks *in vitro*.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	3.497167	0.437146	2.3502
Error	341	63.427007	0.186003	Prob > F
C. Total	349	66.924174		0.0180*

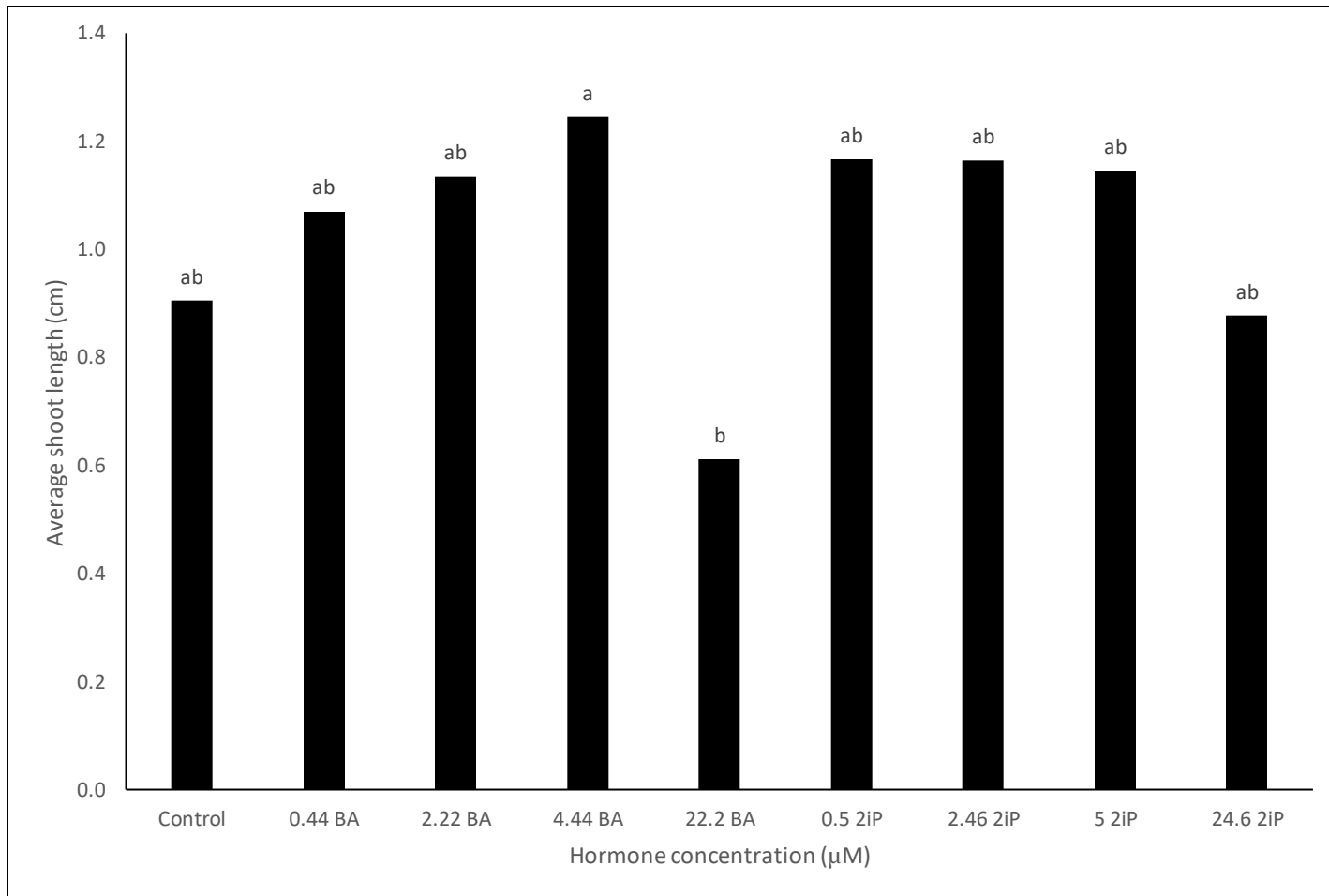


Figure 2. Graph showing average shoot length for four weeks *in vitro* growth of *Vitex agnus-castus* plants in different cytokinin treatments. Treatments that do not share the same letter are significantly different. The α value was set to 0.05.

To analyze the results of the graph, the Tukey Test was used to determine the differences of shoot length between all of the treatments. Based on the test, there were no significant differences of shoot length between the treatments except for one (Table 5). This means that the average length of shoots that were produced was similar in length in all the treatments except between treatment of 4.44 μM BA and treatment of 22.2 μM BA. In these two treatments, the shoots that were produced with 22.2 μM BA are significantly shorter than the shoots produced with 4.44 μM BA (Table 5).

2.4.4 Shoot multiplication for eight weeks of growth.

Analysis of Variance (ANOVA) was completed on the shoots that were produced after eight weeks of growth on different concentrations of BA and 2iP media. The results showed that the treatments had a P-value less than 0.0001 thus making the treatments significant (Table 6). Based on the ANOVA test, a graph bar was constructed based on the mean values in order to visualize the results (Figure 3).

It can be seen that the increase in BA supplements into the media increased the number of shoots that were produced (Figure 3). The pattern of the number of shoots produced for BA media is sigmoidal as it increased from concentration of 0.44 μM BA and peaked at 4.44 μM BA before it declines at 22.2 μM BA. For 2iP, the graph of shoots produced has an irregular pattern. The number of shoots increased when 2iP concentration was increased from 0.5 μM to 2.46 μM but it declines at 5 μM before it increased exponentially at 24.6 μM . To further analyze the results, Tukey Test was done to compare the effects of each hormone concentrations tested with each other (Table 7).

Table 5. Tukey test results of average shoot length after four weeks of growth *in vitro* of *Vitex agnus-castus* plants in different cytokinin treatments.

Hormone	Hormone	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
4.44 μ M BA	22.2 μ M BA	0.3333106	0.0970535	0.030325	0.6362963	0.019
0.5 μ M 2iP	22.2 μ M BA	0.2980315	0.0970535	-0.004954	0.6010173	0.0579
2.5 μ M 2iP	22.2 μ M BA	0.2969751	0.0970535	-0.006011	0.5999608	0.0597
5 μ M 2iP	22.2 μ M BA	0.2878393	0.0976979	-0.017158	0.5928369	0.0817
2.22 μ M BA	22.2 μ M BA	0.2830807	0.0964373	-0.017981	0.5841427	0.0841
0.44 μ M BA	22.2 μ M BA	0.2522563	0.0964373	-0.048806	0.5533183	0.1839
4.44 μ M BA	24.6 μ M 2iP	0.1789821	0.1004175	-0.134506	0.4924698	0.6939
Control	22.2 μ M BA	0.1688559	0.0964373	-0.132206	0.4699179	0.7143
4.44 μ M BA	Control	0.1644547	0.0970535	-0.138531	0.4674404	0.7497
24.6 μ M 2iP	22.2 μ M BA	0.1543285	0.0998221	-0.1573	0.4659573	0.8328
0.5 μ M 2iP	24.6 μ M 2iP	0.143703	0.1004175	-0.169785	0.4571907	0.8852
2.5 μ M 2iP	24.6 μ M 2iP	0.1426466	0.1004175	-0.170841	0.4561343	0.8894
5 μ M 2iP	24.6 μ M 2iP	0.1335108	0.1010405	-0.181922	0.4489434	0.9245
0.5 μ M 2iP	Control	0.1291756	0.0970535	-0.17381	0.4321614	0.9215
2.22 μ M BA	24.6 μ M 2iP	0.1287522	0.0998221	-0.182877	0.440381	0.9339
2.5 μ M 2iP	Control	0.1281192	0.0970535	-0.174867	0.4311049	0.9249
5 μ M 2iP	Control	0.1189835	0.0976979	-0.186014	0.4239811	0.9522
2.22 μ M BA	Control	0.1142248	0.0964373	-0.186837	0.4152868	0.9594
0.44 μ M BA	24.6 μ M 2iP	0.0979278	0.0998221	-0.213701	0.4095566	0.9874
0.44 μ M BA	Control	0.0834004	0.0964373	-0.217662	0.3844624	0.9946
4.44 μ M BA	0.44 μ M BA	0.0810543	0.0970535	-0.221931	0.38404	0.9957
4.44 μ M BA	2.22 μ M BA	0.0502299	0.0970535	-0.252756	0.3532156	0.9999
0.5 μ M 2iP	0.44 μ M BA	0.0457752	0.0970535	-0.257211	0.348761	0.9999
4.44 μ M BA	5 μ M 2iP	0.0454712	0.0983063	-0.261425	0.3523679	0.9999
2.5 μ M 2iP	0.44 μ M BA	0.0447188	0.0970535	-0.258267	0.3477045	0.9999
4.44 μ M BA	2.5 μ M 2iP	0.0363355	0.0976658	-0.268562	0.3412328	1
5 μ M 2iP	0.44 μ M BA	0.035583	0.0976979	-0.269415	0.3405807	1
4.44 μ M BA	0.5 μ M 2iP	0.0352791	0.0976658	-0.269618	0.3401764	1
2.22 μ M BA	0.44 μ M BA	0.0308244	0.0964373	-0.270238	0.3318864	1
0.5 μ M 2iP	2.22 μ M BA	0.0149508	0.0970535	-0.288035	0.3179365	1
Control	24.6 μ M 2iP	0.0145274	0.0998221	-0.297101	0.3261562	1
2.5 μ M 2iP	2.22 μ M BA	0.0138944	0.0970535	-0.289091	0.3168801	1
0.5 μ M 2iP	5 μ M 2iP	0.0101922	0.0983063	-0.296704	0.3170889	1
2.5 μ M 2iP	5 μ M 2iP	0.0091358	0.0983063	-0.297761	0.3160324	1
5 μ M 2iP	2.22 μ M BA	0.0047586	0.0976979	-0.300239	0.3097562	1
0.5 μ M 2iP	2.5 μ M 2iP	0.0010564	0.0976658	-0.303841	0.3059538	1

Table 6. ANOVA analysis of number of shoots produced *in vitro* from *Vitex agnus-castus* plants after eight weeks growth in different cytokinin treatments.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	97.61360	12.2017	7.6431
Error	335	534.80522	1.5964	Prob > F
C. Total	343	632.41881		<.0001*

Based on the Tukey test result (Table 7), none of the 2iP concentrations that were tested had a significant difference in shoot production when compared to the control except for the concentration of 24.6 μM of 2iP. As for BA, the only concentration that did not produce significant result for shoot production when compared to the control was BA with 0.44 μM . The shoot production of BA with concentration of 4.44 μM and 2.22 μM were significant when compared to shoot production of 2iP with concentration of 0.5 μM , 2.46 μM and 5 μM . Lastly, despite producing the most shoots for the 2iP concentration tested, the number of shoots produced by 24.6 μM 2iP were only significantly different than the control, 0.44 μM BA and 0.5 μM 2iP.

2.4.5 Average shoot length for eight weeks of growth.

Analysis of variance (ANOVA) was conducted on the average shoot length of *Vitex agnus-castus* after eight weeks of growth *in vitro*. The analysis showed that the P value has a value smaller than 0.05 thus the treatments were significant (Table 8).

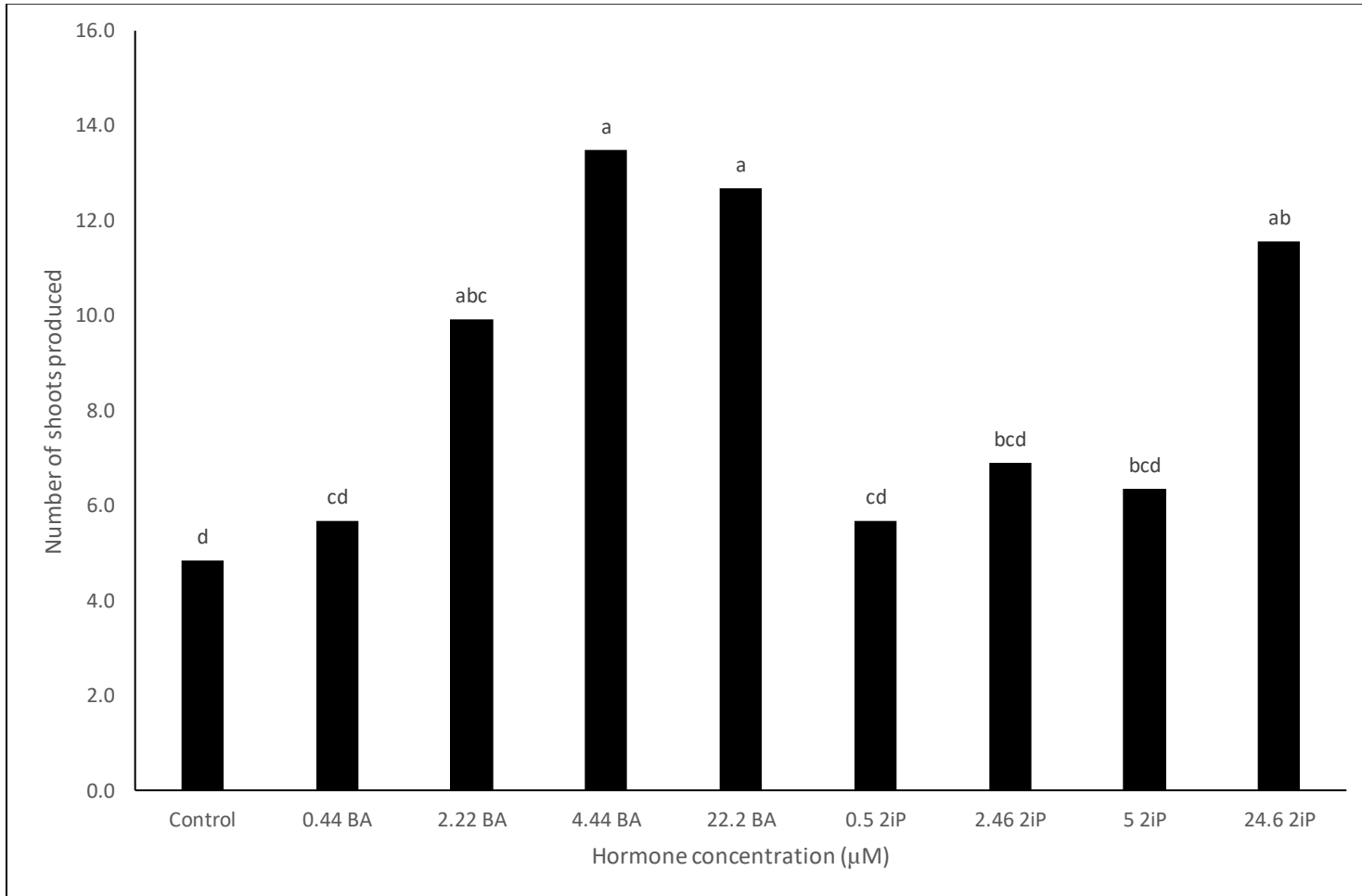


Figure 3. Bar graph of average number of shoots produced *in vitro* from *Vitex agnus-castus* after eight weeks growth in different cytokinin treatments. Treatments that do not share the same letter are significantly different. The α value was set to 0.05.

Table 7. Tukey test analysis of number of shoots produced *in vitro* from *Vitex agnus-castus* after eight weeks growth in different cytokinin treatments.

Hormone	Hormone	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
4.44 μ M BA	Control	1.47213	0.2947283	0.551929	2.392332	<.0001
22.2 μ M BA	Control	1.360393	0.2843326	0.472649	2.248137	<.0001
4.44 μ M BA	0.44 μ M BA	1.28837	0.2947283	0.368168	2.208572	0.0006
4.44 μ M BA	0.5 μ M 2iP	1.287491	0.2982706	0.356229	2.218752	0.0007
24.6 μ M 2iP	Control	1.201607	0.2862206	0.307968	2.095246	0.0011
22.2 μ M BA	0.44 μ M BA	1.176633	0.2843326	0.288888	2.064377	0.0015
22.2 μ M BA	0.5 μ M 2iP	1.175753	0.2880028	0.27655	2.074957	0.0018
4.44 μ M BA	5 μ M 2iP	1.153019	0.2982706	0.221757	2.08428	0.0042
4.44 μ M BA	2.5 μ M 2iP	1.045831	0.3001679	0.108646	1.983017	0.0161
22.2 μ M BA	5 μ M 2iP	1.041281	0.2880028	0.142078	1.940485	0.0103
24.6 μ M 2iP	0.44 μ M BA	1.017847	0.2862206	0.124208	1.911486	0.0127
24.6 μ M 2iP	0.5 μ M 2iP	1.016967	0.2898669	0.111944	1.921991	0.0149
2.22 μ M BA	Control	0.951686	0.2825273	0.069579	1.833794	0.0236
22.2 μ M BA	2.5 μ M 2iP	0.934094	0.2899673	0.028757	1.839431	0.0373
24.6 μ M 2iP	5 μ M 2iP	0.882496	0.2898669	-0.022528	1.787519	0.0624
24.6 μ M 2iP	2.5 μ M 2iP	0.775308	0.2918189	-0.13581	1.686426	0.1677
2.22 μ M BA	0.44 μ M BA	0.767926	0.2825273	-0.114182	1.650034	0.1454
2.22 μ M BA	0.5 μ M 2iP	0.767047	0.2862206	-0.126593	1.660686	0.159
2.22 μ M BA	5 μ M 2iP	0.632575	0.2862206	-0.261064	1.526214	0.4015
2.22 μ M BA	2.5 μ M 2iP	0.525387	0.2881973	-0.374423	1.425198	0.6669
4.44 μ M BA	2.22 μ M BA	0.520444	0.2947283	-0.399758	1.440646	0.7047
2.5 μ M 2iP	Control	0.426299	0.2881973	-0.473512	1.32611	0.8646
22.2 μ M BA	2.22 μ M BA	0.408706	0.2843326	-0.479038	1.296451	0.8826
5 μ M 2iP	Control	0.319112	0.2862206	-0.574528	1.212751	0.9717
4.44 μ M BA	24.6 μ M 2iP	0.270523	0.2982706	-0.660738	1.201785	0.9925
24.6 μ M 2iP	2.22 μ M BA	0.249921	0.2862206	-0.643719	1.14356	0.9942
2.5 μ M 2iP	0.44 μ M BA	0.242539	0.2881973	-0.657272	1.14235	0.9955
2.5 μ M 2iP	0.5 μ M 2iP	0.241659	0.2918189	-0.669459	1.152777	0.996
0.5 μ M 2iP	Control	0.18464	0.2862206	-0.708999	1.078279	0.9993
0.44 μ M BA	Control	0.18376	0.2825273	-0.698348	1.065868	0.9993
22.2 μ M BA	24.6 μ M 2iP	0.158786	0.2880028	-0.740418	1.057989	0.9998
5 μ M 2iP	0.44 μ M BA	0.135351	0.2862206	-0.758288	1.028991	0.9999
5 μ M 2iP	0.5 μ M 2iP	0.134472	0.2898669	-0.770552	1.039495	0.9999
4.44 μ M BA	22.2 μ M BA	0.111737	0.2964593	-0.813869	1.037344	1
2.5 μ M 2iP	5 μ M 2iP	0.107187	0.2918189	-0.803931	1.018306	1
0.5 μ M 2iP	0.44 μ M BA	0.00088	0.2862206	-0.89276	0.894519	1

Table 8. ANOVA result of average shoot length for *Vitex agnus-castus* after eight weeks growth *in vitro* in different cytokinin treatments.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	15.49050	1.93631	6.5643
Error	348	102.65145	0.29498	Prob > F
C. Total	356	118.14196		<.0001*

Further analysis using the Tukey test was done to look at the significant differences of average shoot length produced by each treatment (Figure 4). A table of the Tukey test and the significant level of each treatment against one another was produced (Table 9).

Based on the Tukey analysis (Table 9), shoots produced in 0.44 μM BA and 22.2 μM BA showed significant differences when compared to shoots produced by the control treatment. The shoot length of plants that were grown on media supplemented with 2.22 μM BA and 4.44 μM BA however, did not differ significantly than the control treatment. The same is true for the shoots produced in all of the 2iP media tested. There was no significant difference in the length of shoots produced by any of the 2iP media when compared with the control and the difference is only reported when they are compared with shoots produced in 22.2 μM BA.

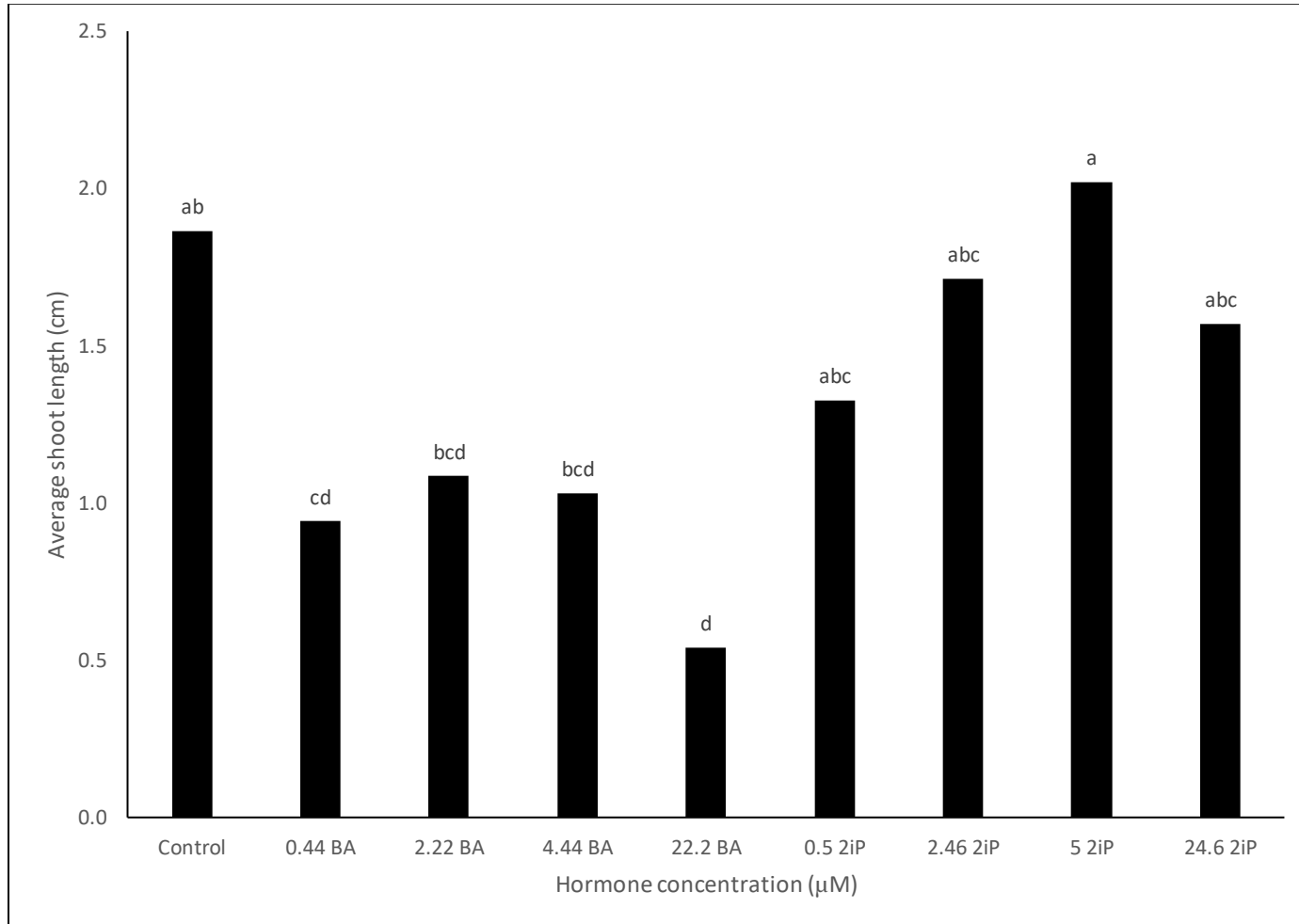


Figure 4. Bar graph of average shoot length (cm) of *Vitex agnus-castus* after eight weeks growth in different cytokinin treatments *in vitro*. Treatments that do not share the same letter are significantly different. The α value was set to 0.05.

Table 9. Tukey test analysis of average shoot length for eight weeks growth of *Vitex agnus-castus* in different cytokinin treatments *in vitro*.

Hormone	Hormone	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
5 µM 2iP	22.2 µM BA	0.6852331	0.1214445	0.306152	1.064315	<.0001
Control	22.2 µM BA	0.6296084	0.1214445	0.250527	1.00869	<.0001
2.5 µM 2iP	22.2 µM BA	0.5728606	0.1214445	0.193779	0.951942	0.0001
24.6 µM 2iP	22.2 µM BA	0.5164356	0.1230321	0.132399	0.900473	0.0011
5 µM 2iP	0.44 µM BA	0.4505451	0.1214445	0.071464	0.829627	0.0074
0.5 µM 2iP	22.2 µM BA	0.415595	0.1214445	0.036514	0.794676	0.0197
5 µM 2iP	4.44 µM BA	0.4053214	0.1222205	0.023818	0.786825	0.0277
Control	0.44 µM BA	0.3949204	0.1214445	0.015839	0.774002	0.0339
5 µM 2iP	2.22 µM BA	0.3798132	0.1214445	0.000732	0.758895	0.0491
Control	4.44 µM BA	0.3496967	0.1222205	-0.031807	0.7312	0.102
2.5 µM 2iP	0.44 µM BA	0.3381726	0.1214445	-0.040909	0.717254	0.1237
Control	2.22 µM BA	0.3241885	0.1214445	-0.054893	0.70327	0.1628
2.22 µM BA	22.2 µM BA	0.3054199	0.1214445	-0.073661	0.684501	0.2284
2.5 µM 2iP	4.44 µM BA	0.2929488	0.1222205	-0.088555	0.674453	0.2889
24.6 µM 2iP	0.44 µM BA	0.2817476	0.1230321	-0.102289	0.665785	0.351
4.44 µM BA	22.2 µM BA	0.2799117	0.1222205	-0.101592	0.661415	0.3509
5 µM 2iP	0.5 µM 2iP	0.2696381	0.1214445	-0.109443	0.648719	0.3948
2.5 µM 2iP	2.22 µM BA	0.2674406	0.1214445	-0.111641	0.646522	0.4065
24.6 µM 2iP	4.44 µM BA	0.2365239	0.1237982	-0.149904	0.622952	0.6069
0.44 µM BA	22.2 µM BA	0.234688	0.1214445	-0.144393	0.613769	0.5916
Control	0.5 µM 2iP	0.2140134	0.1214445	-0.165068	0.593095	0.707
24.6 µM 2iP	2.22 µM BA	0.2110157	0.1230321	-0.173021	0.595053	0.7369
0.5 µM 2iP	0.44 µM BA	0.180907	0.1214445	-0.198174	0.559988	0.8599
5 µM 2iP	24.6 µM 2iP	0.1687975	0.1230321	-0.215239	0.552834	0.9077
2.5 µM 2iP	0.5 µM 2iP	0.1572655	0.1214445	-0.221816	0.536347	0.9324
0.5 µM 2iP	4.44 µM BA	0.1356833	0.1222205	-0.24582	0.517187	0.9725
Control	24.6 µM 2iP	0.1131728	0.1230321	-0.270864	0.49721	0.9918
5 µM 2iP	2.5 µM 2iP	0.1123726	0.1214445	-0.266709	0.491454	0.9914
0.5 µM 2iP	2.22 µM BA	0.1101751	0.1214445	-0.268906	0.489257	0.9925
24.6 µM 2iP	0.5 µM 2iP	0.1008406	0.1230321	-0.283196	0.484878	0.9962
2.22 µM BA	0.44 µM BA	0.0707319	0.1214445	-0.308349	0.449813	0.9997
Control	2.5 µM 2iP	0.0567478	0.1214445	-0.322334	0.435829	0.9999
2.5 µM 2iP	24.6 µM 2iP	0.0564249	0.1230321	-0.327612	0.440462	0.9999
5 µM 2iP	Control	0.0556247	0.1214445	-0.323457	0.434706	0.9999
4.44 µM BA	0.44 µM BA	0.0452237	0.1222205	-0.33628	0.426727	1
2.22 µM BA	4.44 µM BA	0.0255082	0.1222205	-0.355995	0.407012	1

2.4.6 Rooting results for four weeks of growth.

Chi-square test of independence was done to analyze the result of rooting of *Vitex agnus-castus* after four weeks in either IBA or NAA supplemented media (Table 10). Based on the test (Table 11), the percentage with the greatest number of rooted explants after four weeks is 57.5% rooted explants that were grown on the medium with 24.6 μM IBA. The percentage the second highest number of rooted explants was with 52.5% rooted explants and the media used for that were 2.5 μM IBA and 26.9 μM NAA. The lowest percentage of rooted explants were recorded in 5.4 μM NAA with only 22.5% rooted explants while the control explants had 32.5% rooted.

Table 10. Chi square test of rooted *Vitex agnus-castus* explants for four weeks growth *in vitro* in different auxin treatments.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	20.543	0.0085*
Pearson	20.356	0.0091*

Table 11. Percentage of rooted explants of *Vitex agnus-castus* after four weeks growth *in vitro* in different auxin treatments.

Treatment number	Hormone	Concentration used (μM)	Percentage of rooted explants
1	No hormone	0	32.5
2	IBA	0.5	32.5
3	IBA	2.5	52.5
4	IBA	5	40
5	IBA	24.6	57.5
6	NAA	0.54	35
7	NAA	2.7	27.5
8	NAA	5.4	22.5
9	NAA	26.9	52.5

To analyze the roots that were produced after four weeks of growth, analysis of variance (ANOVA) was done on the total length of roots produced (Table 12).

Table 12. ANOVA result of total root length of *Vitex agnus-castus* after four weeks growth *in vitro* in different auxin treatments.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	61.75345	7.71918	3.8393
Error	351	705.71580	2.01059	Prob > F
C. Total	359	767.46925		0.0002*

The ANOVA analysis showed that the treatments had a P value of less than 0.05, thus they are significant. From the analysis, Tukey test was done to compare the root length produced by each treatment to one another (Table 13). In addition, a graph bar was made to visualize the result.

Based on the data (Figure 5), IBA supplements into the media increased the length of roots produced as compared to the roots produced in the control media. The opposite is true for media supplemented with NAA with concentration of 0.54 μM , 2.7 μM and 5.4 μM except for media supplemented with 26.9 μM NAA.

To confirm the significant differences of the length of roots that were produced, the Tukey test showed that only roots produced in media with 24.6 μM IBA media had significant differences when compared with the control, 0.5 μM IBA, 0.54 μM NAA, 2.7 μM NAA and 5.4 μM NAA while the roots produced in other media did not show any significant difference with the control or with other treatments (Table 13).

Table 13. Tukey test of total root length of *Vitex agnus-castus* for four weeks growth *in vitro* in different auxin treatments.

Hormone	Hormone	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
24.6 µM IBA	5.4 µM NAA	1.511586	0.3170636	0.521946	2.501227	<.0001
24.6 µM IBA	2.7 µM NAA	1.195136	0.3170636	0.205496	2.184777	0.0059
24.6 µM IBA	Control	1.100574	0.3170636	0.110933	2.090214	0.0168
24.6 µM IBA	0.5 µM IBA	1.081505	0.3170636	0.091865	2.071146	0.0205
24.6 µM IBA	0.54 µM NAA	1.055461	0.3170636	0.06582	2.045101	0.0266
5 µM IBA	5.4 µM NAA	0.885113	0.3170636	-0.104527	1.874754	0.1215
2.5 µM IBA	5.4 µM NAA	0.850338	0.3170636	-0.139303	1.839978	0.1581
26.9 µM NAA	5.4 µM NAA	0.829212	0.3170636	-0.160429	1.818852	0.184
24.6 µM IBA	26.9 µM NAA	0.682374	0.3170636	-0.307266	1.672015	0.4396
24.6 µM IBA	2.5 µM IBA	0.661248	0.3170636	-0.328392	1.650889	0.4849
24.6 µM IBA	5 µM IBA	0.626473	0.3170636	-0.363168	1.616113	0.5613
5 µM IBA	2.7 µM NAA	0.568664	0.3170636	-0.420977	1.558304	0.6865
2.5 µM IBA	2.7 µM NAA	0.533888	0.3170636	-0.455752	1.523529	0.7561
26.9 µM NAA	2.7 µM NAA	0.512762	0.3170636	-0.476878	1.502403	0.7949
5 µM IBA	Control	0.474101	0.3170636	-0.51554	1.463741	0.8573
0.54 µM NAA	5.4 µM NAA	0.456126	0.3170636	-0.533515	1.445766	0.8821
5 µM IBA	0.5 µM IBA	0.455032	0.3170636	-0.534608	1.444673	0.8835
2.5 µM IBA	Control	0.439325	0.3170636	-0.550315	1.428966	0.9028
0.5 µM IBA	5.4 µM NAA	0.430081	0.3170636	-0.559559	1.419721	0.9131
5 µM IBA	0.54 µM NAA	0.428988	0.3170636	-0.560653	1.418628	0.9142
2.5 µM IBA	0.5 µM IBA	0.420257	0.3170636	-0.569384	1.409897	0.9232
26.9 µM NAA	Control	0.418199	0.3170636	-0.571441	1.40784	0.9252
Control	5.4 µM NAA	0.411012	0.3170636	-0.578628	1.400653	0.932
26.9 µM NAA	0.5 µM IBA	0.399131	0.3170636	-0.59051	1.388771	0.9423
2.5 µM IBA	0.54 µM NAA	0.394212	0.3170636	-0.595428	1.383853	0.9462
26.9 µM NAA	0.54 µM NAA	0.373086	0.3170636	-0.616554	1.362727	0.9609
2.7 µM NAA	5.4 µM NAA	0.31645	0.3170636	-0.673191	1.30609	0.9859
0.54 µM NAA	2.7 µM NAA	0.139676	0.3170636	-0.849964	1.129316	1
0.5 µM IBA	2.7 µM NAA	0.113631	0.3170636	-0.876009	1.103272	1
Control	2.7 µM NAA	0.094563	0.3170636	-0.895078	1.084203	1
5 µM IBA	26.9 µM NAA	0.055901	0.3170636	-0.933739	1.045542	1
0.54 µM NAA	Control	0.045113	0.3170636	-0.944527	1.034754	1
5 µM IBA	2.5 µM IBA	0.034776	0.3170636	-0.954865	1.024416	1
0.54 µM NAA	0.5 µM IBA	0.026045	0.3170636	-0.963596	1.015685	1
2.5 µM IBA	26.9 µM NAA	0.021126	0.3170636	-0.968515	1.010766	1
0.5 µM IBA	Control	0.019069	0.3170636	-0.970572	1.008709	1

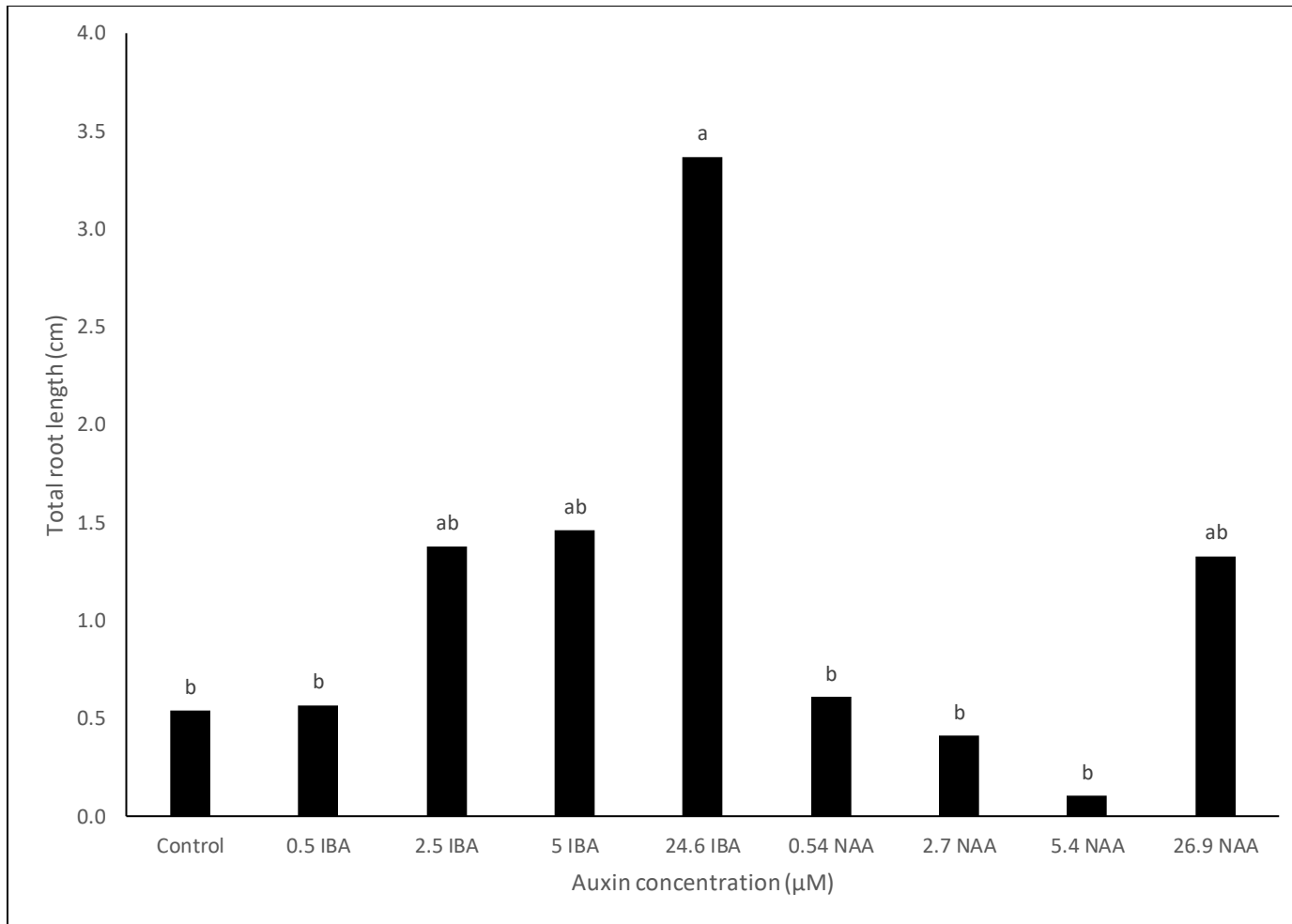


Figure 5. Bar graph of total root length of *Vitex agnus-castus* plants after four weeks growth *in vitro* in different auxin treatments. Treatments that do not share the same letter are significantly different. The α value was set to 0.05.

2.4.7 Rooting result for eight weeks of growth.

To evaluate the rooting percentage of explants, a chi square test was done and the result is summarized in Table 14.

Table 14. Percentage of rooted explants of *Vitex agnus-castus* plants after eight weeks growth *in vitro* in different auxin treatments.

Treatment	Hormone	Concentration used (μM)	Percentage of rooted explants
1	No hormone	0	50
2	IBA	0.5	69
3	IBA	2.5	84
4	IBA	5	90
5	IBA	24.6	86
6	NAA	0.54	69
7	NAA	2.7	77
8	NAA	5.4	80
9	NAA	26.9	71

Table 15. Chi-square test of percentage explants of *Vitex agnus-castus* plants that were rooted *in vitro* for eight weeks in different auxin treatments.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	23.511	0.0028*
Pearson	23.900	0.0024*

The chi-square test had a P value of less than 0.05 demonstrating that the percentage for rooting was significant. For eight weeks of growth, the lowest percentage of rooted explants was observed in explants grown in the control media with only half of the explants successfully rooted while the highest percentage of explants rooted was reported in media supplemented with 5 μM IBA with 90% of explants rooted after eight weeks.

The data for the length of roots of *Vitex agnus-castus* plants was analyzed using ANOVA to compare the significant of length of roots produced in each treatment (Table 16; Figure 6)

The length of roots increased as IBA concentration was increased in the media while the trend is the opposite when NAA concentration was increased. To determine if the length of roots that were produced was significant, the Tukey test showed that the length of roots produced in media 5 μM IBA, 24.6 μM IBA, 5.4 μM NAA, and 26.9 μM NAA are significant when they are compared with the roots that were produced in the control medium. However, the length of roots that were produced in all media were not significantly different when they are compared to other treatments. Based on this observation, it can be concluded that the hormone tested in the media did not have any significant effect on root elongation but only affected root induction.

2.4.8 Acclimatization

Table 17 summarizes the results of the acclimatization protocol. Twenty rooted explants were used for the experiment and 17 explants were successfully transferred into the greenhouse with a survival percentage of 85% (Table 17).

Table 16. Tukey test of total root length of *Vitex agnus-castus* plants for eight weeks growth *in vitro* in different auxin treatments.

Hormone	Hormone	Differences	STD Erro Dif	Lower CL	Upper CL	p-Value
24.6 μ M IBA	Control	2.121981	0.5005271	0.55868	3.685279	0.001
5 μ M IBA	Control	1.956095	0.4871773	0.43449	3.477697	0.0024
2.7 μ M NAA	Control	1.740691	0.4902903	0.20937	3.272016	0.013
5.4 μ M NAA	Control	1.59121	0.4902903	0.05988	3.122534	0.0348
24.6 μ M IBA	0.5 μ M IBA	1.414711	0.5293346	-0.23856	3.067982	0.1619
26.9 μ M NAA	Control	1.346487	0.4935459	-0.19501	2.88798	0.1422
5 μ M IBA	0.5 μ M IBA	1.248825	0.5167296	-0.36508	2.862727	0.2784
24.6 μ M IBA	0.54 μ M NAA	1.221241	0.5293346	-0.43203	2.874513	0.3409
2.5 μ M IBA	Control	1.168015	0.5213392	-0.46028	2.796315	0.3822
5 μ M IBA	0.54 μ M NAA	1.055355	0.5167296	-0.55855	2.669258	0.515
2.7 μ M NAA	0.5 μ M IBA	1.03342	0.5196656	-0.58965	2.656493	0.5525
24.6 μ M IBA	2.5 μ M IBA	0.953966	0.5338354	-0.71336	2.621296	0.6908
0.54 μ M NAA	Control	0.90074	0.5167296	-0.71316	2.514643	0.7192
5.4 μ M NAA	0.5 μ M IBA	0.883939	0.5196656	-0.73913	2.507011	0.7457
2.7 μ M NAA	0.54 μ M NAA	0.839951	0.5196656	-0.78312	2.463024	0.7954
5 μ M IBA	2.5 μ M IBA	0.78808	0.5213392	-0.84022	2.41638	0.8496
24.6 μ M IBA	26.9 μ M NAA	0.775495	0.506728	-0.80717	2.358159	0.8405
0.5 μ M IBA	Control	0.707271	0.5167296	-0.90663	2.321173	0.9087
5.4 μ M NAA	0.54 μ M NAA	0.690469	0.5196656	-0.9326	2.313542	0.9221
26.9 μ M NAA	0.5 μ M IBA	0.639216	0.5227383	-0.99345	2.271886	0.951
5 μ M IBA	26.9 μ M NAA	0.609609	0.4935459	-0.93188	2.151102	0.9481
2.7 μ M NAA	2.5 μ M IBA	0.572676	0.5242494	-1.06471	2.210065	0.975
24.6 μ M IBA	5.4 μ M NAA	0.530772	0.5035576	-1.04199	2.103534	0.98
2.5 μ M IBA	0.5 μ M IBA	0.460744	0.5490558	-1.25412	2.175611	0.9956
26.9 μ M NAA	0.54 μ M NAA	0.445746	0.5227383	-1.18692	2.078416	0.9951
5.4 μ M NAA	2.5 μ M IBA	0.423194	0.5242494	-1.21419	2.060584	0.9966
2.7 μ M NAA	26.9 μ M NAA	0.394204	0.496619	-1.15689	1.945296	0.997
24.6 μ M IBA	2.7 μ M NAA	0.38129	0.5035576	-1.19147	1.954053	0.9978
5 μ M IBA	5.4 μ M NAA	0.364886	0.4902903	-1.16644	1.896211	0.9981
2.5 μ M IBA	0.54 μ M NAA	0.267275	0.5490558	-1.44759	1.982142	0.9999
5.4 μ M NAA	26.9 μ M NAA	0.244723	0.496619	-1.30637	1.795814	0.9999
5 μ M IBA	2.7 μ M NAA	0.215404	0.4902903	-1.31592	1.746729	1
0.54 μ M NAA	0.5 μ M IBA	0.193469	0.5446808	-1.50773	1.894672	1
26.9 μ M NAA	2.5 μ M IBA	0.178472	0.5272954	-1.46843	1.825374	1
24.6 μ M IBA	5 μ M IBA	0.165886	0.5005271	-1.39741	1.729183	1
2.7 μ M NAA	5.4 μ M NAA	0.149482	0.4933837	-1.3915	1.690468	1

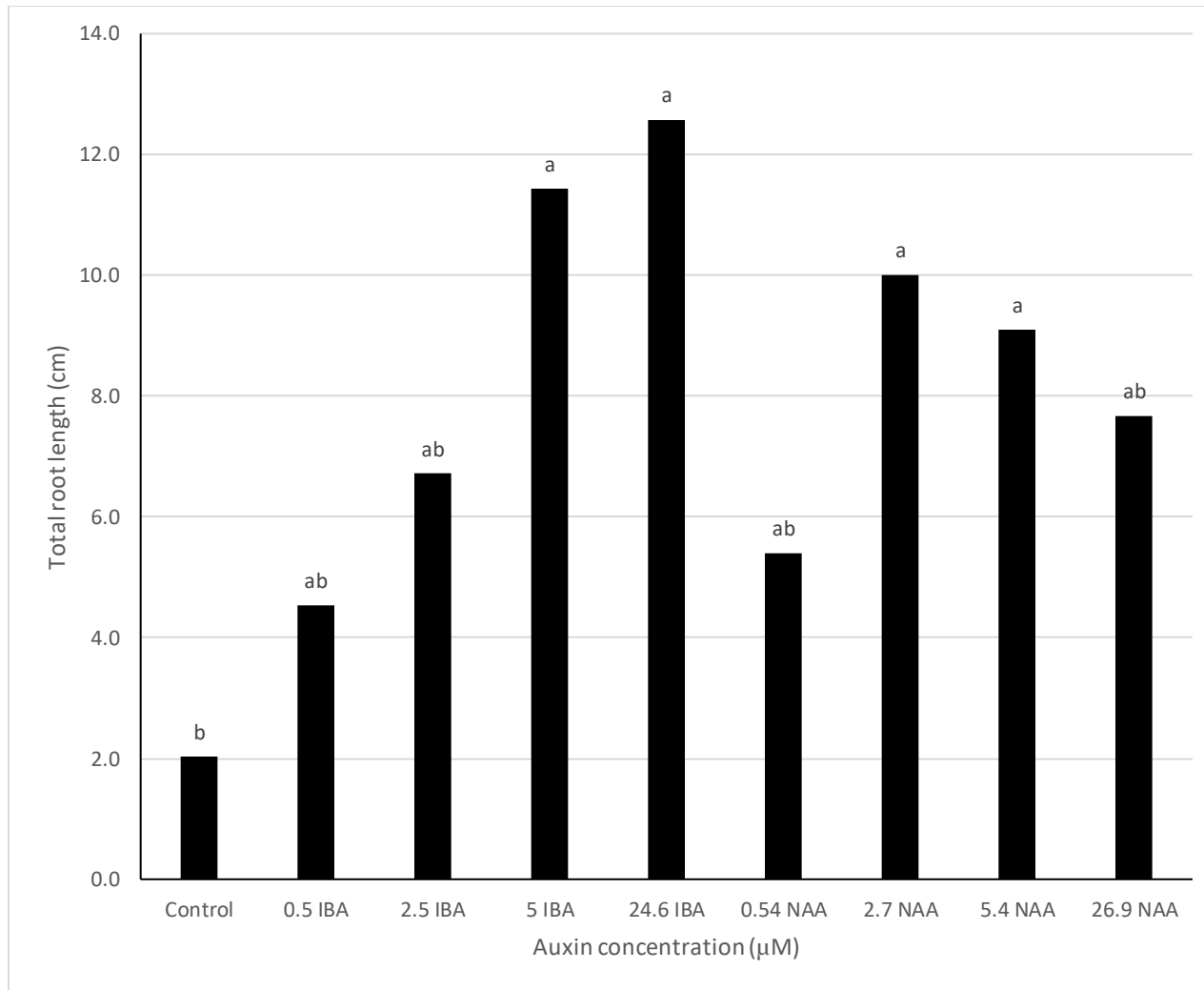


Figure 6. Bar graph of total root length of *Vitex agnus-castus* plants for eight weeks growth *in vitro* in different auxin treatments. Treatments that do not share the same letter are significantly different. The α value was set to 0.05.

Table 17. Percent survival of *Vitex agnus-castus* plants after 5 weeks of acclimation *ex vitro*.

Total number of explants	Number of explants after 5 weeks	Survival percentage %
20	17	85

2.5 Discussion

2.5.1 Surface Sterilization – Stage 1

There were two objectives for the surface sterilization experiment. The first objective was to eliminate any contaminants on the cuttings and the second objective was to ensure the survival of the explants that undergo the sterilization treatment. The contaminants on the cuttings' surfaces were eliminated through physical and chemical methods. The surface sterilization method successfully disinfected 94% of the cuttings that were taken from the greenhouse and the nodes began to grow new shoots after 3 weeks in tissue culture.

The container that was used in this experiment to grow the plants partially contributed to the success rate of sterilizing the cuttings. Prior experiments of surface sterilization used the Magenta™ boxes with 5 cuttings cultured into one box. The success rate was much lower when compared to test tubes (data not included) because the use of test tubes allowed physical isolation of explants after the sterilization method. When Magenta boxes were used, contamination from one explant in the container resulted in the loss of all five explants that were in the same container. Thus, the use of test tubes prevents any recalcitrant spores or contaminants that were not removed during the sterilization procedure to spread to other explants.

2.5.2 Shoot multiplication after four weeks growth– Stage 2

The lowest concentrations that were tested for both cytokinin did not produce significant number of shoots when compared to the control (Table 3). Although 0.44 μM BA and 0.5 μM 2iP were recorded to produce more shoots as compared to the control (Figure 1), the differences were not significant. This shows that concentration of 0.44 μM BA and 0.5 μM 2iP were too low to have a significant impact on shoot production. When BA concentration was increased from 0.44 μM to 2.22 μM and above, the number of shoots that were produced were significant when compared to the control (Table 3). However, the trend is not the same for 2iP. When 2iP concentration was increased from 0.5 μM to 2.46 μM , the number of shoots that were produced were significant when compared to the control. But when 5 μM of 2iP was used, the number of shoots produced was not significant when compared to the control (Table 3). The number of shoots produced in 5 μM 2iP is less than the number of shoots produced in 2.46 μM 2iP (Figure 1). When 2iP concentration was increased to 24.6 μM , the number of shoots produced was significant when compared to the control (Table 3), but the number of shoots that were produced were recorded to be barely 6 shoots produced per explant (Figure 1). The trend of shoot production recorded with BA hormone is as expected since the increase in cytokinin concentration increased the number of shoots produced, but the same trend is not observed with 2iP supplemented media (Figure 1). In addition, the number of shoots that were produced in 2iP media is low when compared to the shoots produced in BA media. The highest concentration of 2iP that was tested, 24.6 μM , was recorded to produce similar number of shoots as in media supplemented with 2.22 μM BA. These two reasons might suggest that hormone 2iP is not compatible with *Vitex* or higher concentration is required to produce significant shoot production.

To compare the treatments with each other, 4.44 μM BA and 22.2 μM BA produced significant number of shoots when compared with the control, 0.44 μM BA, 0.5 μM 2iP and 5 μM 2iP (Table 3). For 2iP media, none of the concentrations that were tested produced significant number of shoots when compared with any of the BA or 2iP media. The highest concentration of 2iP tested, 24.6 μM 2iP, produced significant number of shoots only to the control but not with any other media tested (Table 3).

As for the length of shoots produced after 4 weeks growth in cytokinins, the shoot length produced in BA media is similar in media with 0.44 μM and 2.22 μM while slightly longer shoots were produced in 4.44 μM and shorter shoots were produced in the 22.2 μM medium when compared to other BA media (Figure 2). As for shoots grown in 2iP media, the length of shoots in 0.5 μM 2iP was similar with 2.46 μM 2iP while 5 μM 2iP produced shorter shoots and 24.6 μM 2iP produced the shortest shoots when compared to other 2iP media (Figure 2). Based on statistics analysis of the shoot length (Table 5), the shoots produced in both BA and 2iP media were not significantly different than the control. Only shoots produced in 4.44 μM BA reported to be significantly different than shoots produced in 22.2 μM BA, but this is insignificant since the length of shoots in both media is not significantly different than the control. Based on this, it can be concluded that the length of shoots produced in all media were not significantly affected by the cytokinins after four weeks of growth.

2.5.3 Shoot multiplication for 8 weeks growth – Stage 2

After 8 weeks of growth *in vitro*, BA demonstrated to be more effective for shoot production of *Vitex* when compared to 2iP. None of the 2iP media had significant shoot production when

compared to the control except for 24.6 μM 2iP (Table 7). Shoot production of BA with a concentration of 4.44 μM showed significant differences when compared to all of the 2iP media other than 24.6 μM of 2iP. This indicate that BA with concentration of 4.44 μM is the optimum concentration to use to promote shoot production in *Vitex*.

To compare *Vitex* shoot production after four weeks of growth with eight weeks of growth, the production is very similar to one another (Figures 1 and Figure 3). The number of shoots produced by BA is always higher when compared to shoots produced by 2iP media. BA with 4.44 μM always showed the greatest number of shoot production after four weeks of growth as well as after eight weeks. The highest concentration of 2iP tested only showed significant differences with the control plants in four weeks of growth but not after eight weeks of growth. Based on the data, it can be concluded that BA is better at promoting shoot production in *Vitex* than 2iP and the best concentration to use is 4.44 μM .

When the average shoot length was analyzed (Figure 4), it showed shoots that were produced in BA media to be shorter than the ones grown in the control treatment. This may be because the energy expenditure was invested in shoot production rather than shoot elongation. The significant difference of shoot length reported for the 22.2 μM BA concentration is expected because more shoots were produced. However, the increase in total shoot length is not proportional to the number of shoots produced. The data used to analyze the average shoot length was taken by dividing the total length of all shoots produced and averaging it with the number of shoots produced. When the increase in total shoot length is not proportional to the increase in the number of shoots produced,

the average shoot length calculated will be low thus leading to a significant difference when compared to the control treatment.

For BA treatments of 4.44 μM and 22.2 μM , the average shoot length was not significantly different than the control treatment (Table 9). It is safe to conclude that the average shoot length is similar to the control. The same is true for the shoots produced in all of the media supplemented with 2iP. The shoots that were produced in 2iP media were also similar in length or did not differ significantly when compared to the control. This is unexpected because looking at the low shoot production count, explants in 2iP media did not have to invest too much energy in shoot production thus, should have significant shoot elongation.

2.5.4 Rooting – Stage 3

Two auxins, IBA and NAA, were evaluated for their effect on *in vitro* rooting of *Vitex*. There were four concentrations of each auxin and they were evaluated for four weeks and eight weeks. After four weeks of growth in the rooting media, the medium that reported the highest percentage of rooted explants was 24.6 μM IBA with 57.5% of the plants rooted (Table 10). The length of the roots that were produced in the same medium was observed to be significantly longer than the roots that were produced in the other treatments.

After eight weeks of growth in the rooting media, the highest percentage of rooted explants was recorded in media supplemented with 5 μM IBA (Table 14). This treatment showed 90% of the explants rooted while plants in the control medium (no auxin) had the lowest percentage of rooting with only 50% of the explants rooted. When the length of roots that were produced in these media were compared, the media supplemented with 5 μM IBA, 24.6 μM IBA, 5.4 μM NAA and 26.9 μM NAA were significantly different to the control (Table 16). However, the length of roots that

were produced in all media were not significantly different when they are compared to each other. Based on this observation, it can be concluded that the hormone tested in the media did not have any significant effect on root elongation but only affected root induction.

2.5.5 Acclimatization

Acclimatization of explants was achieved by controlling the humidity of the growing environment at Stage 4. Rooted explants were taken off tissue culture media and the roots were washed thoroughly to avoid any fungal growth from any sucrose residue. During the first week of acclimatization, the humidity of the growing environment was not altered and the explants were allowed to adjust to the new growing condition under a plastic cover without sucrose supplement. This allowed the explants to start or increase their photosynthetic machinery. In the second week of acclimatization, the humidity of the growing environment was decreased starting with a vertical slit of 0.5 cm in the plastic cover. The length of the slit was doubled in size every four days to gradually decrease the humidity of the growing environment. Some damaged leaves were observed during the second week but the damage was minimal.

From the explants that were tested during the acclimatization experiment, 85% of them were successfully introduced into the greenhouse. In the greenhouse, the plants were placed under shade cloth to limit light exposure before they are exposed to full sunlight.

2.6 Summary

The experiments designed in the research achieved their goals. The surface sterilization method successfully introduced greenhouse cuttings of *Vitex agnus-castus* into sterile environment with 94% success rate.

For shoot multiplication, the cytokinin BA proved to be more effective when compared to 2iP. The optimum concentration to use was 4.44 μM of BAP and this was proven in both four weeks as well as eight weeks observation. As for the average shoot length that was produced in different cytokinin media, the average shoot length was similar in all the treatments for four weeks observation including the control. For eight weeks observation, the average shoot length for BA media with a concentration of 4.44 μM was similar to the control, thus shoot production did not diminish the length of shoots produced. As for 22.2 μM BA, it produced less shoots than 4.44 μM BA in both four weeks and eight weeks observations although the difference is not significant. However, in eight weeks growth, there is a significant difference in shoot length when the two media is compared and 22.2 μM BA produced shorter shoots as compared to 4.44 μM BA media.

For rooting, observations after four weeks of growth showed that 24.6 μM IBA had the highest percentage of rooted explant but eight weeks observation showed IBA with 5 μM had the highest percentage of rooted explants. As for the length of roots produced, four weeks of observation showed that IBA with 24.6 μM produced a significant increase in the length of roots, but eight weeks of observation showed that both 5 μM IBA and 24.6 μM IBA produced significant amounts of roots when compared to the control. Based on the percentage of rooted explants and the length

of roots produced, it can be concluded that IBA is better at inducing root as compared to NAA and the concentration to use is 5 μ M and the plants should be grown in the media for eight weeks.

Lastly, the acclimatization protocol successfully introduced 85% of rooted tissue culture explants into the greenhouse.

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CHAPTER 3: *IN VITRO* MUTAGENESIS OF *VITEX AGNUS-CASTUS*

3.1 Abstract

The goal of this project was to determine the lethal dose of 50% survival of *Vitex agnus-castus* plants after exposure to oryzalin *in vitro* using a procedure called chemical mutagenesis. Mutagenesis is the process by which the genetic information of an organism is changed, resulting in a mutation. It may occur spontaneously in nature, or it may occur as a result of exposure to mutagens. It can also be achieved experimentally using laboratory procedures. There are many methods available to mutate plants experimentally from chemical, physical, and molecular biological techniques. Mutants are very useful in a breeding program as they are a source of novel phenotypes which could lead to the development of new cultivars. This research documents the effects of *Vitex agnus-castus* when it is exposed to the mutagen, oryzalin, at different concentrations and at several exposure times. The result of this research will allow future plant breeders to use oryzalin to produce mutant *Vitex* plants in tissue culture. In this study, tissue cultured cuttings of *Vitex agnus-castus* were exposed to oryzalin at three different concentrations

with three different exposure times, and observed for 16 weeks. Morphological changes and the number of cuttings that survived the treatments were recorded to determine the lethal dose of oryzalin to *Vitex*. From the observations, it was found that the lethal dose of oryzalin that causes 50% of death to *Vitex* in tissue culture to be at 0.3 millimolar of oryzalin at 30 minutes of exposure time. Higher concentration or longer exposure time increased the percentage of death of the cuttings. After 16 weeks, the surviving explants exposed to oryzalin were maintained in tissue culture for further research and evaluation.

3.2 Introduction

Vitex agnus-castus, also known as the chaste tree or monk's pepper, is an important medicinal herb. There have been several studies conducted to evaluate and quantify its medicinal properties (Van die *et al.*, 2012); it is also available as over-the-counter herbal supplement. In addition to its medicinal properties, *Vitex* also has promising ornamental plant qualities. The palmately compound leaves of the shrub are fragrant and it produces showy flowers in loose panicles during late summer (Gilman and Watson, 1994). The flowers attract pollinators, but the plants do not attract deer. The shrub can easily grow in many different types of soils with good drainage and it is not affected by any major pests or diseases.

Despite the many attractive attributes of *Vitex*, there are three weaknesses that hinder its popularity (Dirr, 2015). The first is the plant's aggressive growth habit. The plant can grow up to approximately 1.3 meters in one growing season. In colder climate, the growth is limited; however, in warmer climates, the shrub can grow up to six meters in height and width. The aggressive or rapid growth habit causes *Vitex* to require a large amount of maintenance in order for it to be used in a landscape. The second weakness of *Vitex* is the lack of flower colors that are available.

Currently there are only two flower colors available commercially: purple-blue and pink. The limited flower colors that are available reduce the sales potential of this lovely plant. Lastly, the plant is not cold tolerant in zones above USDA Zone 7. Not being cold tolerant limits the landscape use of *Vitex* to warmer climate.

Vitex has the potential to be a great ornamental plant and a novel product to be introduced into the ever-growing horticulture market. The weaknesses of *Vitex* can be mitigated with a breeding program to improve its popularity. The long-term goal of this research is to improve the ornamental qualities of *Vitex* through the use of chemical mutagenesis *in vitro*. Before this can be accomplished, a protocol to mutate *in vitro* plants must first be outlined.

Chemical mutagenesis is the process by which the genetic information of an organism is changed, resulting in a mutation. It may occur spontaneously in nature, or it may occur as a result of exposure to mutagens using laboratory procedures. There are several classes of chemicals that have been identified to induce mutation. Different chemicals are able to induce different type of mutations and some examples are alkylating agents, nitroso compounds, base analogues and many more. One of the chemicals that has been identified to be a successful mutagen in the class of dinitroaniline sulfonamide is called oryzalin.

Oryzalin, known by many different commercial names, is an herbicide with the chemical name of 4-(dipropylamino)-3,5-dinitrobenzenesulfonamide. The effect of oryzalin's mutagenicity was demonstrated in a study published in 1987 (Morejohn *et al.*, 1987). That publication demonstrated that oryzalin was able to disrupt the tubulin polymerization and affect the segregation of

chromosome during cell division in anaphase. The same study also compared the effectiveness of oryzalin to colchicine and found that oryzalin is more effective at disrupting the tubulin formation in plants as compared to colchicine. Because of this, treatment with oryzalin is able to alter the ploidy level of plants by altering the sets of chromosomes in the daughter cells of actively dividing cells.

There are many benefits to increasing the number of chromosome sets in plants and it has been used in many breeding programs to improve plants of interest. Increasing the ploidy levels can lead to altered or enhanced morphology such as the rhododendron produced from oryzalin treatment by Contreras et al. that was reported to enhanced the size of the leaves, flowers and the pollen as compared to the diploid (Contreras *et al.*, 2007). These improvements have been reported in other induced polyploids such the Chinese sage (Gao *et al.*, 1997), tetraploid watermelons (Jaskani *et al.*, 2005) and many more. Increasing the ploidy level of plants have also been reported to produce plants that is short and more compact (Chen *et al.*, 2006). These new desirable traits in plants have the chance to improve their ornamental properties. Because treatment with oryzalin was reported to be able to produce new phenotypes in plants, it was an ideal candidate to be used to improve *Vitex* ornamental properties by either enhancing the size of the flowers or leaves, or creating a more compact plant.

In order to use oryzalin in a breeding program, the factors that affect the success of chemical mutagenesis must be understood. Altering the chromosome sets of a cell affects the viability of the cells. The cells with extra chromosomes in the nuclei will require more energy in order to divide and be maintained, thus usually causing the cell to die. Using too high of a concentration or too

long of exposure time to the mutagen will cause too much cell death and lead to the death of the plants. In order to successfully obtain a plant with altered ploidy level, the right dosage of the mutation agent needs to be determined, and this involves determining the correct combination of the agent's optimum concentration and exposure time to use with the desired plant.

In addition to the dosage of the mutagen, the second factor that need to be considered is the physiological state of the plant. The study done by Morejohn *et al.* (Morejohn *et al.*, 1987) showed that oryzalin only affects actively dividing cells by disrupting the tubulin polymerization during cell division while nondividing cells are left unaffected. Because of this, the plants to be used in chemical mutagenesis must be actively growing and dividing or the treatment with oryzalin will not be effective.

The third factor that will affect the success of chemical mutagenesis is the cell's doubling time. The doubling time is the time it takes for a cell to go through one cell cycle. Since oryzalin only affects dividing cells, the exposure time to oryzalin must be at least longer than the doubling time of the plant's cells. By ensuring the exposure time to be longer than the doubling time of the cells, it will be possible to disrupt chromosome segregation in pluripotent cells in the growing points or the apical meristem.

The fourth and last factor that needs to be taken into consideration is the penetrance of the mutagen for the growing condition and the explant itself. To be effective, the mutagen must be able to penetrate the many layers of cells and reach the totipotent cells inside the plant. When the growing conditions are *in vitro*, the plants will not have epicuticular wax on their surface (Sutter and

Langhans, 1979) and this might help the mutagen to penetrate several layers of the plants' surface. However, Kermani *et al.* (Kermani *et al.*, 2003) and Allum *et al.* (Allum *et al.*, 2007) suggested that the absorption of the mutagen is only through the cut made and not through the plant's surface thus recommend small size explants to be used and the cut surface to be made close to the meristem cells for chemical mutagenesis experiment.

Because of the many factors with chemical mutagenesis, the lethal dose or the survival percentage are commonly used as an indicator to gauge the success of mutagen treatments. It is important to determine the correct combination of concentration and exposure time for specific mutagens to be used with plants of interest, while other factors are constant. This project aims to pave the way to improve the ornamental features of *Vitex* with the chemical mutagen oryzalin by reporting the survival percentages of *in vitro* plants that are exposed to different concentrations of oryzalin at different exposure times.

3.3 Materials and Methods

Plants originated from cuttings of *Vitex agnus-castus* plants that were grown under standard greenhouse conditions. Shoot tips were introduced into tissue culture by soaking in 10% bleach (8.25% NaOCl) solution for 15 minutes then rinsing three times with sterile water. The plants were maintained in Murashige and Skoog (MS; (Murashige and Skoog, 1962)) medium with vitamin supplements with sucrose at 30 g/l, agar at 6 g/l, benzylaminopurine (BA) supplement of 4.44 μ M and pH calibrated to 5.70- 5.75 before autoclaving. The plants were transferred onto fresh media every four weeks to ensure they were actively growing and dividing.

To determine the lethal dose of 50% survival (LD_{50}) for oryzalin on *Vitex*, three different concentrations with three different exposure times for each concentration were tested. The concentrations tested were 0.3 mM, 1.3 mM and 2.8 mM oryzalin while the time of exposure for each concentration tested were 30 minutes, 60 minutes and 90 minutes. A total of 12 treatments were prepared with three treatments designated as controls with no exposure to oryzalin; the plants were soaked in sterile water with shaking for 30, 60 or 90 minutes. Ten plants of 2.5 cm in length with their apical meristem attached were used for each combination of exposure time. There were 10 explant replications per treatment. The experiment was repeated twice and the summary of the treatments is summarized in Table 18 for easy reference.

After exposing the plants to the treatments, the plants were transferred to fresh medium every four weeks and observed for 16 weeks. For the first month, the plants were grown on MS media supplemented with 4.44 μ M BA before they were transferred into half strength MS media in the second month. At the start of the third month, the explants were subcultured into full strength MS media followed by full strength MS media supplemented with 2.22 μ M BA on the last month. Observation on the plants and the number of surviving plants were recorded every four weeks before they are transferred into fresh medium. Dead tissues were removed from the plants while carefully preserving the surviving growing points before they are transferred onto fresh medium. After 16 weeks, surviving plants were maintained in half strength MS media supplemented with 2.22 μ M BA for further research use.

3.3.1 Results analysis

The data obtained for shoot multiplication and rooting experiments were analyzed using JMP PRO version 12 software produced by SAS Institute Incorporated (JMP®, Version 12. SAS Institute Inc). Each treatment in the experiments were given a treatment number in order to perform statistical analysis (Table 18).

Table 18. Summary of the treatment descriptions for the 12 treatments received by the *Vitex agnus- castus* plants.

Treatment number	Treatment detail
1	Control explants soaked in sterile water for 30 minutes
2	Control explants soaked in sterile water for 60 minutes.
3	Control explants soaked in sterile water for 90 minutes.
4	Explants were exposed to 0.3 mM oryzalin for 30 minutes.
5	Explants were exposed to 0.3 mM oryzalin for 60 minutes.
6	Explants were exposed to 0.3 mM oryzalin for 90 minutes.
7	Explants were exposed to 1.3 mM oryzalin for 30 minutes.
8	Explants were exposed to 1.3 mM oryzalin for 60 minutes.
9	Explants were exposed to 1.3 mM oryzalin for 90 minutes.
10	Explants were exposed to 2.8 mM oryzalin for 30 minutes.
11	Explants were exposed to 2.8 mM oryzalin for 60 minutes.
12	Explants were exposed to 2.8 mM oryzalin for 90 minutes.

3.4 Results

After plants were treated with different concentrations of oryzalin at different exposure times, they were observed for physiological changes every four weeks and the survival rate of the plants was recorded for 16 weeks (Figure 7).

For the first month after exposure to oryzalin, the plants were observed to be green and healthy with their leaves intact (Figure 7A). The obvious differences between the treated plants and the control plants was that control plants began to produce axillary shoots after four weeks while there were no new shoots or buds can be seen on the treated plants.

During the second month after exposure, the apical meristems of the treated plants began to turn color from a healthy green color to yellow (Figure 7B). As time progressed, the yellowing apical meristem turned brown and eventually black and dry. By the end of the second month, the apical meristem looked dry and dead while the edges of the leaves started to turn yellow. Some plants progressed faster than others and started to either have yellowing stem or started to die in the second month.

In the third month, the damaged yellowing leaves progressed and suffered. The leaves started to turn brown and desiccated while the stems turned yellow (Figure 7C). The cell death on the plants was observed to be moving inwards towards the center of the plant beginning from the base, the apical meristem, and the margin of the leaves towards the center of the plants. Most plants lost their leaves and most death was recorded in the third month.

In the fourth and last month of observation, the surviving plants typically had yellow stems and some had swollen stems(Figure 7D). Some of the treated plants started to recover from the treatment with oryzalin and some growth could be observed from the growing points or the nodes. After four months, the control plants had grown to the top of the magenta boxes with many new shoots produced, while the treated plants remained small with no leaves and only starting to produce new shoots.

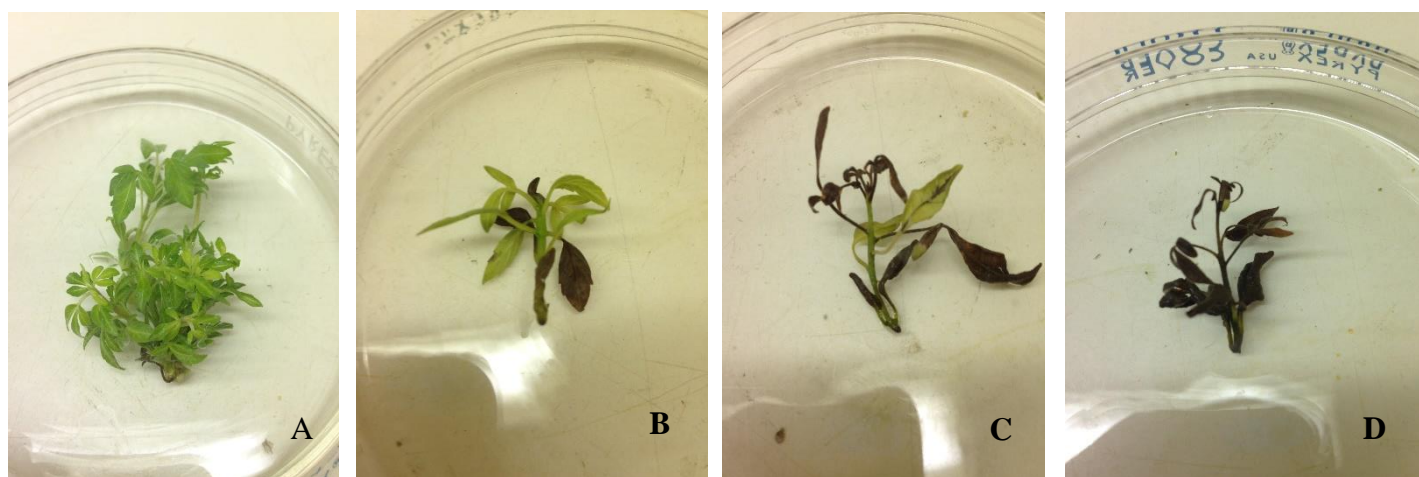


Figure 7. Progression of *Vitex agnus-castus* plants after oryzalin exposure from the first month (A) to the fourth month (D).

Data of plant survival after 16 weeks of observation was summarized in Table 19 and a bar graph was made to visually show the survival percentage based on the treatments received by the plants in Figure 8.

Table 19. Result of survival percentage of *in vitro* plants of *Vitex agnus-castus* for different treatments received.

Treatment number	Oryzalin concentration (milliMolar)	Exposure time (minute)	Survival percentage after 16 weeks.
1	0	30	100%
2	0	60	100%
3	0	90	100%
4	0.3	30	55
5	0.3	60	50
6	0.3	90	25
7	1.3	30	20
8	1.3	60	40
9	1.3	90	10
10	2.8	30	40
11	2.8	60	30
12	2.8	90	10

All of the control plants of 30 minutes, 60 minutes and 90 minutes with no exposure to oryzalin had 100% survival while treated plants had varying percentage of survival (Table 19, Figure 8).

The greatest percentage of survival of treated plants was observed in plants which received the lowest dosage of oryzalin exposure of 0.3 mM concentration for 30 minutes. The lowest percentage of survival was observed in 1.3 mM oryzalin for 90 minutes exposure and 2.8 mM oryzalin at 90 minutes exposure (Table 19). The trend for highest and lowest concentrations of oryzalin was that survival was lowest at the longest time exposure of 90 minutes and survival was greatest at the shortest time exposure of 30 minutes. Plants from the 1.3 mM oryzalin treatment had the greatest survival rate at the 60 minute treatment.

The data obtained was analyzed to determine which treatment significantly affected the survival of the plants. In order to observe the interaction between oryzalin concentration and exposure time to plant survival, logistic regression test was done. The result showed that the interaction of the two variables was not significant with a P value of 0.6304 (Table 20).

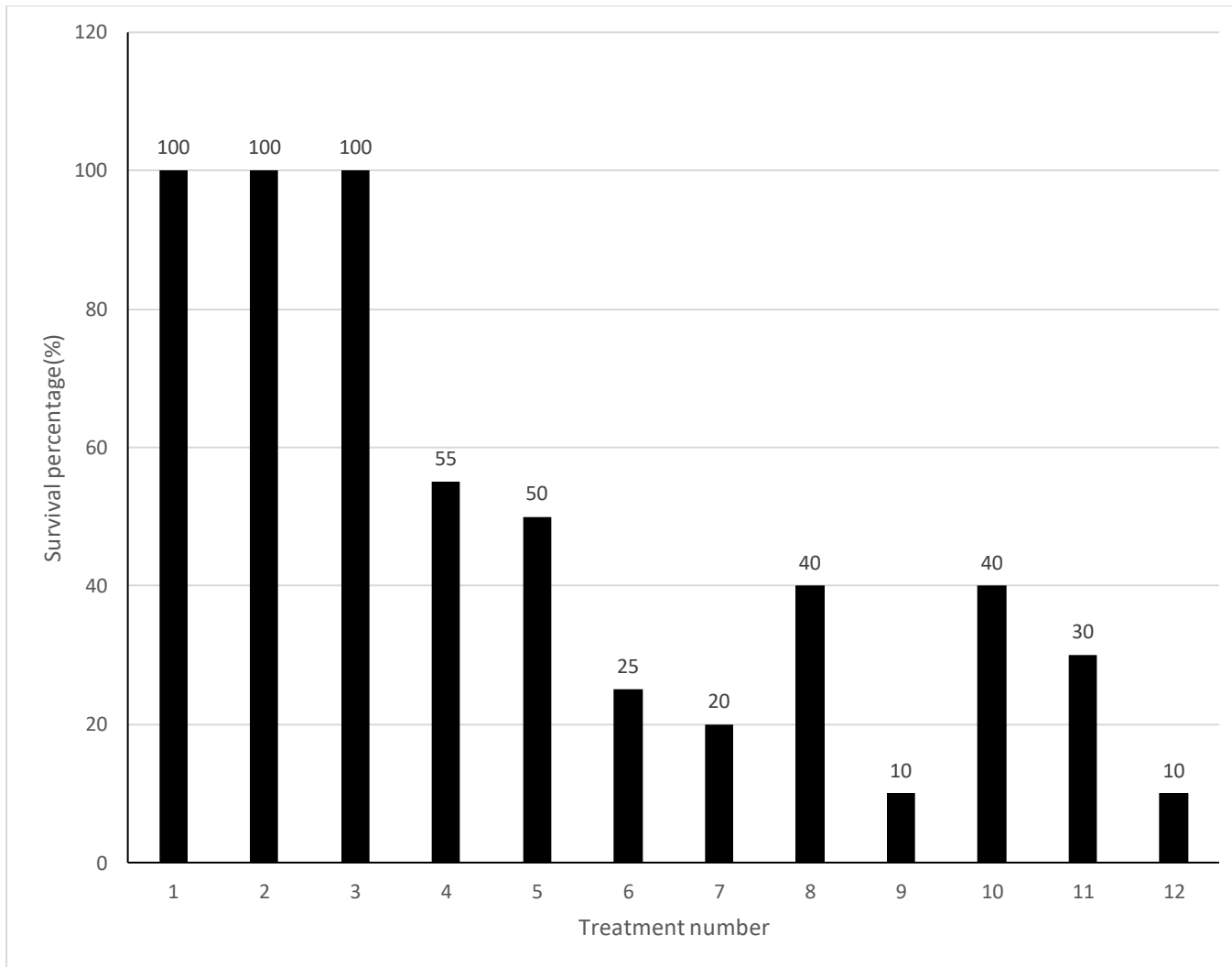


Figure 8. Bar graph of *in vitro* *Vitex* plants survival percentage for different oryzalin treatments.

Table 20. Result of logistic regression test between the interaction of oryzalin concentration and exposure time to survival of *Vitex agnus-castus* explants.

Variable	Degree of freedom	L-R ChiSquare	Prob> ChiSQ
Oryzalin concentration x exposure time	4	2.5797719073	0.6304

A chi-square test of independence was done in order to determine the significance of the interaction of the two variables on survival separately. First, oryzalin concentration and plant survival was analyzed.

The test measured plant survival based on the oryzalin concentration tested across all exposure times. The result of plant survival percentage for each of the concentration tested were recorded in Table 21 and the P value of the chi-square test was reported in Table 22.

Table 21. Result of plant survival at different oryzalin concentrations across all oryzalin exposure times tested on *Vitex agnus-castus* plants *in vitro*.

Oryzalin concentration (mM)	Plants survival percentage.
0.3	43
1.3	23
2.8	27

Table 22. Result of chi-square test of independence on all three concentrations against plant survival percentage of *Vitex agnus-castus*.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	6.305	0.0427*
Pearson	6.429	0.0402*

Plant survival percentage was the highest at concentration of 0.3 mM of oryzalin with 43% plants survived and the lowest survival percentage was observed when 1.3 mM of oryzalin was used with 23% plants survived. The highest concentration of oryzalin with 2.8 mM recorded 27% of plants survived (Table 21). The chi-square test reported a P value of 0.0427 which is significant (Table 22).

The P value of the independence test between 0.3 mM oryzalin and 1.3 mM of oryzalin showed that the difference in plant survival is 0.0194 which is significant (Table 23).

Table 23. The chi-square test of independence result between 0.3 mM oryzalin concentration and 1.3 mM of oryzalin concentration on tissue cultured plants of *Vitex agnus-castus*.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	5.463	0.0194*
Pearson	5.400	0.0201*

The P value of the independence test between 1.3 mM oryzalin and 2.8 mM of oryzalin showed that the difference in plant survival is 0.6732 which is not significant (Table 24).

Table 24. The chi-square test of independence result between 1.3 mM oryzalin concentration and 2.8 mM of oryzalin concentration on tissue cultured plants of *Vitex agnus-castus*.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	0.178	0.6732
Pearson	0.178	0.6733

The same chi-square test of independence was done to determine the significance of exposure time to the plant survival. The test measured plant survival based on exposure time across all concentrations tested. The result of percent plant survival for each of the exposure time tested were recorded in Table 25 and the P value of the chi-square test was reported in Table 26.

Table 25. Result of plants survival at different exposure time across all oryzalin concentrations tested.

Exposure time (minutes)	Plant survival percentage.
30	38
60	40
90	15

Table 26. Result of chi-square independence test of exposure time on plant survival on tissue cultured plants of *Vitex agnus-castus*.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	11.828	0.0027*
Pearson	10.939	0.0042*

For 30 minutes of exposure to oryzalin, 38% of the plants survived while 60 minutes of exposure had 40% of plants survived and lastly 90 minutes of exposure to oryzalin had only 15% of plants survived (Table 25). The chi-square test gave a P value of 0.0027 for the interaction between exposure time and plant survival which is less than 0.05 (Table 26). The interaction was shown to be significant.

Results of plant survival between 30 minutes of exposure time and 60 minutes exposure time yielded a P value of 0.8516 which is not significant (Table 27).

Table 27. The chi-square test of independence result between 30 minutes exposure time and 60 minutes exposure time on tissue cultured plants of *Vitex agnus-castus*.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	0.035	0.8516
Pearson	0.035	0.8516

Results from Table 28 showed that the independence test between 60 minutes of exposure time and 90 minutes of exposure time yield a P value of 0.0019 which is significant.

Table 28. The chi-square test of independence result between 60 minutes exposure time and 90 minutes exposure time on tissue cultured plants of *Vitex agnus-castus*.

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	9.674	0.0019*
Pearson	9.404	0.0022*

3.5 Discussion

All control plants that were treated with sterile water, not oryzalin, survived 100%. This was expected because sterile water should have no effect on a plant's survival. Plants that were treated with oryzalin were expected to have a lower survival percentage than the control plants because oryzalin is an herbicide and the effect of ploidy manipulation does affect a cell's viability, and thus, the plant's viability too.

The experiment was designed to observe the effects of three concentration of oryzalin at three exposure time on the survivability of *V.agnus-castus*. Thus, it was important to first analyze the significance of the combination of the two variables on the survival of the plants. To do this, logistic regression test was used. Based on the test, the combination of the two variables was not significant enough to reject the null hypothesis of the test. Because the combination of oryzalin concentration and exposure time on plant survival could not be shown to be significant, it is not possible to compare the significance of one treatment against another treatment (Table 20).

Despite the result obtained from logistic regression test, it can be observed that there is a correlation between oryzalin concentration and exposure time on plant survival (Figure 8). To determine the significance of the variables on plant survival, statistical analysis of independence test was done to observe how each variable affect the survival of the plants.

The first test of the independence test showed that oryzalin concentration does significantly affect the survivability of the plants (Table 21). From table 21, it can be observed that 0.3 mM of oryzalin had 43% survival while 1.3 mM had 23% survival and 2.8 mM had 27% explants survived. Since

1.3 mM oryzalin and 2.8 mM oryzalin had similar survival percentage, the significant difference must be between 0.3 mM of oryzalin and 1.3 mM of oryzalin since the survival percentage decreased significantly from 43% to 23% when oryzalin concentration was increased. This was confirmed when the same test was done by comparing the survival percentage of 0.3 mM of oryzalin concentration with 1.3 mM of oryzalin concentration (Table 23) and comparing the survival percentage of 1.3 mM of oryzalin concentration with 2.8 mM of oryzalin concentration (Table 24). Based on Table 23, the increase of oryzalin concentration from 0.3 mM to 1.3 mM significantly affected the survival percentage of the plants but the increase from 1.3 mM of oryzalin to 2.8 mM did not significantly affect the survival percentage of the plants (Table 24). It can be concluded that oryzalin concentration does significantly affect plant survival and the difference in survival is significant when oryzalin concentration was increased from 0.3 mM to 1.3 mM (Table 22 and Table 23).

As for the effect of exposure time on plant survival, the test of independence showed that exposure time does affect plant survival (Table 26). Plant survival reported for 30 minutes of exposure time was similar to 60 minutes of exposure time with 38% and 40% survival percentage, respectively, while survival of 90 minutes exposure time was only 15% (Table 25). The P value of the chi-square test obtained when plant survival of 30 minutes was compared to plant survival of 60 minutes showed that the differences in plant survival between the two exposure time was not significant (Table 27). However, the P value of the chi-square test between the exposure time of 60 minutes with 90 minutes showed that the difference is significant (Table 28). Based on the chi-square tests, it showed that exposure time does have an effect on explant survival (Table 26) and

exposure time change from 60 minutes to 90 minutes will significantly affect explant survival (Table 28).

An important trend that was observed from this experiment was that as the concentration of oryzalin increased from 0.3 mM to 1.3 mM, the percent survival of plants decreased (Table 21). The same is also true for exposure time. When exposure time was increased from 60 minutes to 90 minutes, the percent survival of plants decreased significantly (Table 25). The trend of decreased survival percentage when the concentration of oryzalin is increased is expected as the increased concentration of the toxic compound, oryzalin will cause more damage to more cells in the plants thus reducing the viability of the plants. It is also expected that the increase in exposure time to oryzalin will decrease the survival percentage of plants since more exposure time will allow more chemical to be absorbed into the plant or react with the plant cells thus causing more damage as compared to shorter exposure time.

3.6 Conclusion

This experiment successfully achieved its objective and demonstrated the effects of concentration and exposure time of oryzalin on *Vitex agnus-castus*. The results were as expected: the increase in concentration or the increase in exposure time increased the mortality of plants. The result of this experiment will enable the use of oryzalin for chemical mutagenesis in future research by providing the predicted mortality associated with the dosage of oryzalin. This chemical can be used to create mutants; however, further research is required to determine the success of the treatments in altering the chromosome sets in the surviving explants.

3.7 Literature Cited

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CHAPTER 4 CONCLUSION

There were two goals to be achieved for this thesis: the first goal was to outline the micropropagation protocol of *Vitex agnus-castus* and the second goal was to document the effects of oryzalin exposure to *V. agnus-castus* and to determine the LD₅₀ for oryzalin on *Vitex agnus-castus*.

In this thesis, each stage of the micropropagation protocol was explored and successfully outlined. The stages of micropropagation were outlined starting from Stage 1, which is introducing the plant into sterile environment, to Stage 4, the acclimatization stage. Stage 0, the plant selection stage was not included in this research. The starting material for the micropropagation protocol was young plants, three to five months old, that germinated from seeds in a greenhouse environment. These plants were young, healthy, and relatively clean without any pests on them. It was not in the interest of this project to work with any specific cultivar of *Vitex*, but rather to serve as a general protocol to clone any *Vitex agnus-castus*.

In stage 1, cuttings of *Vitex* were taken from the greenhouse and transported into the laboratory before they were sterilized with bleach solution. The protocols for transporting, washing, and the medium used for stage 1 was outlined. The experiment done for stage 1 of micropropagation using *Vitex* grown in the greenhouse reported a 94% success rate (Table 1). The sterile cuttings of *Vitex* from the experiment was later used for stage 2 of micropropagation which is the multiplication stage.

In stage 2, the sterile cuttings of *Vitex* were grown in two different cytokinin media with different concentrations for each cytokinin for either four weeks or eight weeks. The basal medium used in stage 2 was the same for all treatments in this experiment; only the type of cytokinin and the concentration of cytokinin were varied. The media in these experiments were prepared with full strength MS powder with vitamins with 30 g/l sucrose, 6 g/l agar, and the pH was calibrated to 5.70-5.75. The cuttings used for the experiments were also standardized. Each cutting was cut to approximately 2.5 cm in length with the apical meristem removed before they were placed in the cytokinin media. For each cytokinin used, four different concentrations were tested in order to determine the most suitable cytokinin to be used and the optimum concentration to be used to multiply *Vitex*. The number of shoots produced and the length of the shoots produced after four and eight weeks of growth were calculated to determine the best cytokinin for *Vitex* micropropagation. The results of the experiment were analyzed using ANOVA to compare each treatment with one another and a total of four ANOVA analysis were done. The trend of growth for the number of shoots produced in different cytokinin after four weeks and eight weeks of growth is very similar (Figure 1 and Figure 3). The number of shoots that were produced in BA media followed a sigmoidal curve as the concentration of BA was increased, while the number of shoots produced in 2iP media produced an irregular pattern as the concentration of 2iP was increased. The length of shoots that were produced in all treatments did not differ significantly for four weeks growth, when compared to the control plants (Figure 2 and Table 5). After eight weeks of growth, the shoots produced in BA media were recorded to be shorter than the shoots produced in 2iP media (Figure 4) but only shoots produced in 0.44 μM BA and 22.2 μM BA differ significantly in length than the control plants (Table 9). Based on the greatest number of shoots that were produced after four weeks and eight weeks of growth, both showed that BA is better at

inducing shoots on *Vitex* as compared to 2iP (Figure 1 and Figure 3). BA with 4.44 μM showed the greatest number of shoot production after four weeks and eight weeks growth (Figure 1 and Figure 3) while ANOVA analysis on the length of shoots produced with 4.44 μM BA does not significantly differ from the control plants (Table 5 and Table 7). The highest concentration of BA tested, 22.2 μM is not an optimum concentration to be used because although the number of shoots produced in the media is comparable to the number of shoots produced in media with 4.44 μM BA, the length of shoots produced in 22.2 μM BA is significantly shorter than the shoots in the control media after eight weeks of growth (Table 9).

For stage 3, the rooting stage, two auxins were tested at four different concentrations to determine the best auxin to use to induce rooting. The same basal medium was used: full strength MS with vitamins, 30 g/l sucrose and 6 g/l agar before the pH was calibrated to 5.70-5.75. Similar to stage 2, the cuttings were cut to approximately 2.5 cm in length to ensure uniformity but the apical meristem was left intact before the cuttings were placed in media supplemented with auxins. The cuttings were grown in the auxin-supplemented media for four weeks and eight weeks before the number of rooted cuttings and the length of the roots were recorded. To analyze the result, chi-square test of independence and ANOVA analysis were done. From four weeks observation, the highest percentage of rooted cuttings was observed to be only 58% from media supplemented with 24.6 μM IBA (Table 10) while the highest percentage of rooted cuttings were recorded to be 90% with media supplemented with 5 μM IBA after eight weeks of growth (Table 14). From this observation, it can be surmised that four weeks of growth is not sufficient to induce rooting using the two auxins tested. As for the length of roots produced after four weeks, the longest roots were recorded in media supplemented with 24.6 μM IBA (Figure 5). From figure 5, it can be observed

that the trend of root length increased as the concentration of IBA was increased. The same trend can be observed after eight weeks growth with IBA (Figure 6). However, the growth trend for root length in NAA-supplemented media is irregular after four weeks of growth (Figure 5) and after eight weeks of growth, it can be observed that the increase in NAA concentration decreased the length of roots produced (Figure 6). After eight weeks growth, the greatest number of cuttings rooted in media supplemented with 5 μ M IBA (Table 14) and since the length of roots produced in 5 μ M IBA is comparable to the length of roots produced in 24.6 μ M IBA (Figure 6), the best concentration of IBA to use is 5 μ M instead of 24.6 μ M.

The last stage of micropropagation, the acclimatization stage, was outlined. The plants chosen for the experiment were rooted plants that were maintained in 5 μ M IBA and the greenhouse medium used for stage 4 was pasteurized 50:50 perlite: peat moss greenhouse media. The roots of the plants were thoroughly washed to remove any tissue culture media before the plants were placed in the 50:50 medium. The acclimatization stage started in the laboratory and the plants were slowly acclimated by first allowing them to adjust to grow in medium without sucrose supplement before gradually reducing the humidity of the growing environment. Based on the protocol outlined for this stage, 85% of the plants were successfully taken out of tissue culture and into the greenhouse (Table 17).

The second goal of the thesis was to document the effect of oryzalin exposure to *Vitex agnus-castus in vitro*. For this experiment, the stock plants were prepared by using the result obtained from the multiplication protocol outlined in the micropropagation experiment. Plants were grown in 5 μ M BA to create enough stock plants for the mutagenesis experiment. The stock plants were

maintained in the same 5 μ M BA medium to ensure that they are actively growing before they are exposed to oryzalin. For this experiment, three concentrations of oryzalin were tested with three different exposure times. After exposing the plants to oryzalin, physiological changes and mortality of the plants were recorded for a total of 16 weeks of observation. To analyze the data, two statistical methods were used. The first statistical method used was logistic regression test and the second method was chi-square test of independence. The logistic regression test failed to show a significance difference of interaction between the two variables tested, the concentration of oryzalin used and the exposure time on survival of the plants (Table 20). Despite the result of the first test, chi-square tests that were conducted on the concentration of oryzalin and exposure time individually were able to show that the concentration of oryzalin used and exposure time do have significant effects on the survival of the plants (Table 22 and Table 26). The chi-square test showed that when oryzalin exposure was increased from 0.3 mM to 1.3 mM, the effect on the survival of the plants was significant (Table 23). As for exposure time, the chi-square test showed that increasing the exposure time from 60 minutes to 90 minutes significantly reduced the number of surviving plants (Table 28). Based on the chi-square tests, it can be concluded that increasing concentration of oryzalin as well as increasing exposure time will decrease the number of surviving plants. After 16 weeks, the surviving plants exposed to oryzalin were saved for future research.

Based on the results obtained, the project successfully met its two objectives. At the end of the research, the micropropagation protocol for *Vitex agnus-castus* was outlined and the effects of oryzalin exposure to *Vitex agnus-castus* were recorded. The results of this research will allow future research to be able to propagate *Vitex* in tissue culture and to use oryzalin for mutagenesis of *Vitex*.

CHAPTER 5: LITERATURE CITED

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