POLYPLOIDY INDUCTION AND TISSUE CULTURE TECHNIQUES OF *HIBISCUS MOSCHEUTOS*

by

ZHITONG LI

(Under the Direction of John M. Ruter)

ABSTRACT

The native herbaceous plant, *Hibiscus moscheutos* L., was used in this project. Polyploidy induction was attempted via soaking seeds and seedlings in colchicine and oryzalin. Both colchicine and oryzalin were effective in inducing tetraploids. Triploids were obtained from the traditional method of crossing tetraploids with diploids. Triploid and tetraploid plants showed varying degrees of difference from diploid plants when comparing morphological traits. Shoots of *H. moscheutos* were also treated in colchicine and oryzalin in tissue culture for polyploidy induction. Only one tetraploid was successfully induced in tissue culture in varying concentrations of mitotic inhibitors during different exposure durations. Glutamine was added in tissue culture media to compare with some common PGRs in stimulating shoot elongation. After seven weeks of culture, glutamine promoted shoot elongation inconsistently in different studies. More experimentation should be attempted with other amino acids as a nitrogen source for promoting plant growth *in vitro*.

INDEX WORDS: polyploidy, *Hibiscus moscheutos*, colchicine, oryzalin, seedling soaking, sterility, ornamental plant breeding, tissue culture, glutamine

POLYPLOIDY INDUCTION AND GROWTH REGULATIONS OF *HIBISCUS MOSCHEUTOS*

by

ZHITONG LI

B.S., Beijing Forestry University, 2012

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfilment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2016

POLYPLOIDY INDUCTION AND GROWTH REGULATIONS OF *HIBISCUS MOSCHEUTOS*

by

ZHITONG LI

Major Professor: John M. Ruter

Advisory Committee: Donglin Zhang
Scott A. Merkle

Electronic Version Approved:

Suzanne Barbour
Dean of Graduate School
University of Georgia
December 2016

DEDICATION

I dedicate this thesis to my mom, Hongwei Zhu, who raised me up and always be there for me. I also dedicate this thesis to my fiancé, Daniel Greenwell. Thank you for your support, both in my work and life.

ACKNOWLEGEMENTS

Dr. John Ruter for his patience with me, and his direction and support through this whole process.

My advisory committee, Dr. Donglin Zhang and Dr. Scott Merkle, for giving me access to their labs and giving me guidance on research planning and thesis writing. I appreciate their encouragement along the way.

Vickie Waters, Heather Gladfelter, Meg Green, Jinying Dong, Sue Dove, John Doyle and Paul Montello – great technicians, for providing help when I was in need and giving advice on lab, greenhouse and field work.

Graduate students and undergraduate student workers, I appreciate all the care and help from them and I am truly thankful for having so many friends.

The crew at Horticulture Farm, for assisting my research in the greenhouse and in the field.

The staff at Trial Gardens, for providing help for my seed germination study.

Dr. Savithri Nambeesan, for letting me use her lab for tissue culture studies.

Dr. Anish Malladi, for giving me advice on flow cytometry.

Dr. Richard Chandler, for advice on data analysis.

Dr. Cecelia McGregor, for giving me advice on tetraploidy induction techniques.

Deborah Franco, Mary Jane Scarborough and Vickie Edwards – administrative staff, for assistance on all coordination matters.

TABLE OF CONTENTS

Page

LIST OF TABLES

page

LIST OF FIGURES

page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

History of Polyploidy Induction

Polyploidy in plants has long been observed as occurring in natural populations or through artificial induction. People have been exploring the benefits and methods of manipulating ploidy levels since the early 20th century. Blakeslee and Avery (1937) successfully applied colchicine to double chromosomes of plants, through inhibiting the separation of mitotic spindles during the mitosis process. Subsequently, the dinitroaniline herbicide oryzalin was also identified as an effective mitotic inhibitor that assisted in the formation of polyploids (BARTELS AND HILTON, 1973), which later became used more due to its nontoxicity to human beings. Induction of polyploidy has long been used in agricultural crops (BLAKESLEE AND AVERY, 1937). Those working with horticultural species, ornamental plants in particular, later adopted the technique to promote cultivar improvement (DERMEN, 1961; JONES, 2008; KEHR, 1996).

Effects of Polyploidy Induction

Polyploids are characterized as having enlarged cells resulting in a variety of morphological, cytological and physiological alterations (DERMEN, 1961; EIGSTI AND DUSTIN, 1955; HANCOCK, 1997). In breeding ornamental plants, induction of polyploidy has been widely applied to enlarge plant organs (CAPORALI et al., 2014; KEHR, 1996; RANNEY, 2006), improve plant vigor (GHANI et al., 2014; RANNEY, 2006), overcome sterility of F1 hybrids following interspecific hybridization (RANNEY, 2006; VAN TUYL AND JEU, 1997; VAN TUYL AND LIM, 2003), and develop sterile cultivars with ornamental traits (CONTRERAS AND RUTER, 2009;

RANNEY, 2006). Polyploids often have larger and thicker flowers (CAPORALI et al., 2014), longer blooming period (KEHR, 1996), and thicker and darker leaves with an altered length to width ratio (DIXIT AND CHAUDHARY, 2014). Leaves of polyploids often have larger stomata (STANYS et al., 2006; WU et al., 2011) both in their length and width (YENCHON AND TE-CHATO, 2014), higher numbers of chloroplasts per guard cell (JEONG AND HYUN, 2013), and sometimes altered shapes (CONTRERAS et al., 2010). Some induced polyploid plants may produce larger fruits with altered shapes (WU et al., 2012). Polyploid plants may have altered metabolic rates which are associated with slower growth and maturation (COMAI, 2005; LEVIN, 1983), enhanced resistance to environmental stresses (OTTO, 2007; YANG et al., 2014) and diseases (CARLIER, 1974; HUMPHREYS et al., 2010), decreased pollen viability (SHAO et al., 2003), as well as reduced seed production (CONTRERAS AND RUTER, 2009; CONTRERAS et al., 2009).

Beyond enlargement, induced polyploid plants may also possess decreased, but favorable characteristics. For example, tetraploid *Dendrobium nobile* produces larger, but fewer, flowers per pseudobulb and more, but smaller pseudobulbs (VICHIATO et al., 2014). The induced octaploid *Hibiscus acetosella* 'Panama Red' remains more compact (CONTRERAS et al., 2009); the induced tetraploid *Humulus lupulus* develops thinner and shorter shoots (TROJAK-GOLUCH AND SKOMRA, 2013).

Methods of Polyploidy Induction

Of the methods applied for polyploidization of plants, immersion of seeds in doubling agents was the first to exhibit satisfactory performance (BLAKESLEE AND AVERY, 1937; LEHRER et al., 2007; RUBULUZA et al., 2007). Seedlings or apical shoots can also be immersed in doubling agents at different concentrations and periods of time (PEREIRA et al., 2014; RANNEY, 2006). Oryzalin in warm agar solution applied on apical shoots successfully induced tetraploid

*Rhododendron*s from their diploid progenitors (JONES, 2008). Treating older plants often results in a greater percentage of cytochimeras (RANNEY, 2006), while treatment of younger plant material might result in a higher mortality rate (SOONTHORNKALUMP et al., 2014).

Besides using colchicine or oryzalin as the major induction chemical, adding SilEnergy® (organosilicate surfactant) (CONTRERAS et al., 2010), Tween® 20 (polyoxyethylene sorbitan monolaurate) or DMSO (dimethyl sulfoxide) can effectively increase the rate of polyploidization (HUMPHREYS et al., 2010; MORGAN, 1976). However, there is a trade-off in that adding DMSO to colchicine solution increases the rate of polyploidy production, but with a negative effect on plant survival (HAMILL et al., 1992; PEREIRA et al., 2014).

Apical shoots can also be treated *in vitro* for polyploidization (COLA et al., 2014), as can seeds, seedlings or roots (SHI et al., 2014). Tissue culture techniques have been widely incorporated into polyploidy induction for the convenience of regeneration (GALLONE et al., 2014; SHI et al., 2014), as well as a faster growth rate of the polyploids (SAKHANOKHO AND KELLEY, 2009).

Polyploidy Induction in *Hibiscus*

"Hibiscus" typically brings to mind the tropical *Hibiscus rosa-sinensis*, with colorful and showy flowers, but *Hibiscus moscheutos* (Malvaceae), also known as hardy hibiscus, is an equally beautiful species that is native and hardy in the eastern region of North America (FLORA OF NORTH AMERICA EDITORIAL COMMITTEE, 1993+; WINTERS, 1970). A popular cultivar on the market with large flower petals and dwarf compact stature is 'Luna Red'. These valued characteristics may be further improved through polyploidy induction, with the possibility that tetraploids and triploids will be superior to the original diploids. The first goal of this study is to define the optimal method for inducing tetraploids from diploid *H. moscheutos* 'Luna Red' by

determining survival rates and the ploidy levels after transformation. It is expected that the convertion process will result in a variety of changes in morphology and physiology. Thus, we are seeking to discover the unknown differences among diploids, triploids and tetraploids of *H. moscheutos*.

In previous years we have successfully obtained several hybrid diploid plants with mixed *H. moscheutos* lineage. The protocol for 'Luna Red' will help determine the protocol for the hybrid selections in terms of doubling agent concentration and time of treatment. The optimal method will be employed to produce tetraploids with the hope of finding a number of desirable characteristics.

Hibiscus, especially *H. rosa-sinensis* and *H. syriacus*, have long been cultured and bred to have different ploidy levels, with a variety of genotypes (WILSON, 1994). Many of the hibiscus cultivars on the market, such as 'Diana', 'Helene', 'Minerva', and 'Aphrodite', are tetraploids or sterile triploids (EGOLF, 1970; EGOLF, 1981; EGOLF, 1986; EGOLF, 1988). Though not much polyploidy breeding work has been done on *H. moscheutos*, based on its similar genetics to other hibiscus species (WILSON, 1994), it may be easier to induce polyploidy in herbaceous *H. moscheutos* than in woody *Hibiscus* species such as *H. rosa-sinensis* and *H. syriacus* (OLSEN, 2006). Therefore, there is great potential to develop new *H. moscheutos* germplasm through induction of polyploidy.

In vitro Propagation of Hibiscus

There have been many cases reporting proliferating hibiscus *in vitro*, using seeds (AYADI et al., 2011) and nodal shoots (AIRO et al., 2009; CHRISTENSEN et al., 2008). A variety of culture media have been tested (WEST AND PREECE, 2004) for *Hibiscus*, with MS (Murashige and Skoog, 1962) medium being widely applied to proliferate shoots (AIRO et al., 2009; AYADI et al., 2011;

CHRISTENSEN et al., 2008), and ½ MS used as rooting medium (AIRO et al., 2009). Additionally, benzyladenine (BA) (AIRO et al., 2009) and thidiazuron (TDZ) (JENDEREK et al., 1998; WEST AND PREECE, 2004) have been successfully used to induce the formation of callus and adventitious buds.

Effect of Glutamine and Some Other Amino Acids in Tissue Culture

Amino acids in the glutamine/glutamate family (glutamine, glutamate, proline, arginine) not only function as building blocks of proteins or nucleotides (HORTON et al., 2006), they can also serve as nitrogen sources (FLAIG AND MOHR, 1992; OKUMOTO et al., 2016), or as transportation media to incorporate ammonia into other amino acids (HORTON et al., 2006; OKUMOTO et al., 2016). These amino acids initiate and accelerate ammonia and nitrite entering into organic nitrogen metabolism. Under catalysis of glutamine synthetase, glutamine is reduced to glutamate, and can be further modified into proline or arginine (HORTON et al., 2006; SHAHSAVARI, 2011). Because of this, amino acids serve as an efficient nitrogen source through direct synthesis of proteins (EFZUENI ROZALI et al., 2014), and an efficient assimilation approach during long distance metabolic transportation (OKUMOTO et al., 2016), particularly for plants in tissue culture environments (SAAD, 2012).

In tissue culture basal media, inorganic nitrogen is supplied in sufficient quantities (MURASHIGE AND SKOOG, 1962). Supplementing tissue culture basal media with glutamine enables higher nitrogen intake due to an increase of both nitrogen sources and assimilation ability (OKUMOTO et al., 2016). Glutamine has been used in media for both dedifferentiation and redifferentiation processes (HABIB et al., 2015): callus induction (CAI et al., 2013), shoot differentiation (CHEN et al., 2015; LI et al., 2014; PERVEEN AND MANSURI, 2015), faster rooting (LIU et al., 2015; TOPPO et al., 2012) and somatic embryogenesis (YAPO et al., 2011).

Polyploidy Induction Research Objectives

Hibiscus moscheutos 'Luna Red' possesses good ornamental value, however it can be further improved while maintaining its original traits. Fruit set follows blooming which begins during the second half of summer, while unsightly brown capsules form and remain on the stalks. Therefore, breeding for sterile selections is a primary goal. By manipulating plants into a higher ploidy level, we expect to see a "Gigas effect" (ACQUAAH, 2007). With sawflies (*Atomacera decepta*) being the most damaging pest affecting *H. moscheutos* for several decades (CRANSHAW, 2004; TIPPINS, 1965), it could be of great interest to increase resistance by increasing leaf density through polyploidy induction. A cultivar with good ornamental qualities and increased resistance to pests is of horticultural importance.

The ultimate goal of this project is to discover an effective method of inducing tetraploids from diploid *H. moscheutos*, and eventually to obtain tetraploid and triploid forms of advanced hybrids from previously selected crosses. The inbred cultivar *H. moscheutos* 'Luna Red' was used as a template to work out a protocol, determining an optimal combination of chemical concentration and period of treatment to induce tetraploids through soaking seeds and seedlings. Phenotypic data of diploid, triploid and tetraploid plants was compared for the reference of future work.

Literature Cited

Acquaah, G., 2007. *Principles of Plant Genetics and Breeding*. Blackwell Pub., Malden, MA; Oxford.

Airo, M., Giardina, G., Farruggia, G., and Zizzo, G.V., 2009. In Vitro Propagation of *Hibiscus rosa-sinensis* (L.), p. 107-112. In: Romano, A. (ed.), Iii International Symposium on Acclimatization and Establishment of Micropropagated Plants. Int Soc Horticultural Science, Leuven 1.

Ayadi, R., Hamrouni, L., Hanana, M., Bouzid, S., Trifi, M., and Khouja, M.L., 2011. *In vitro* Propagation and Regeneration of an Industrial Plant Kenaf (*Hibiscus cannabinus* L.). *Industrial Crops and Products* 33:474-480.

Bartels, P.G. and Hilton, J.L., 1973. Comparison of Trifluralin, Oryzalin, Pronamide, Propham, and Colchicine Treatments on Microtubules. *Pest. Biochem. Physiol.* 3:462-472.

Blakeslee, A.F. and Avery, A.G., 1937. Methods of Inducing Doubling of Chromosomes in Plants by Treatment with Colchicine. *Journal Heredity* 28:393-411.

Cai, X.D., Wang, G.Y., and Cao, W.J., 2013. *In vitro* Induction and Proliferation of Callus from Immature Cotyledons and Embryos of *Juglans regia* cv. 'Xiangling'. *Not. Bot. Horti Agrobot. Cluj-Na.* 41:378-384.

Caporali, S., Hammami, S.B.M., Moreno-Alias, I., Rapoport, H.F., Chiancone, B., Germana, M.A., and Rosati, A., 2014. Effects of Tetraploidy on Olive Floral and Fruit Biology. *Scientia Horticulturae* 179:198-203.

Carlier, L., 1974. Comparison of Feeding Diploid and Tetraploid Italian and Perennial Ryegrass Cultivars from RvP. Universiteit Gent, Belgium, Ph.D.

Chen, Z., Tang, J., and Li, H., 2015. Method for Culturing Peony Plant Tissue, Involves Selecting Robust Growth Peony Branches, Preparing Medium, Performing Disinfection and Vaccination Followed by Removing Section, Subculturing Shoots, Transferring to Medium, and Culturing. University of Chongqing Arts & Science (UYCH-Non-standard).

Christensen, B., Sriskandarajah, S., Serek, M., and Muller, R., 2008. In vitro Culture of *Hibiscus rosa-sinensis* L.: Influence of Iron, Calcium and BAP on Establishment and Multiplication. *Plant Cell Tissue and Organ Culture* 93:151-161.

Cola, G.P.A., Marques, A.M., Damasceno, S., Carvalho, C.R., and Clarindo, W.R., 2014. *In Vitro* Polyploidization in *Solanum lycopersicum* Mill. 'Santa Cruz Kada Gigante'. *Cytologia* 79:351-358.

Comai, L., 2005. The Advantages and Disadvantages of Being Polyploid. *Nature Reviews Genetics* 6:836-846.

Contreras, R. and Ruter, J., 2009. An Oryzalin Induced Polyploid from a Hybrid of *Hibiscus acetosella* x *H. radiatus (Malvaceae)* Exhibits Reduced Fertility and Altered Morphology. *HortScience* 44:1177.

Contreras, R.N., Ruter, J.M., and Hanna, W.W., 2009. An Oryzalin-Induced Autoallooctoploid of *Hibiscus acetosella* 'Panama Red'. *J. Am. Soc. Hortic. Sci.* 134:553-559.

Contreras, R.N., Ruter, J.M., and Schwartz, B.M., 2010. Oryzalin-induced Tetraploidy in *Cryptomeria japonica* (Cupressaceae). *HortScience* 45:316-319.

Cranshaw, W., 2004. Leaf Chewers, p. 84, Garden Insect of North America. Prinston University Press.

Dermen, H., and S. L. Emsweller, 1961. The Use of Colchicine in Plant Breeding. *ARS Mimeographed Leaflet* 34:10.

Dixit, V. and Chaudhary, B.R., 2014. Colchicine-Induced Tetraploidy in Garlic (*Allium sativum* L.) and Its Effect on Allicin Concentration. *J. Horticult. Sci. Biotechnol.* 89:585-591.

Efzueni Rozali, S., Rashid, K.A., and Mat Taha, R., 2014. Micropropagation of an Exotic Ornamental Plant, Calathea crotalifera, for Production of High Quality Plantlets. *The Scientific World Journal* 2014:12.

Egolf, D.R., 1970. *Hibiscus syriacus* 'Diana', a New Cultivar (Malvaceae). *Baileya* 17:75-78.

Egolf, D.R., 1981. 'Helene' Rose of Sharon (Althea). *HortScience* 16:226-227.

Egolf, D.R., 1986. 'Minerva' Rose of Sharon (Althea). *HortScience* 21:1463-1464.

Egolf, D.R., 1988. 'Aphrodite' Rose of Sharon (Althea). *HortScience* 23:223.

Eigsti, O.J. and Dustin, P., 1955. *Colchicine in Agriculture, Medicine, Biology, and Chemistry*. Iowa State College Press, Ames.

Flaig, H. and Mohr, H., 1992. Assimilation of Nitrate and Ammonium by the Scots Pine (Pinus sylvestris) Seedling under Conditions of High Nitrogen Supply. *Physiologia Plantarum* 84:568-576.

Flora of North America Editorial Committee, 1993+. *Flora of North America, Vol. 6*. Oxford University Press, New York and Oxford.

Gallone, A., Hunter, A., and Douglas, G.C., 2014. Polyploid Induction *in vitro* Using Colchicine and Oryzalin on *Hebe* 'Oratia Beauty': Production and Characterization of the Vegetative Traits. *Scientia Horticulturae* 179:59-66.

Ghani, M.A., Sun, Q., Li, J.X., Cao, L.W., Rao, L.L., Zou, X.X., and Chen, L.P., 2014. Phenotypic and Genetic Variation Occurred during Wide Hybridisation and

Allopolyploidisation between *Brassica rapa* and *Brassica nigra*. *Scientia Horticulturae* 176:22-31.

Habib, D., Zia, M., Bibi, Y., Abbasi, B.H., and Chaudhary, M.F., 2015. Response of Nitrogen Assimilating Enzymes during *in vitro* Culture of *Argyrolobium roseum*. *Biologia* 70:478-485.

Hamill, S.D., Smith, M.K., and Dodd, W.A., 1992. *In Vitro* Induction of Banana Autotetraploids by Colchicine Treatment of Micropropagated Diploids. *Australian Journal of Botany* 40:887-896.

Hancock, J.F., 1997. The Colchicine Story. *HortScience* 32:1011-1012.

Horton, H.R., Moran, L.A., Scrimgeour, K.G., Perry, M.D., and Rawn, J.D., 2006. Amino Acid Metabolism, p. 520-529. In: Carlson, G. (ed.), Principles of Biochemistry (Fourth Edition). Pearson Prentice Hall, New Jersey.

Humphreys, M., Feuerstein, U., Vandewalle, M., and Baert, J., 2010. Ryegrasses, p. 223-224. In: Boller, B., Posselt, U. K., and Veronesi, F. (eds.), Handbook of Plant Breeding: Fodder Crops and Amenity Grasses. Springer New York.

Jenderek, M.M., Olney, A.J., and Kolosowski, S., 1998. Effect of Different Cytokinin Types and Concentrations on Micropropagation of Eastern Hibiscus. *In Vitro Cellular and Developmental Biology Animal* 34:60A.

Jeong, K.S. and Hyun, K.H., 2013. A Tetraploid Induction in *Hypericum patulum* Thunberg by Colchicine Soaking Treatment. *Korean Journal of Plant Reources.* 26:284-288.

Jones, J.R., Ranney, Thomas G., Eaker, Thomas A., 2008. A Novel Method for Inducing Polyploidy in Rhododendron Seedlings. *Journal American Rhododendron Soceity*:130-135.

Kehr, A.E., 1996. Breeding for a Purpose. *American Rhododendron Society Bulliten*:131-141.

Lehrer, J., Brand, M., and Lubell, J., 2007. Induction of Tetraploidy in Japanese Barberry (*Berberis thunbergii* DC.) Seedlings through Exposure to Colchicine and Oryzalin. *HortScience* 42:901-901.

Levin, D.A., 1983. Polyploidy and Novelty in Flowering Plants. *Am. Nat.* 122:1-25.

Li, J.Y., Fan, Z.Q., and Wu, B., 2014. Callus Induction and Plant Regeneration from Immature Cotyledon of Camellia azalea, p. 157-162. In: Zhang, Q. and Jin, X. (eds.), Vi International Symposium on the Taxonomy of Cultivated Plants. Int Soc Horticultural Science, Leuven 1.

Liu, Y., Tong, X., Hui, W.K., Liu, T., Chen, X.Y., Li, J., Zhuang, C.X., Yang, Y.S., and Liu, Z.L., 2015. Efficient Culture Protocol for Plant Regeneration from Petiole Explants of Physiologically Mature Trees of *Jatropha curcas* L. *Biotechnol. Biotechnol. Equip.* 29:479-488.

Morgan, W.G., 1976. A Technique for the Production of Polyploids in Grasses. *Euphytica*:443-446.

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

Okumoto, S., Funck, D., Trovato, M., and Forlani, G., 2016. Editorial: Amino Acids of the Glutamate Family: Functions Beyond Primary Metabolism, Frontiers in Plant Science.

Olsen, R.T., 2006. Utilizing Polyploidy for Developing Improved Nursery Crops: Restoring Fertility in Wide Hybrids, Limiting Fertility of Invasive Species, Embryo Culture of Triploids, Pest Resistance, and Inheritance of Ornamental Traits. North Carolina State University, Ann Arbor, 3269438 Ph.D.

Otto, S.P., 2007. The Evolutionary Consequences of Polyploidy. *Cell* 131:452-462.

Pereira, R.C., Ferreira, M.T.M., Davide, L.C., Pasqual, M., Mittelmann, A., and Techio, V.H., 2014. Chromosome Duplication in *Lolium multiflorum* Lam. *Crop Breeding and Applied Biotechnology* 14:251-255.

Perveen, A. and Mansuri, S., 2015. Rapid Propagation of a Biodiesel Plant Cassava (Manihot esculenta Crantz) through Tissue Culture. *International Journal of Biology and Biotechnology* 12:369-372.

Ranney, T.G., 2006. Polyploidy: From Evolution to New Plant Development. *Combined Proceeding of International Plant Propagators Society* 56:137-142.

Rubuluza, T., Nikolova, R.V., Smith, M.T., and Hannweg, K., 2007. *In vitro* Induction of Tetraploids in *Colophospermum mopane* by Colchicine. *S. Afr. J. Bot.* 73:259-261.

Saad, A.I.M.E., A.M., 2012. Plant Tissue Culture Media, p. 31. In: Leva, D. A. (ed.), Recent Advances in Plant in vitro Culture. InTech.

Sakhanokho, H.F. and Kelley, R.Y., 2009. Influence of salicylic acid on in vitro propagation and salt tolerance in Hibiscus acetosella and Hibiscus moscheutos (cv 'Luna Red'). *Afr. J. Biotechnol.* 8:1474-1481.

Shahsavari, E., 2011. Impact of Tryptophan and Glutamine on the Tissue Culture of Upland Rice. *Plant Soil Environ.* 57:7-10.

Shao, J., Chen, C., and Deng, X., 2003. In vitro Induction of Tetraploid in Pomegranate (*Punica granatum*). *Plant Cell Tiss Organ Cult* 75:241-246.

Shi, H., Yu, W., Zhang, G., Tsang, P.E., and Chow, C.F.S., 2014. Induction of Polyploid Hairy Roots and Its Plant Regeneration in *Pogostemon cablin*. *Chinese Journal of Biotechnology* 30:1235-1246.

Soonthornkalump, S., Chuenboonngarm, N., Soontornchainaksaeng, P., Jenjittikul, T., and Thammasiri, K., 2014. Effect of Colchicine Incubation Time on Tetraploid Induction of *Kaempferia rotunda*. *Acta Horticulturae*:89-92.

Stanys, V., Weckman, A., Staniene, G., and Duchovskis, P., 2006. In vitro Induction of Polyploidy in Japanese Quince (*Chaenomeles japonica*). *Plant Cell Tiss Organ Cult* 84:263-268.

Tippins, H.H., 1965. The Sawfly *Atomacera decepta*, a Pest of *Hibiscus*. *Journal of Economic Entomology* 58:161-161.

Toppo, D.D., Singh, G., Purshottam, D.K., and Misra, P., 2012. Improved *in vitro* Rooting and Acclimatization of *Jatropha curcas* Plantlets. *Biomass Bioenerg.* 44:42-46.

Trojak-Goluch, A. and Skomra, U., 2013. Artificially Induced Polyploidization in *Humulus lupulus* L. and Its Effect on Morphological and Chemical Traits. *Breed. Sci.* 63:393-399.

Van Tuyl, J.M. and Jeu, M.J.D., 1997. Methods for Overcoming Interspecific Crossing Barriers, p. 273-292. In: Sawhney, V. K. and Shivanna, K. R. (eds.), Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press, New York.

van Tuyl, J.M. and Lim, K.B., 2003. Interspecific Hybridisation and Polyploidisation as Tools in Ornamental Plant Breeding, p. 13-22. In: Forkmann, G., Hauser, B., and Michaelis, S. (eds.), Proceedings of the International Symposium on Classical Versus Molecular Breeding of Ornamentals. International Society Horticultural Science, Leuven 1.

Vichiato, M.R.D., Vichiato, M., Pasqual, M., Rodrigues, F.A., and de Castro, D.M., 2014. Morphological Effects of Induced Polyploidy in *Dendrobium nobile* Lindl. (*Orchidaceae*). *Crop Breeding and Applied Biotechnology* 14:154-159.

West, T. and Preece, J.E., 2004. Effects of Thidiazuron and Nutrient Salt Formulations on Micropropagation of Hardy Hibiscus (*Hibiscus moscheutos* L.), p. 293-296. In: Fernandez, T. and Davidson, C. G. (eds.), Nursery Crops Development, Evaluation, Production and Use. International Society Horticultural Science, Leuven 1.

Wilson, F.D., 1994. The Genome Biogeography of *Hibiscus* L. section *Furcaria* DC. *Genetic Resources and Crop Evolution* 41:13-25.

Winters, H.F., 1970. Our Hardy Hibiscus Species as Ornamentals. *Economic Botany* 24:155-164.

Wu, J.-H., Ferguson, A.R., Murray, B.G., Jia, Y., Datson, P.M., and Zhang, J., 2012. Induced Polyploidy Dramatically Increases the Size and Alters the Shape of Fruit in *Actinidia chinensis*. *Annals of Botany* 109:169-179.

Wu, Y.X., Yang, F.H., Zhao, X.M., and Yang, W.D., 2011. Identification of Tetraploid Mutants of *Platycodon grandiflorus* by Colchicine Induction. *Caryologia* 64:343-349.

Yang, C.W., Zhao, L., Zhang, H.K., Yang, Z.Z., Wang, H., Wen, S.S., Zhang, C.Y., Rustgi, S., von Wettstein, D., and Liu, B., 2014. Evolution of Physiological Responses to Salt Stress in Hexaploid Wheat. *Proc. Natl. Acad. Sci. U. S. A.* 111:11882-11887.

Yapo, E.S., Kouakou, T.H., Kone, M., Kouadio, J.Y., Kouame, P., and Merillon, J.M., 2011. Regeneration of Pineapple (*Ananas comosus* L.) Plant through Somatic Embryogenesis. *J. Plant Biochem. Biotechnol.* 20:196-204.

Yenchon, S. and Te-chato, S., 2014. Polyploidy Induction of *Dendrobium formosum* by Colchicine Treatment *in vitro*. *Acta Horticulturae*:81-88.

CHAPTER 2

INDUCTION OF POLYPLOID *HIBISCUS MOSCHEUTOS* THROUGH SOAKING OF SEEDS AND SEEDLINGS[1]

[1]Li, Z. and J. Ruter. To be submitted to *HortScience*.

Abstract

Hibiscus moscheutos L. is an herbaceous hibiscus native to eastern North America that has been a popular landscape and container plant exhibiting large and colorful flowers in the summer. However, unsightly fruit develop and remain on the stalks at the end of the blooming season, which greatly decreases the ornamental value. Thus, breeding for sterility was attempted through ploidy level manipulation to reduce formation and growth of seed stalks, and to improve blooming vigor and longevity.

Colchicine and oryzalin were used as mitotic inhibitors to induce tetraploid breeding lines that could be used to develop sterile triploids. Pre-scarified seeds and germinated seedlings of the cultivar 'Luna Red' were soaked in three different concentrations of each doubling agent for three different durations. Exposure to a low concentration of colchicine solution for a long time or to a low concentration of oryzalin for a short period were found to be effective in yielding a high number of tetraploids with a low rate of mortality. Triploids were obtained from the traditional method of crossing tetraploids with diploids.

Triploid and tetraploid plants showed a decrease in height with a more compact shape. Leaves of tetraploid plants were more ruffled, with an increase in overall leaf thickness, but were not different from leaves of diploids and triploids in regards to leaf mass per area. Triploid plants bloomed longer, but had smaller flowers than diploid plants. While the whole planting was infected by aerial Phytophthora, diploid, tetraploid and triploid plants were significantly different in their tolerances: all diploid branches were infected, but only one triploid branch had a slight Phytophthora infection, and the transmission remained slow. Flowers of tetraploid plants failed to produce pollen, while flowers of triploid plants produced only non-viable pollen grains and fruit aborted after pollination, which led to infertility of induced triploids.

Introduction

Hibiscus moscheutos L., often called swamp rose-mallow or hardy hibiscus, is a perennial plant native to wetland areas in the eastern region of North America (USDA zone 5a-10b) (FLORA OF NORTH AMERICA EDITORIAL COMMITTEE, 1993+; WINTERS, 1970). As a landscape ornamental, it has long been bred for compact stature, large flowers in various colors, unique leaf colors and shapes. In the mid-Atlantic region, flowers are produced daily from July to September (SPIRA, 1989); however unsightly fruit eventually develop and remain on the stalks until the end of the season. This unsightliness greatly decreases the ornamental value. Thus, breeding for sterility was attempted to avoid formation and growth of seed stalks, and to improve blooming vigor and longevity.

Induction of polyploid genotypes is an effective means of producing enhanced characteristics and increased sterility. In breeding ornamental plants, polyploidy induction has been widely applied to enlarge plant organs (CAPORALI et al., 2014; KEHR, 1996; RANNEY, 2006), improve plant vigor (GHANI et al., 2014; RANNEY, 2006), and develop sterile cultivars with ornamental traits (CONTRERAS AND RUTER, 2009; RANNEY, 2006). Polyploids often have larger and thicker flowers (CAPORALI et al., 2014), longer blooming period (KEHR, 1996), and thicker and darker leaves with altered length to width ratio (DIXIT AND CHAUDHARY, 2014). Beyond enlargement, induced polyploid plants may also possess decreased, yet favorable characteristics. For example, the induced octaploid *Hibiscus acetosella* 'Panama Red' remained more compact (CONTRERAS et al., 2009) while the induced tetraploid *Humulus lupulus* developed thinner and shorter shoots (TROJAK-GOLUCH AND SKOMRA, 2013).

Following Blakeslee and Avery's (1937) successful application of colchicine to inhibit mitosis and to double the number of chromosomes in plants, chemical treatment has been widely

used in the formation of polyploids. Colchicine and the dinitroaniline herbicide oryzalin (BARTELS AND HILTON, 1973) are commonly used as doubling agents for induction of tetraploids. Of the methods applied to induce polyploidization, immersion of seeds in doubling agents was the first to exhibit satisfactory performance (BLAKESLEE AND AVERY, 1937; LEHRER et al., 2007; RUBULUZA et al., 2007). Inducing polyploids by submerging germinated seedlings has been reported in lilac (FIALA, 1988) and hypericum (OLSEN et al., 2006), but not in the genus *Hibiscus*.

In this study we sought to determine a protocol using *Hibiscus moscheutos* 'Luna Red' to induce tetraploids, and to further raise sterile triploid clones. Following identification of ploidy levels from induction treatments, diploid, triploid and tetraploid plants were evaluated regarding various morphological traits.

Materials and methods

Plant materials

A popular inbred *Hibiscus moscheutos* cultivar on the market, one with large flower petals and dwarf compact stature, is 'Luna Red' (LEUE, 2005). Half of the seeds (purchased from Ball® Horticulture) were treated immediately after soaking and before germination occurred; the remaining half of the seeds were allowed to germinate first and then were treated. To facilitate harvesting whole seedlings for soaking treatment, seeds were sown in pure sand and covered by a 0.5 cm layer of vermiculite. Seedlings were ready to be treated once they reached the 'cotyledon stage' (CONTRERAS et al., 2009) where cotyledons are fully expanded and shoot meristems are forming.

Treatment Methods

Seed treatment studies

Fresh *H. moscheutos* 'Luna Red' seeds were allowed to imbibe water for 24 hours prior to any treatment. Seed soaking experiments were divided into two separate studies utilizing colchicine and oryzalin. Each study was designed as a two-way factorial linear model with three chemical concentrations and three soaking periods, and with eight replicates in each experimental unit and 5 seeds per replicate. Water-imbibed seeds were treated in 0.05%, 0.1%, or 0.2% (w/v) colchicine (≥95% (HPLC) powder, Sigma-Aldrich®, St. Louis, MO) solution for 12, 24, or 36 hours, and 100, 125, or 150 μM oryzalin (Surflan® A.S., Southern Agricultural Insecticides, Inc., Hendersonville, NC) suspension for 6, 12, or 24 hours. Seeds were rinsed under running tap water for one hour to thoroughly remove chemical residue after chemical soaking. During the treatment, all seeds were placed in wide-neck reagent bottles on a rotational shaker (Lab-Line® Instruments Inc., No. 3590, Tripunithura, Kochi, India) at a speed of 120 rpm. A control group, having received a 24-hour water soaking and 1-hour water rinsing, was added.

Seedling treatment studies

H. moscheutos 'Luna Red' seeds were germinated under mist in sand. When seeds had germinated and reached the 'cotyledon stage' (prior to emergence of the first true leaf), the germinated seedlings were removed from the sand and treated in chemicals. Seedling-soaking experiments were also carried out as two studies using colchicine and oryzalin. Each study was designed as a two-way factorial linear model with three levels of chemical concentration and three levels of exposure duration; after adding a control group, there were 10 experimental units for each study. Seedlings were exposed in 0.025%, 0.05%, or 0.1% (w/v) colchicine solution for

6, 12, or 24 hours or in oryzalin suspension (100, 125, or 150 μM) for 6, 12, or 24 hours Within each experimental unit, there were eight replicates and five seedlings per replicate. Seedlings were soaked as previously described. All treated seedlings were rinsed under running tap water for one hour to remove any residue. Seedlings were then wrapped in damp task wipers (Kimwipes®, Kimberly-Clark Professional®, Roswell, GA) in Petri dishes (VWR® international, Radnor, PA) to be transferred to the greenhouse.

Greenhouse production

After treatments, seeds and seedlings were immediately moved to the greenhouse. Seeds were sown in potting media (Jolly Gardener® Pro-line™ C/B Growing Mix, Jolly Gardener Products Inc., Poland, ME) supplemented with 0.6 $mg \cdot m^{-3}$ Micromax micronutrients (Everris®, Geldermalsen, the Netherlands) and 4 $mg \cdot m^{-3}$ 15-9-12 slow release fertilizer (Osmocote® Plus, Marysville, OH), covered by a thin layer of potting media in 10 cm containers. Seedlings were planted in potting media supplemented with micronutrients and slow release fertilizer in 10-cm containers. Five seeds / seedlings were planted in each container. Containers were placed in flats in a randomized complete block design with 10 blocks. The flats were placed in a shaded area for 6 weeks, and then in a partially shaded area for 4 weeks until being moved to full sun light conditions. The greenhouse temperature was set to 25 ℃ during the daytime and 20 ℃ during the night, with natural lighting. Plants were fertilized with 200 $mg \cdot L^{-1}$ 20-10-20 water-soluble fertilizer (Jack's Professional®, JR Peters Inc, Allentown, PA) once every week.

Seed germination was initially evaluated at six weeks after treatment. Some seeds died during the germination process and survival data was taken on both treated seeds and seedlings at eight weeks after treatment. As the plants expanded in size, they were transplanted into potting

media with Micromax® mix in 2.8 L containers. A ploidy level test was performed after treated plants were well developed with regular, continuous growth, but before initiation of flower buds.

Ploidy Level Analysis

The ploidy levels of treated plants were determined by measuring the fluorescence intensity of stained nuclei relative to the ploidy of a known control plant via flow cytometry. The analysis was conducted on a CyFlow® Ploidy Analyser flow cytometer (Partec GmbH, Münster, Germany). At most, 1 cm^2 leaf tissue of the second spreading leaf from each plant was sampled and then chopped with a sharp stainless razor blade (Electron Microscopy Sciences, PA) into 1-2 mm^2 particles in nuclei extraction buffer (CyStain® UV Precise P, Sysmex Partec, Germany) and allowed to rest for 30 seconds in the solution. The sample particles were removed through straining through a 40-µm mesh filter, and nuclei staining buffer (CyStain® UV Precise P, Sysmex Partec, Germany) was added into the remaining solution with intact DNA for at least two minutes.

When the solution was taken up through the flow cytometer, histograms were automatically generated. Prior to scanning any samples of treated plants, the reference fluorescence of stained nuclei was set by scanning a known diploid sample, and this was the basis on which diploid, triploid, tetraploid, or mixaploid samples were identified.

Tetraploid x Diploid Crosses

Hibiscus moscheutos flowers bloom for only one day (SPIRA, 1989), and fresh pollen is produced between 08:00 and 10:00 in the morning on a sunny day (SHIMAMURA et al., 2005). Hand pollination was conducted in the greenhouse during the blooming season of *H.*

moscheutos. Flowers of diploid 'Luna Red' plants with abundant pollen were collected daily to cross with flowers of identified tetraploids between eight and ten o'clock in the morning. Pollen was gently rubbed on the stigma of tetraploids until fully covering the stigma. A pollination tag was placed around the flower pedicel with information of accession number, maternal and paternal parents, and pollination date. Over 1,300 crosses were made and data was recorded with regard to whether seeds aborted or matured.

Seeds from tetraploid x diploid crosses were harvested and cleaned from mature capsules. The seed number of each specific cross was counted, and all seeds were germinated in potting media supplemented with micronutrients and Osmocote® fertilizer (15-9-12 slow release fertilizer) in 18-cm containers. Seeds germinated gradually and seedlings with over three true leaves were tested for ploidy level via flow cytometry, using methods referred to in 'Ploidy Level Analysis'. Identified triploids were then transplanted individually into 7.5-cm containers for further evaluation.

The main pollen source was diploid 'Luna Red' plants, and pollen from seven red-foliage hybrid diploid plants (Hib 2015-1, Hib 2015-5, Hib 2015-9, Hib 2015-12, Hib 2015-13, Hib 2015-14, Hib 2015-16) were also utilized. Later, the hybrid triploid plants were also planted out in the field for evaluation.

Cuttings were taken from tetraploids before they entered dormancy. After being dipped in 300 $mg \cdot L^{-1}$ K-IBA (Indole-3-butyric acid potassium salt, Sigma-Aldrich®, St. Louis, MO) solution for 3 seconds at the base, cuttings were planted in potting media in 10-cm containers on 25 ℃bottom heat under shaded mist bench for further root development. Mist bench was set for 5 s of misting every 8 minutes. When roots were well established, cuttings were removed from mist and transferred to regular greenhouse conditions for winter. Diploid 'Luna Red' and

putative triploid seeds were germinated in flats and germination percentages were recorded. After ploidy level determination of triploid plants, diploid and triploid seedlings were transferred in 10-cm containers and kept in the greenhouse until the growing season.

Characteristic Measurement

Planting in the field took place on 3 May 2016. Well established diploid, triploid and tetraploid plants were trimmed back to a height of 15 cm. Prior to planting, pre-emergent herbicides Surflan® (40.4% oryzalin, Surflan® A.S., Southern Agricultural Insecticides Inc., Hendersonville, NC) and Gallery® (Isoxaben, Dow® AgroSciences, Indianapolis, IN) was sprayed in the field at a rate of 0.4 $L \cdot ha^{-1}$ and 2.0 $kg \cdot ha^{-1}$, respectively, and field soil was amended with 15-0-15 fertilizer at a rate of 9.3 $kg \cdot ha^{-1}$ (Tri County Fertilizer and Specialty, Honea Path, SC) and Epsom® salts (magnesium sulfate, Saltworks®, Woodinville, WA,) at a rate of 96.0 $kg \cdot ha^{-1}$. Plants were placed in rows 1.8 m apart and 1.2 m apart within each row. The field was maintained using 16-23 $ml \cdot L^{-1}$ Honcho® (glyphosate, Monsanto Agrochemical Company, Greater St. Louis, MO) for weed control. Drip irrigation was implemented for 1-2 hours every week, but the frequency was adjusted as needed.

Under open field conditions, morphological data was taken on all plants of different ploidy levels. After the plants reached full bloom, plant heights were measured. Leaf characteristics were measured on fully expanded leaves, recording leaf area using a Li-3100C leaf area meter (Li-Cor® Biosciences, Lincoln, NE), leaf thickness using a micrometer (6" Digital Caliper, Pittsburgh®, Camarillo, CA), leaf greenness using a chlorophyll meter (Minolta SPAD-502, Spectrum® Technologies, Inc., Aurora, IL), and leaf mass per area. Leaf mass per area (LMA) ($mg \cdot cm^{-2}$) is the ratio of leaf dry mass to its area size, which can be used to assess

leaf density and resistance to herbivory (DE LA RIVA et al., 2016; LAMBERS et al., 1998). LMA was measured by sampling 20 leaf punches (0.85 cm in diameter) taken from each plant with a hole puncher. Leaf tissue was oven-dried at 104 °C for 17 hours before measuring the dry weight of each sample. Floral characteristics measured were blooming period (start, peak, end), flower diameter, petal redness using an anthocyanin content meter (ACM-200plus, Opti-Sciences, Hudson, NH), and pollen presence and viability. Pollen viability was tested by observing pollen stainability. This was done by staining pollen with 1% acetocarmine solution at 25 °C for 3 hours. Pollen viability was also identified by observing pollen tube elongation in Brewbaker and Kwack (BK) agar pollen germination media (BREWBAKER AND KWACK, 1963) after 24 hours of culture at 25 °C in a growth incubator (ThermoFisher® Scientific, Precision™, Waltham, MA). Tolerance to aerial Phytophthora (*Phytophthora spp.*) was visually rated in each ploidy level group and compared between groups. On a scale of zero to three, "0" = no phytophthora infection, "1" = light phytophthora infection, "2" = medium phytophthora infection, and "3" = severe phytophthora infection.

Data Analysis

Estimation of survival rate and tetraploid transformation rate were made using binomial regression, and morphological traits comparisons were conducted using one-way analysis of variance (ANOVA) and Tukey's test among ploidy levels. All statistical analysis was performed on R computing software (R, 2015).

Results

Analysis of tetraploid induction

Following chemical soaking, seeds had very low germination rates, 2.78% and 3.33% respectively, for colchicine and oryzalin treatment studies. Therefore, statistical analysis was not conducted on these two studies. While it is difficult to compare the toxicity between colchicine and oryzalin due to different concentration measurements, germination was observed only for 0.05% colchicine solution treatments for 12 or 24 hours exposure duration, and on oryzalin treatments for 6 hours (Table 2.1). A low concentration of colchicine or a short exposure period to oryzalin solution contributed to a higher rate of survival. A total of 28 plants were examined via flow cytometry and only three tetraploids and eleven mixaploids were identified for both colchicine and oryzalin treatments.

After being treated in colchicine or oryzalin solutions, seedlings were transplanted in potting soil, but remained stunted for 2-3 weeks (HANCOCK, 1997). During the last stage of stagnation, colchicine-soaked seedlings started showing signs of swollen and splitting bases, and lesions formed (Figure 2.1a). On the seedlings which survived, the plants soon resumed growth and developed leaves and stems more quickly than seedlings in the control group; seedlings that did not survive died with one or multiple visible lesions at the stem base. Oryzalin herbicide causes seedling browning and death due to an inhibitory effect on root development (TOMLIN, 1997). Hibiscus seedlings that survived the 'chemical shock' developed regular stems and leaves, but seedlings that died during or slightly after the stagnation stage underwent root growth inhibition and died from root destruction (Figure 2.1b).

Both colchicine and oryzalin effectively induced tetraploids from soaking germinated seedlings of *H. moscheutos*; survival rate and tetraploid transformation rate, however, varied

greatly depending on the concentration and exposure duration under each chemical study. In the colchicine-soaking study, a binomial linear regression was fitted ($R^2 = 0.487$) for seedling survival percentages with two significant main effects colchicine concentration ($p=0.001$) and exposure duration ($p<0.001$). A significant interaction effect ($p=0.01$) appeared between two main variables (Table 2.2 and Figure 2.2). The highest percentage (97.5%) of seedlings survived at the lowest concentration (0.025%) and the shortest exposure duration (6 hours); and the survival percentage dropped to 40.0% at the highest colchicine concentration (0.1%) when treated for the longest time (24 hours) (Table 2.2). The survival of germinated seedlings decreased as colchicine increased in concentration and also as exposure duration increased, although in different magnitudes. Survival percentage decreased at a similar pace at colchicine concentration of 0.025% and 0.05%; meanwhile, when the concentration went up to 0.1%, there was a sudden decrease in survival percentages (Figure 2.2). With regard to exposure duration, survival percentage decreased as the duration of exposure went up; however, the decrease was accelerated when exposure duration was greater than 12 hours, particularly when exposure duration was associated with lower concentrations (0.025% and 0.05%). Decrease in survival rate was similar according to exposure duration at the concentration of 0.1%.

Examination of the ploidy level of each surviving seedling by flow cytometry indicated that the highest conversion percentage was 20.0%, induced under 0.1% colchicine for 24 hours, and the lowest conversion percentage was 10.0%, induced by 0.1% colchicine solution for 6 hours (Table 2.2). Tetraploid conversion percentage was not significantly affected by colchicine concentration, exposure duration or their interaction in this two-factor linear model.

Oryzalin treated seedlings displayed lower survival rates overall in contrast to colchicine-treated seedlings. The highest survival percentage was 57.5% and the lowest survival rate was

0% (Table 2.3). Results of a binomial regression analysis revealed that there was statistical significance for oryzalin concentration (p=0.03), exposure duration (p=0.002) and their interaction effect (p=0.03) (Table 2.3 and Figure 2.3). Seedling survival percentage decreased as oryzalin concentration increased or as the exposure duration increased. At six hours of chemical exposure, the lowest oryzalin concentration (100 μM) yielded the highest survival rate, but there is a more severe drop with exposure duration for the lowest oryzalin concentration than for the other concentrations (Figure 2.3). Between 6 and 12 hours of chemical exposure, the variable interaction entered into play, and a higher percentage of seedlings survived under treatments of 150 μM oryzalin solutions. Survival percentages of plants treated with 100 μM and 125 μM oryzalin decreased drastically between 6 and 12 hours of exposure, and continued to decrease as the exposure duration reached 20 hours (Figure 2.3). The rate of decrease in survival percentage was less severe for the 150 μM oryzalin treatments than the other treatments.

Some of the low efficiencies of oryzalin treatments for conversion of tetraploids were due to low survival percentage (Table 2.3). Oryzalin concentration (p=0.008) and exposure duration (0.007) were both significant as main effects, as was their interaction (p=0.01). Percentages of tetraploid conversion decreased steadily as the exposure duration increased under 150 μM oryzalin concentration, while there was a more sharply decreasing curve for concentrations of 100 μM or 125 μM (Figure 2.4). At six hours of exposure duration, the lower concentration contributed to a higher tetraploid conversion, possibly due to a larger number of surviving seedlings (Table 2.3 and Figure 2.4). Above 6 hours of exposure duration, the prediction line appeared to show an optimal efficiency in induction of tetraploidy at an oryzalin concentration of 150 μM (Figure 2.4).

Tetraploid x diploid crosses

Though only a few crosses were viable and eventually produced seeds, tetraploids were receptive to diploid pollen. A gamete containing two sets of chromosomes provided by tetraploid parents combines with a gamete containing one set of chromosomes provided by diploid pollen and forms a triploid zygote (ACQUAAH, 2007). Crosses were made between flowers of identified tetraploid plants and diploid flowers of 'Luna Red' and of seven red-foliaged *H. moscheutos* selections. Hundreds of crosses were attempted, but only 33 crosses yielded viable seeds, of which 15 crosses were between an induced tetraploid and a diploid 'Luna Red' (Table 2.4).

After seeds germinated, the ploidy level of every seedling was confirmed via flow cytometry. It appeared that some of the expected triploid seeds reverted back to diploids at some point. Eight triploid seedlings were confirmed from crosses after colchicine induction, while 22 triploid seedlings were confirmed from crosses of oryzalin induced plants (Table 2.4). Throughout the whole experiment, an inbred diploid cultivar was adopted and induced into autotetraploids, that is, plant material which contains four sets of similar gametes (ACQUAAH, 2007). Autotetraploidy was developed only for short-term enhancements and thus could be adopted towards breeding purposes (RANNEY, 2006), but this state was difficult to maintain and usually got lost in the flow of plant evolution due to its instability (LEVIN, 1983; SOLTIS et al., 2014). Therefore, such a situation could be a result of harvesting supposed triploid seeds from a flower produced on a diploid branch, a branch which had earlier reverted from a confirmed tetraploid plant. This possible reversion acted against the harvest gain of triploid seeds; however, more seeds coming from an oryzalin-induction background were maintained as triploids as opposed to those of a colchicine-induction background (Table 2.4).

Characteristic measurement and comparison

Morphological measurements were recorded after diploid, triploid and tetraploid plants were well established in the field. Instead of being 'enlarged', tetraploid and triploid plants appeared to be further dwarfed after being induced from their compact, diploid homologs (Table 2.5). Leaves of tetraploid plants displayed a highly ruffled leaf texture due to an increase in thickness over leaves of diploids, but there was no difference in LMA. Tetraploids had a shorter stature than diploids, with darker green and more ruffled leaves (Figure 2.5); meanwhile triploids were the shortest among the three groups, with large leaves (Table 2.5). Leaves of tetraploids possessed a leathery texture, while leaves of diploids and triploids had a papery texture (Figure 2.5).

Tetraploids started blooming slightly earlier ($p=0.394$) than diploids, and triploids started slightly later ($p=0.593$) than diploids, but the differences were not statistically significant. Triploids, however, exhibited a longer blooming period than diploids ($p<0.001$). This corresponded with the hypothesis of having an extended blooming period for polyploids with reduced fertility. Opposite to the common 'Gigas' effect (ACQUAAH, 2007; CAPORALI et al., 2014), flowers of tetraploids and triploids displayed a decrease of flower diameter (Figure 2.6), and triploid flower petals faded to a lighter red color ($p=0.003$) while tetraploids and diploids maintained the same level of anthocyanin content (Table 2.5).

As a common fungal disease, aerial Phytophthora (*Phytophthora spp.*, identified by the University of Georgia Disease Clinic) infected the research field on a large scale, including all experimental plants (Figure 2.7). Observed symptoms were leaf wilting and eventually abscission, seed pod and flower bud abortion, and browning of branches from the base. Although the first infected plant found was a tetraploid, tetraploids withstood the disease transmission well

and were not severely damaged until every plant in the diploid line was infected to a severe level. The highest Phytophthora tolerance was found on triploids ($p<0.001$) (Figure 2.8). Among the 27 plants in the field, only two infected branches on one triploid plant were found and the infection did not transmit elsewhere over time.

Reduced fertility

During blooming season, diploid, triploid and tetraploid plants were planted in an open field under conditions conductive to open pollination. *Hibiscus moscheutos* is a self-compatible, outcrossing plant, which produces pollen and fruit in abundance (FLORA OF NORTH AMERICA EDITORIAL COMMITTEE, 1993+). An induced tetraploid, however, was sterile in pollen production due to its sterile nature as an autopolyploid (SOLTIS et al., 2014). Flowers of tetraploid plants may rarely develop pollen on anthers, but in most situations, only clean or deformed anthers were on display, with no pollen shedding (Figure 2.9). Triploid flowers produced abundant pollen, and in 1% acetocarmine solution, pollen grains stained a dark red color (Figure 2.10). However, when pollen was cultured in BK (Brewbaker and Kwack) agar media, no pollen tube germination was observed on pollen from triploid plants while pollen tubes of diploids started elongating after 4 hours (Figure 2.11).

Both diploid and tetraploid plants produced normal fruit, from successful pollination until the fruit matured and dehisced. In an open pollination environment, fruit formed on triploids and were set for 1-3 days until self-abortion occurred (Figure 2.12). Through the whole blooming and seed production season, no fruit were found growing or maturing on triploid plants at any stage of the blooming season.

Discussion

Results of chemical polyploidization in the seed and seedling experiments indicated a higher efficiency was obtained by treating germinated seedlings for tetraploid induction. A low concentration of oryzalin exhibited a higher efficiency than colchicine at any tested level in inducing tetraploidy, due firstly to its lower toxicity compared to colchicine (BLAKESLEE AND AVERY, 1937), and secondly to its stability, as fewer plants reverted from tetraploid or triploid in the next generation. Though instability is typical among induced autotetraploids (RANNEY, 2006; SOLTIS et al., 2014), continuous vegetative propagation maintained the ploidy levels. For a longer term, adding genetic lines from other series or species will avoid reversion to diploids on a large scale.

Tetraploid and triploid plants displayed a decrease in height, as expected (CONTRERAS et al., 2009); and infertile triploid plants maintained a longer blooming time without interruption from seed production. Although leaves of tetraploid plants were thicker, greener and more leathery than diploid plants, the test on leaf mass per area (LMA) showed no difference between leaves of tetraploid and diploid plants. LMA is known to be an important morphological trait, as well as an indicator of physiological traits, such as potential growth rate (DE LA RIVA et al., 2016). Thus, induced tetraploids and triploids did not show a difference in vegetative growth rate, and also remained the same leaf tissue density, but a greater leaf thickness in tetraploid plants was observed due to its more ruffled leaf texture.

Induced tetraploid and triploid plants displayed no improvement in flower size but showed a decrease. Flowers of triploids also showed a decrease in the redness of flower petals from diploid plants. This failure to maintain a desirable trait was partially accounted for as a result of a high degree of inbreeding. Therefore, incorporating more distinct parents should

encourage higher degree of hybrid vigor in promoting the expression of 'enlargement effects' (ACQUAAH, 2007) on phenotypic traits. Reduced pollen production was observed in tetraploid plants, while both pollen and seed production were observed in triploid plants. Triploid plants exhibited an extension in blooming period as a result of disabled seed production.

This study generated a seedling-soaking protocol manipulating *Hibiscus moscheutos* ploidy levels, and found that, on an induced inbred triploid, there was an increase in tolerance to disease damage and a decrease in seed production along with an extended period for blooming. More than merely creating new selections for cultivar development, polyploidy induction in *H. moscheutos* opens the gate for future breeding endeavors. The next step for this study is to implement this protocol on other available diploid *H. moscheutos*. With the combination of 'Gigas effect' and heterosis, we are anticipating both a decrease in fertility and an improvement of ornamental traits.

Literature Cited

Acquaah, G., 2007. *Principles of Plant Genetics and Breeding*. Blackwell Pub., Malden, MA; Oxford.

Bartels, P.G. and Hilton, J.L., 1973. Comparison of Trifluralin, Oryzalin, Pronamide, Propham, and Colchicine Treatments on Microtubules. *Pest. Biochem. Physiol.* 3:462-472.

Blakeslee, A.F. and Avery, A.G., 1937. Methods of Inducing Doubling of Chromosomes in Plants by Treatment with Colchicine. *Journal Heredity* 28:393-411.

Brewbaker, J.L. and Kwack, B.H., 1963. The Essential Role of Calcium Ion in Pollen Germination and Pollen Tube Growth. *American Journal of Botany* 50:859-865.

Caporali, S., Hammami, S.B.M., Moreno-Alias, I., Rapoport, H.F., Chiancone, B., Germana, M.A., and Rosati, A., 2014. Effects of Tetraploidy on Olive Floral and Fruit Biology. *Scientia Horticulturae* 179:198-203.

Contreras, R. and Ruter, J., 2009. An Oryzalin Induced Polyploid from a Hybrid of *Hibiscus acetosella* x *H. radiatus (Malvaceae)* Exhibits Reduced Fertility and Altered Morphology. *HortScience* 44:1177.

Contreras, R.N., Ruter, J.M., and Hanna, W.W., 2009. An Oryzalin-Induced Autoallooctoploid of *Hibiscus acetosella* 'Panama Red'. *J. Am. Soc. Hortic. Sci.* 134:553-559.

De la Riva, E.G., Olmo, M., Poorter, H., Ubera, J.L., and Villar, R., 2016. Leaf Mass per Area (LMA) and Its Relationship with Leaf Structure and Anatomy in 34 Mediterranean Woody Species along a Water Availability Gradient. *Plos One* 11:e0148788.

Dixit, V. and Chaudhary, B.R., 2014. Colchicine-Induced Tetraploidy in Garlic (*Allium sativum* L.) and Its Effect on Allicin Concentration. *J. Horticult. Sci. Biotechnol.* 89:585-591.

Fiala, J.L., 1988. Use of Colchicine in Inducing Polyploids, p. 187-188, Lilacs: the Genus *Syringa*. Timber Press, Portland, OR.

Flora of North America Editorial Committee, 1993+. *Flora of North America, Vol. 6*. Oxford University Press, New York and Oxford.

Ghani, M.A., Sun, Q., Li, J.X., Cao, L.W., Rao, L.L., Zou, X.X., and Chen, L.P., 2014. Phenotypic and Genetic Variation Occurred during Wide Hybridisation and Allopolyploidisation between *Brassica rapa* and *Brassica nigra*. *Scientia Horticulturae* 176:22-31.

Hancock, J.F., 1997. The Colchicine Story. *HortScience* 32:1011-1012.

Kehr, A.E., 1996. Breeding for a Purpose. *American Rhododendron Society Bulliten*:131-141.

Lambers, H., Chapin, F.S.I., and Pons, T.L., 1998. Grwoth and Allocation, p. 308, Plant Physiological Ecology. Springer.

Lehrer, J., Brand, M., and Lubell, J., 2007. Induction of Tetraploidy in Japanese Barberry (*Berberis thunbergii* DC.) Seedlings through Exposure to Colchicine and Oryzalin. *HortScience* 42:901-901.

Leue, E., 2005. *Hibiscus moscheutos* 'Bahlibred'. Ball Horticulture, West Chicago, IL.

Levin, D.A., 1983. Polyploidy and Novelty in Flowering Plants. *Am. Nat.* 122:1-25.

Olsen, R.T., Ranney, T.G., and Werner, D.J., 2006. Fertility and Inheritance of Variegated and Purple Foliage Across a Polyploid Series in *Hypericum androsaemum* L. *J. Am. Soc. Hortic. Sci.* 131:725-730.

R, R.D.C.T., 2015. R: A Language and Environment for Statistical Computing, Version 3.2.2. R Foundation for Statistical Computing, Vienna, Austria.

Ranney, T.G., 2006. Polyploidy: From Evolution to New Plant Development. *Combined Proceeding of International Plant Propagators Society* 56:137-142.

Rubuluza, T., Nikolova, R.V., Smith, M.T., and Hannweg, K., 2007. *In vitro* Induction of Tetraploids in *Colophospermum mopane* by Colchicine. *S. Afr. J. Bot.* 73:259-261.

Shimamura, R., Kachi, N., Kudoh, H., and Whigham, D.F., 2005. Visitation of a Specialist Pollen Feeder *Althaeus hibisci* Olivier (Coleoptera : Bruchidae) to Flowers of *Hibiscus moscheutos* L. (Malvaceae). *J. Torrey Bot. Soc.* 132:197-203.

Soltis, D.E., Visger, C.J., and Soltis, P.S., 2014. The Polyploidy Revolution Then...and Now: Stebbins Revisited. *American Journal of Botany* 101:1057-1078.

Spira, T.P., 1989. Reproductive Biology of *Hibiscus moscheutos* (Malvaceae), p. 247-256. In: Bock, J. H. and Linhart, Y. B. (eds.), The Evolutionary Ecology of Plants. Westview Press, Boulder, Colorado, USA.

Tomlin, C.D.S.e., 1997. *The Pesticide Manual - World Compendium*. British Crop Protection Council, Surrey, England.

Trojak-Goluch, A. and Skomra, U., 2013. Artificially Induced Polyploidization in *Humulus lupulus* L. and Its Effect on Morphological and Chemical Traits. *Breed. Sci.* 63:393-399.

Winters, H.F., 1970. Our Hardy Hibiscus Species as Ornamentals. *Economic Botany* 24:155-164.

Tables and Figures

Table 2.1. Summary of results for soaking seeds of *Hibiscus moscheutos* 'Luna Red' in colchicine or oryzalin solutions.

Mitotic Inhibitor	Chemical Rate	Exposure duration (h)	No. of Treated Seeds	No. of Survival	No. of Tetraploid Conversion	No. of Mixaploid Conversion
Control	n/a	n/a	40	35	0	0
Colchicine	0.05%	12	40	10	1	3
Colchicine	0.05%	24	40	3	0	1
Colchicine	0.05%	48	40	0	0	0
Colchicine	0.1%	12	40	0	0	0
Colchicine	0.1%	24	40	0	0	0
Colchicine	0.1%	48	40	0	0	0
Colchicine	0.2%	12	40	0	0	0
Colchicine	0.2%	24	40	0	0	0
Colchicine	0.2%	48	40	0	0	0
Oryzalin	100 μM	6	40	7	0	2
Oryzalin	100 μM	12	40	0	0	0
Oryzalin	100 μM	24	40	0	0	0
Oryzalin	150 μM	6	40	2	0	2
Oryzalin	150 μM	12	40	0	0	0
Oryzalin	150 μM	24	40	0	0	0
Oryzalin	200 μM	6	40	6	2	3
Oryzalin	200 μM	12	40	0	0	0
Oryzalin	200 μM	24	40	0	0	0

Table 2.2. Summary of results for soaking germinated seedlings of *Hibiscus moscheutos* 'Luna Red' in colchicine solutions.

Colchicine Concentration (w/v)	Exposure duration (h)	No. of Treated Seedlings	Survival Rate (%)[a]	Tetraploid Conversion Rate (%)
0.025%	6	40	97.5	12.5
0.025%	12	40	92.5	22.5
0.025%	24	40	72.5	17.5
0.05%	6	40	87.5	10.0
0.05%	12	40	62.5	17.5
0.05%	24	40	52.5	17.5
0.1%	6	40	50.0	12.5
0.1%	12	40	37.5	10.0
0.1%	24	40	40.0	20.0
Analysis of variance [b]				
Concentration			***	NS
Exposure duration			***	NS
Concentration x Exposure duration			*	NS

a. Survival rate was assessed 6 weeks following initial treatment.
b. NS-not significant, * significant at $p \leq 0.05$, *** significant at $p \leq 0.001$

Table 2.3. Summary of results for soaking germinated seedlings of *Hibiscus moscheutos* 'Luna Red' in oryzalin solutions.

Concentration (µM)	Exposure duration (h)	No. of Treated Seedlings	Survival Rate (%)[a]	Tetraploid Conversion Rate (%)
100	6	40	57.5	30.0
100	12	40	5.0	0
100	24	40	0	0
125	6	40	55.0	27.5
125	12	40	15.0	5.0
125	24	40	2.5	0
150	6	40	47.5	20.0
150	12	40	5.0	2.5
150	24	40	5.0	5.0
Analysis of variance [b]				
Concentration			**	**
Exposure duration			*	**
Concentration x Exposure duration			*	*

a. Survival rate was assessed 6 weeks following initial treatment.
b. * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$

Table 2.4. Crosses of *Hibiscus moscheutos* between tetraploids and diploids. Number of seeds was the number of putative triploid seeds from all harvest seed pots, and number of triploids was the number of true triploids after flow cytometry examination.

Cross #	♀	♂	# Harvested Seeds	# of Triploids
1	C1, 6h, T[a]	Luna Red	13	0
2		Hib 2015-1[b]	5	0
3		Hib 2015-5	8	0
4		Hib 2015-12	1	0
5	C1, 12h, T	Luna Red	161	0
6		Hib 2015-1	17	0
7		Hib 2015-5	6	0
8	C1, 24h, T	Luna Red	87	0
9		Hib 2015-1	29	0
10		Hib 2015-9	47	0
11		Hib 2015-13	106	0
12		Hib 2015-16	41	0
13	C2, 6h, T	Luna Red	83	0
14	C2, 12h, T	Luna Red	104	0
15	C2, 24h, T	Luna Red	60	0
16		Hib 2015-1	31	3
17		Hib 2015-13	15	0
18	C3, 6h, T	Luna Red	68	0
19		Hib 2015-13	9	0
20	C3, 12h, T	Luna Red	73	0
21	C3, 24h, T	Luna Red	36	0
22		Hib 2015-1	4	2
23	C3, 24h, T	Hib 2015-12	7	3
24		Hib 2015-14	3	0
25	O1, 6h, T	Luna Red	28	4
26		Hib 2015-12	3	1
27	O2, 6h, T	Luna Red	22	0
28		Hib 2015-12	57	6
29	O2, 12h, T	Luna Red	2	1

(Table continued on next page)

(Table continued from last page)

Cross #	♀	♂	# Harvested Seeds	# of Triploids
30	O3, 6h, T	Luna Red	50	4
31		Hib 2015-12	10	1
32	O3, 24h, T	Luna Red	18	6
33	O3, 6h, T	Luna Red	5	0

a. Tetraploids were listed by the treatment: "C" means treated by colchicine while "O" means treated by oryzalin; the number following C or O indicates chemical concentration, "1" means the lowest and "3" means the highest; "6h", "12h" or "24h" indicates chemical exposure duration for 6 hours, 12 hours or 24 hours.

b. The inbred cultivar 'Luna Red' and seven red-foliage hybrid selections (Hib 2015-1, Hib 2015-12, etc.) were used as paternal parents.

Table 2.5. Morphological characteristics were measured on diploid, triploid and tetraploid *Hibiscus moscheutos* 'Luna Red' in the field. Tukey's HSD test was performed and comparisons were made between triploid and diploid, and between tetraploid and diploid plants.

Ploidy level	Plant height (cm)	Leaf area (cm^2)	Leaf greenness index	Leaf thickness	Leaf mass per area ($mg \cdot cm^{-2}$)	Leaf stomata length (µm)	First bloom (days after planting)	Blooming period (days)	Flower diameter (cm)	Flower anthocyanin content index
2x	54	58	39.4	0.28	13.8	2.43	58	68	19.1	18.4
3x	43	85	38.5	0.31	12.6	n/a [a]	62	87	17.2	12.4
4x	46	65	46.1	0.35	12.4	3.18	55	65	16.2	18.2
Treatment[b]										
3x vs. 2x	***	***	NS	NS	NS	n/a	NS	***	***	**
4x vs. 2x	***	NS	***	***	NS	NS	NS	NS	***	NS

a. Leaf stomata length was not determined for triploid plants.

b. ***- significant at $p \leqslant 0.001$, **- significant at $p \leqslant 0.01$, NS-not significant, n/a-not applied.

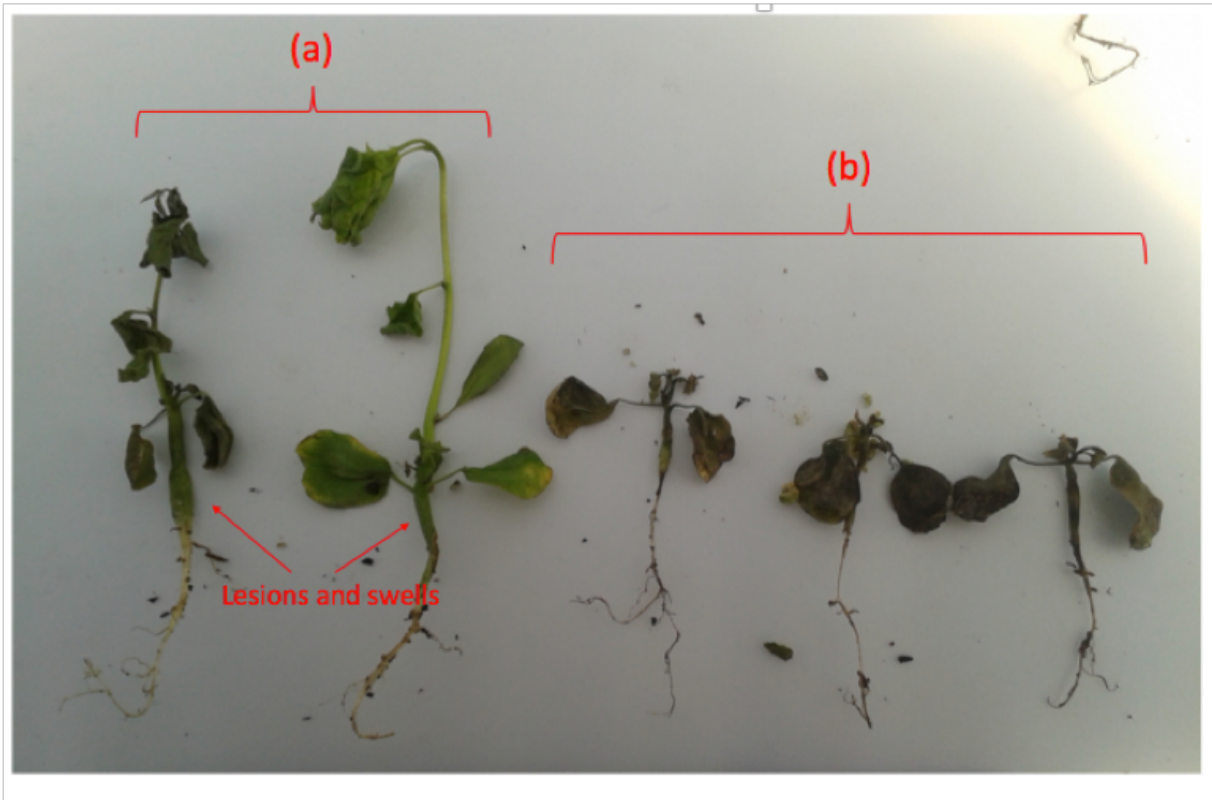

Figure 2.1. Germinated seedlings of *Hibiscus moscheutos* 'Luna Red' after colchicine or oryzalin treatments. (a) Two seedlings treated by colchicine with swollen stem bases and lesions on the stems. (b) Three seedlings treated by oryzalin with inhibited root growth.

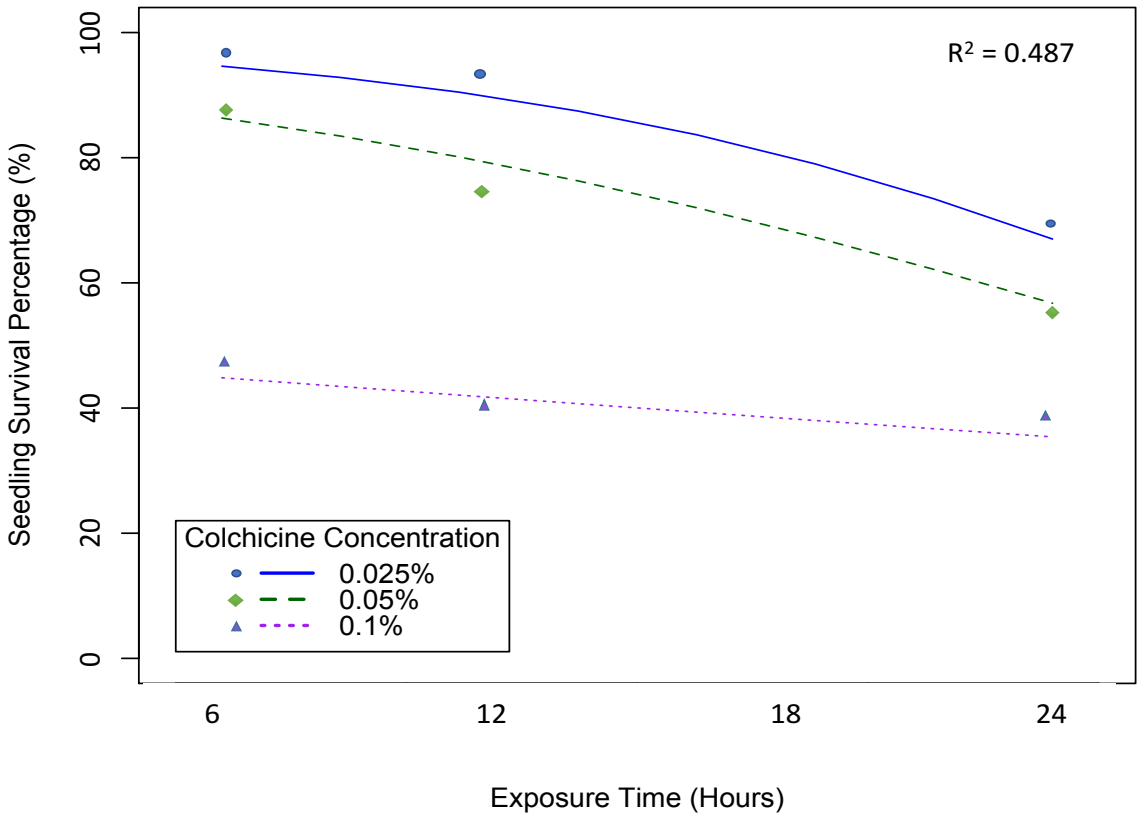

Figure 2.2. Survival percentage of *Hibiscus moscheutos* 'Luna Red' germinated seedlings after soaking in 3 concentrations of colchicine for 3 exposure durations. There were significant main effects on both colchicine concentration and exposure duration, as well as an interaction effect between them (R^2=0.487) (Table 2.2).

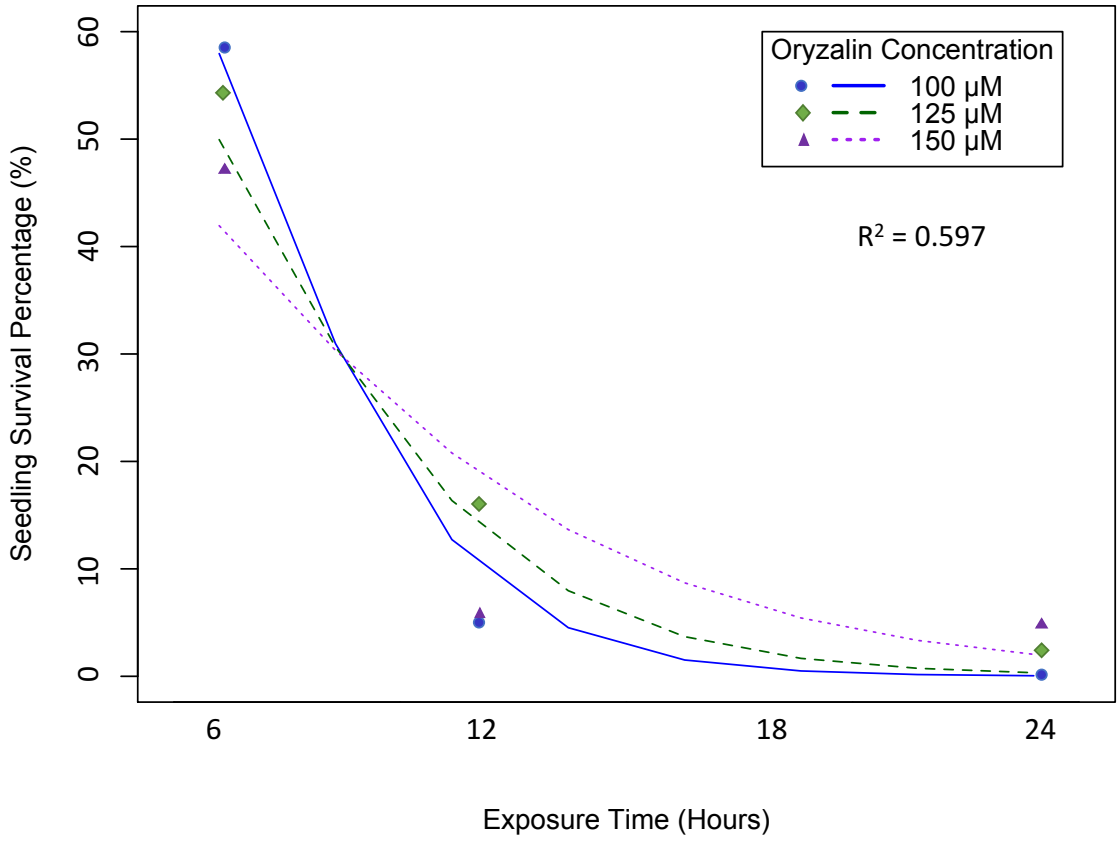

Figure 2.3. Survival percentage of *Hibiscus moscheutos* 'Luna Red' germinated seedlings after treatments of three oryzalin concentrations and three different exposure durations. A binomial regression was conducted with an overall fit of $R^2 = 0.597$.

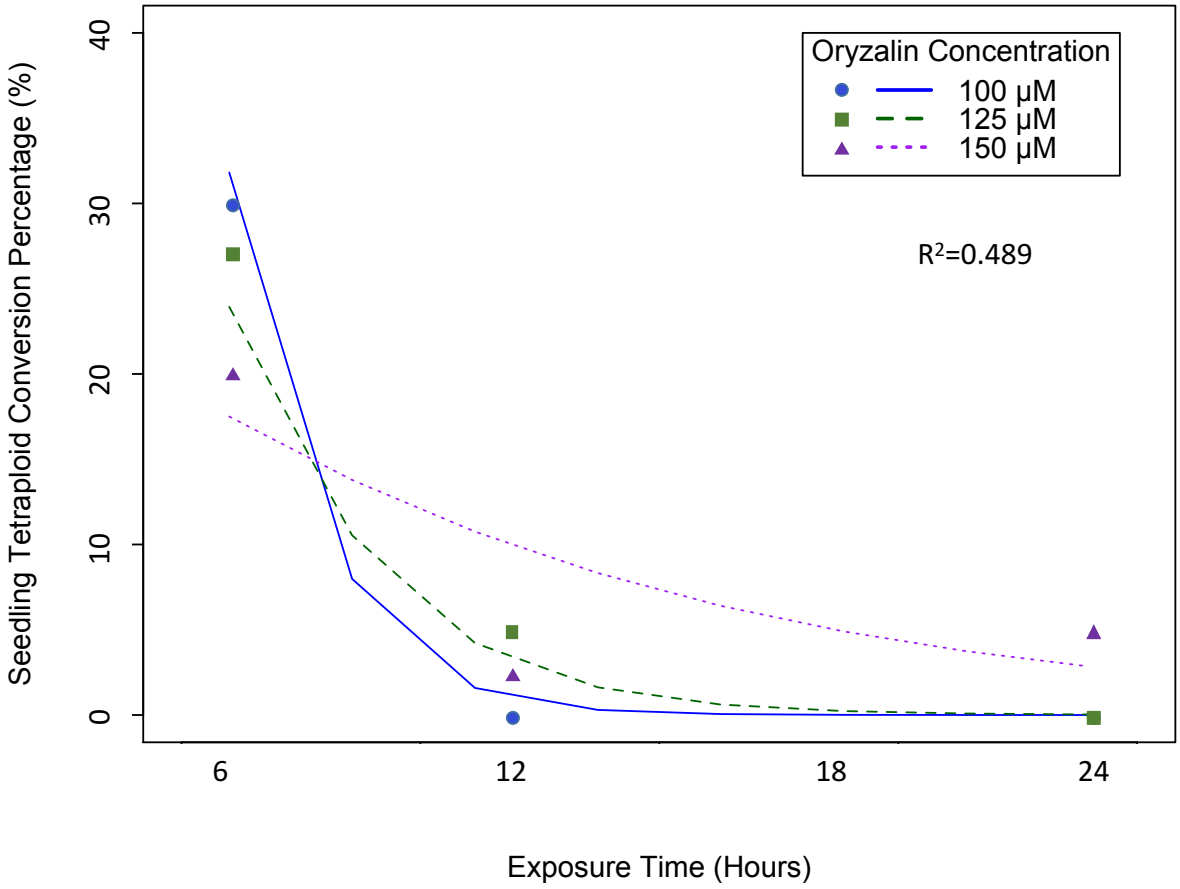

Figure 2.4. Tetraploid conversion percentage of *Hibiscus moscheutos* 'Luna Red' germinated seedlings following soaking in combinations of three oryzalin concentrations and three exposure durations. A binomial regression was conducted with an overall fit of $R^2 = 0.489$.

Figure 2.5. Diploid and induced tetraploid leaves of *Hibiscus moscheutos* 'Luna Red'. Tetraploid leaves remained similar in size to those of diploids, but showed a darker green and more leathery texture than diploids.

Figure 2.6. Diploid flowers were larger, but tetraploid flowers of *Hibiscus moscheutos* 'Luna Red' displayed more ruffled petals.

Figure 2.7. Effect of aerial Phytophthora infection (*Phytophthora spp.*) on *Hibiscus moscheutos* 'Luna Red'.

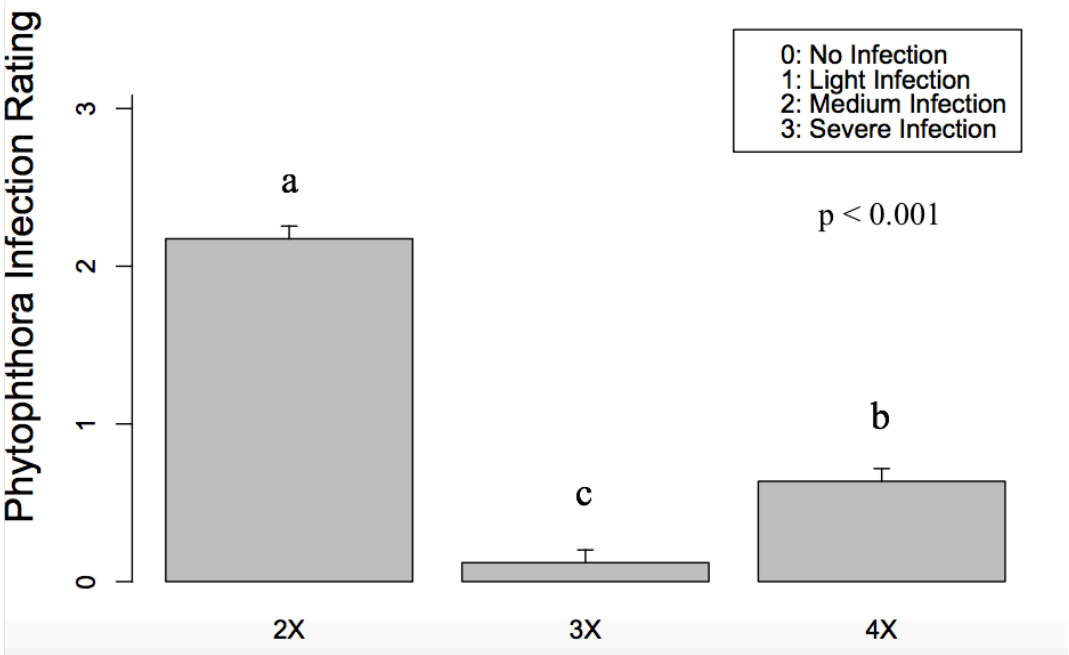

Figure 2.8. Aerial Phytophthora infection index on diploid, triploid and tetraploid of *Hibiscus moscheutos* 'Luna Red'. Different letters indicate significant differences according to Tukey's HSD test at P ⩽ 0.05.

Figure 2.9. Tetraploid flowers of *Hibiscus moscheutos* 'Luna Red' showed low pollen presence, and sometimes reduced anther size.

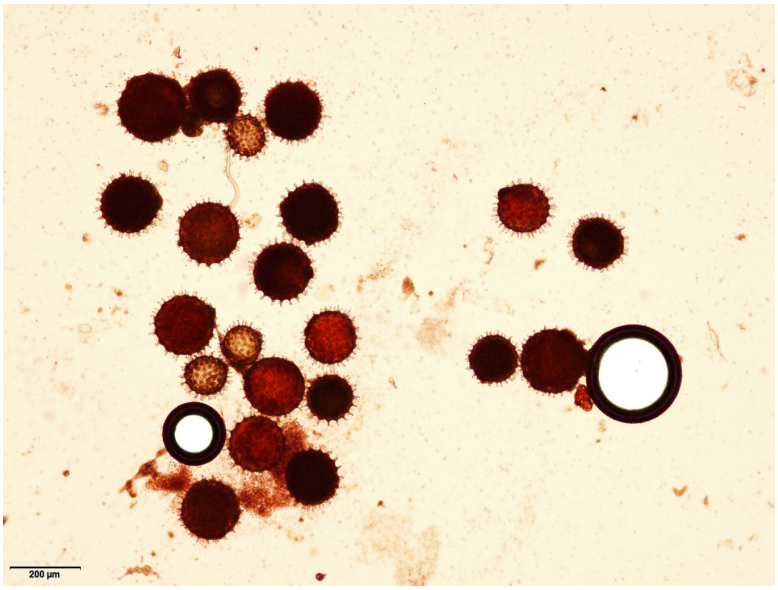

Figure 2.10. Pollen of triploid *Hibiscus moscheutos* 'Luna Red' was stained with 1% acetocarmine. Viability was shown through the stained pollen.

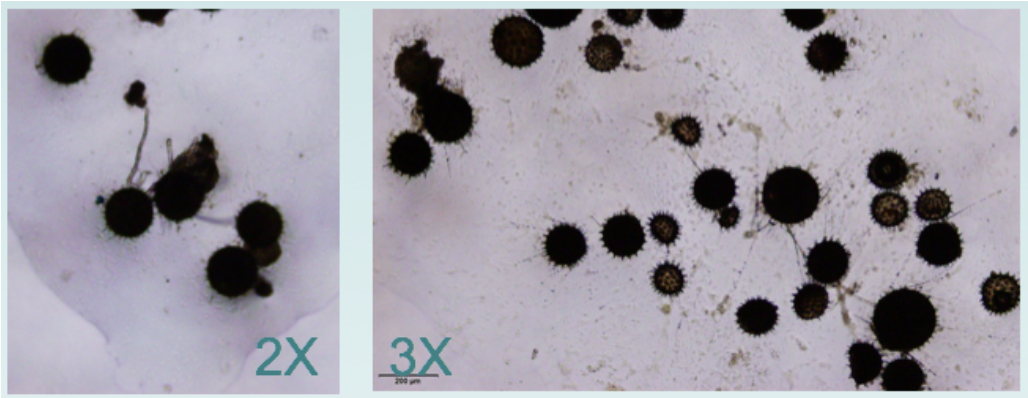

Figure 2.11. Pollen of diploid and triploid *Hibiscus moscheutos* 'Luna Red' was germinated in (Brewbaker and Quack) BK pollen germination agar media. Pollen collected on diploid flowers showed pollen tube growth after 12-hour culture. No pollen collected from flowers of triploids showed any growth during any culture stage.

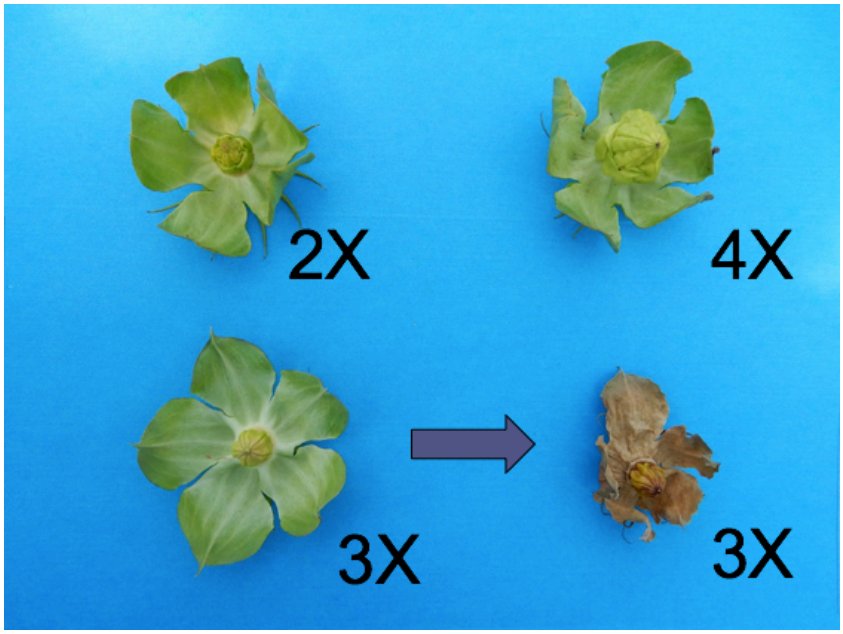

Figure 2.12. Fruit development of diploid, triploid and tetraploid *Hibiscus moscheutos* 'Luna Red'. Both fruit on diploid and tetraploid plants developed normally. Triploid plants displayed fruit setting for the first few days, but the fruit eventually aborted.

CHAPTER 3

INDUCTION OF POLYPLOID *HIBISCUS MOSCHEUTOS* IN TISSUE CULTURE[2]

[2] Li, Z. and J.M. Ruter. To be submitted to *Acta Horticulture*.

Abstract

Commercial breeding of hardy hibiscus (*Hibiscus moscheutos*) has been focused on compact stature and enhanced floral characteristics. In recent years, interest has arisen in breeding for purple foliage, as well as sterility. A purple-foliage selection (Hib14-113) was treated using colchicine and oryzalin as mitotic inhibitors to induce tetraploid breeding lines. After Hib14-113 plants were introduced into tissue culture and multiplied, 10 two-node shoots per treatment were immersed in 0%, 0.025%, 0.05%, or 0.1% (w/v) colchicine solutions for 12, 24, or 36 hours, and 0, 100, 125, or 150 μM oryzalin solutions for 3, 6, or 9 hour durations. Following treatment, shoots were transferred to MS basal medium, and a separate group of shoots was transferred to MS medium with supplemental GA_3. A third group of shoots were soaked in 0, 12.5, 25, or 50 μM of sterile oryzalin solutions for 2, 4, or 6 hours and transferred to MS medium afterwards. Treated shoots were the first to display stunted growth. Treatments with lower oryzalin concentrations and shorter exposure durations showed fewer stagnation effects and an increase in survival percentage from 10% to 23.5%. Flow cytometry was performed to examine the ploidy level. One tetraploid was successfully induced by soaking in 150 μM oryzalin solutions for 6 hours and culturing in GA_3 amended medium. Mixaploids were also obtained. These mixaploids may possibly be used to develop tetraploids through self-pollination.

Introduction

As a native perennial, hardy hibiscus (*Hibiscus moscheutos)* has been widely used as a landscape and container plant, and has been widely bred to produce various cultivars. Commercial breeding work has been focused on generating variation in plant size and flower color (SCHOELLHORN, 2004), but in recent years, interest has focused on having purple foliage (FALSTAD, 2009; FALSTAD, 2015; HURD, 2012; SMITH, 2008). A goal of our breeding program is to have purple foliage selections in combination with compact forms and large, showy flowers.

Hardy hibiscus blooms during the summer, from June to early September in Georgia. However, starting early August, seed production begins; blooming slows down and eventually shuts down as plants produce unsightly brown seed capsules remaining on top of the pedicels. Thus, induction of sterility was attempted to reduce seed production and to further increase ornamental value of flowers and the length of blooming period.

For ornamental plants specifically, polyploidy induction has been widely used for the enlargement effect on plants (ACQUAAH, 2007) and reduction of fertility (CONTRERAS AND RUTER, 2009; RANNEY, 2006). Polyploid plants often possess larger and thicker flowers (CAPORALI et al., 2014) with a longer blooming period (KEHR, 1996), larger and thicker leaves (DIXIT AND CHAUDHARY, 2014), improved stress and disease resistance (HUMPHREYS et al., 2010; OTTO, 2007; YANG et al., 2014), and reduced seed production (CONTRERAS AND RUTER, 2009; CONTRERAS et al., 2009). While maintaining the existing characteristics, such as leaf color, increasing ploidy levels of purple foliage selections would be a further step toward reduced fertility and advanced morphological traits.

The purpose of this study was to induce polyploidy of a purple foliage selection in tissue culture. Common mitosis inhibitors colchicine (BLAKESLEE AND AVERY, 1937) and oryzalin (BARTELS AND HILTON, 1973) were used as polyploidy induction agents.

Materials and Methods

A purple foliage selection of *Hibiscus moscheutos*, Hib 2014-113, from the breeding program of Dr. John Ruter, University of Georgia, GA, was used in this experiment. Hib 2014-113 is a dwarf, purple-leaf hardy hibiscus with large, rosy-color flowers. Axillary buds were collected as explants from one healthy, field-grown plant. Whole buds were surface disinfected with 70% (w/v) alcohol for 5 s and half-strength bleach (sodium hypothlorite 8.25%, Great Value®, Bentonville, AK) for 3 min, followed by three rinses with sterile distilled water. Surface-sterilized buds were cultured on Murashige & Skoog (MS) basal medium with Gamborg's vitamins (Sigma-Aldrich®, St. Louis, MO) supplemented with 30 $g \cdot L^{-1}$ sucrose (Sigma-Aldrich®, St. Louis, MO) and 7 $g \cdot L^{-1}$ agar (Fisher Scientific®, Hampton, NH) (pH = 5.7), with 10 ml gel media in each 25 x 150 mm borosilicate glass test tube (Pyrex®, Thomas Scientific, Swedesboro, NJ). Shoots were cultured under 16-h photoperiod and 98 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (measured with Li-250A light meter, Li-Cor®, Lincoln, NE) of fluorescence light (F40T12/DX, Philips® Lightning, Amsterdam, the Netherlands).

Healthy, vigorous *in vitro* shoots were selected and the middle parts of their stems were cut into multiple two-node segments. Segments were inserted vertically into 10 ml MS medium with vitamins supplemented with 30 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ agar (pH = 5.7) in 25 x 150 mm borosilicate glass test tubes for seven days and allowed to form a thin layer of callus on the top

and bottom. Segments were then removed from the medium and rinsed with sterile distilled water in preparation for chemical treatment.

Colchicine (≥95% (HPLC) powder, Sigma-Aldrich®, St. Louis, MO) solution was prepared using filter sterilization with 0.2 μm pore size filter (Whatman®, Maidstone, UK). Oryzalin (Surflan® A.S., Southern Agricultural Insecticides Inc., Hendersonville, NC) solution underwent heat sterilization in an autoclave (TOMLIN, 1997). Ten clean shoots per treatment were soaked in 0, 0.025%, 0.05%, or 0.1% (w/v) of sterile colchicine solution for 12, 24, or 36 hours on a rotational shaker (Lab-Line® Instruments Inc., No. 3590, Tripunithura, Kochi, India) at a speed of 120 rpm. A separate group of 10 clean shoots per treatment was soaked in 0, 100 μM, 125 μM, or 150 μM of sterile oryzalin solution for 3, 6, or 9 hours. After treatment, shoots were rinsed in sterile distilled water and transferred to MS medium.

A second experiment was conducted using the same soaking method with the same concentrations of colchicine and oryzalin solutions and the same exposure duration, but after treatment, shoot segments were transferred onto MS basal medium with 5 μM gibberellic acid (GA_3) (Sigma-Aldrich®, St. Louis, MO).

Later, a third experiment was carried out with adjustments to the treatment methods. Only oryzalin was used as polyploidy induction agent. Two-node segments were soaked in 0, 12.5, 25, or 50 μM of sterile oryzalin solution for 2, 4, or 6 hours. Chemical was rinsed off in sterile distilled water and segments were transferred to MS medium for culture.

Two-node segments from each chemical treatment were placed under a 16-h photoperiod and 97.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of fluorescence light, and were arranged using a randomized complete block design with ten blocks. Data was taken on survival percentage of treated shoots, after they overcame the stagnation stage. Shoots that survived and later resumed growth were transferred in

½ - strength MS medium supplemented with 0.1 $g \cdot L^{-1}$ IBA (Sigma-Aldrich®, St. Louis, MO) and 1 $g \cdot L^{-1}$ activated charcoal for root development. Well-rooted plants were transplanted into potting media (Jolly Gardener® Pro-line™ C/B Growing Mix, Jolly Gardener Products Inc., Poland, ME) amended with 0.6 $mg \cdot m^{-3}$ Micromax® micronutrients (Everris®, Geldermalsen, the Netherlands) and transferred to a growth chamber (ThermoFisher® Scientific, Precision™, Waltham, MA) with a 16-h photoperiod and 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (measured with Li-250A light meter, Li-Cor®, Lincoln, NE) of fluorescence light (F40T12/DX, Philips® Lightning, Amsterdam, the Netherlands), and constant 25 °C temperature.

Leaf tissue was collected from each plant and prepared for ploidy analysis via flow cytometry on a CyFlow® Ploidy Analyser flow cytometer (Partec GmbH, Münster, Germany). A diploid sample was first examined as standard reference, and plant samples to be tested were compared with diploids for ploidy determination. Up to 1 cm^2 leaf tissue was sampled from the second spreading leaf of each shoot treatment. Leaf tissue was then gently cut with a sharp stainless razor blade (Electron Microscopy Sciences, PA) into 1-2 mm^2 particles in 400 µl nuclei extraction buffer (CyStain® UV Precise P, Sysmex Partec, Germany) and remained in the solution for approximately 30 seconds. The sample particles and the solution were separated using a 40µm mesh filter, while 1600 µl nuclei staining buffer (CyStain® UV Precise P, Sysmex Partec, Germany) was added into the remaining solution for at least 2 minutes. When the solution was processed through the flow cytometer, histograms were automatically generated, showing the fluorescence intensity of stained nuclei in each sample. The relative means of the fluorescence of samples were then examined and compared to those of a control plant with known ploidy. Tetraploid and mixaploid plants were selected and remained in a growth chamber for 4-6 weeks before being moved to the greenhouse.

Results

After being transferred from soaking solutions to MS medium, two-node segments (Figure 3.1) treated with the control solution with no chemical started showing signs of shoot elongation, while chemical-treated shoots showed stagnated growth. Eight weeks later, a few shoots started showing signs of browning and necrosis (Figure 3.2). Gradual shoot necrosis continued for 25 weeks. Some of the plants treated with colchicine had lesions at the base of shoots, callus formed at the lesion, and eventually the shoot died with a swollen and distorted stem. Oryzalin-treated shoots did not show any browning or change of shape during stagnation. Some chemical-treated shoots gradually overcame growth stagnation and developed new leaves; while many of them did not show any new growth or any sign of death until 25 weeks after the initial treatment. While control shoots had a survival rate of 90%, the overall survival rate for colchicine treated shoots was 7.8% (Table 3.1), and for oryzalin treated shoots, survival was 13.3% (Table 3.2). After flow cytometry was performed, all treated plants, either by colchicine or oryzalin, were identified as mixaploids.

Compared to the first experiment, shoots in the control treatment of the second experiment grew faster and produced greener leaves, while chemical-treated shoots remained stunted for 4-6 weeks until necrosis occurred. The survival rate of oryzalin-treated plants increased to 21.1% (Table 3.4). Only one colchicine-treated plant survived, giving a survival rate of 1.1% (Table 3.3). After chemical-treated shoots resumed growth, leaves showed regular growth and greenness in culture. One oryzalin-treated shoot was converted to a tetraploid (Table 3.4) and others were identified as mixaploids.

In the third experiment, shoots in control groups developed new leaves, while chemical-treated shoots remained green, but stagnated, in culture. In contrast to the first two experiments,

the stagnation stage was not as long, and the survival rate of treated shoots also increased to 23.5% (Table 3.5). Many shoots produced new growth while some shoots turned brown and died. The overall survival rate was higher than in the other two experiments. After examination via flow cytometry, all chemical-treated plants were identified as diploids, and no converted tetraploids were found.

Discussion

An advanced selection of hardy hibiscus, Hib 2014-113, was multiplied and treated by colchicine or oryzalin to induce tetraploidy in tissue culture. Chemical-treated shoots showed long periods of growth stagnation after being transferred into culture media. There was an increase in survival percentage when GA_3 was added into growth medium, and as oryzalin concentration and its exposure duration decreased. Tetraploid conversion percentage, however, did not change with the addition of GA_3 to then medium. One tetraploid was obtained from an oryzalin treatment cultured in GA_3-supplemented MS medium. Other treated shoots were only converted to mixaploids or remained diploids. However, GA_3 did not encourage shoot elongation or decrease the length of stagnation period comparing to the previous study with no PGR added. In a separate experiment (Chapter Four), shoots cultured with BAP added to the media were observed producing large numbers of axillary shoots, thus BAP can be further studied as an alternative PGR to promote shoot initiation and ultimately to overcome the long stagnation period.

Polyploidy induction efficiency increased when meristem regions were treated (XI-LING et al., 2011). Soaking shoots, instead of callus where cell division was highly active, led to a relatively low survival and low tetraploid conversion percentage. Induced callus should be used

for ploidy level manipulation treatments for a more effective result. However, this approach requires the ability to regenerate adventitious shoots or somatic embryos from the callus to obtain polyploid plants. A number of studies have reported success regarding the induction of tetraploids in tissue culture through treating callus (HEBERT et al., 2010; MEYER et al., 2009) or embryogenic cells (SAKHANOKHO et al., 2009).

Plant samples reacted positively to a lower oryzalin concentration by showing a higher survival percentage, but the length of treatment period did not significantly affect survival percentage or tetraploid conversion (MEYER et al., 2009). One suggestion is to lower the chemical concentration and correspondingly to extend the immersion period from hours to days (CHUNG et al., 2014; HEBERT et al., 2010). While plants responded differently to mitotic inhibitors, colchicine induces better results under long-term treatments and oryzalin functions more effectively in short treatments (DENAEGHEL et al., 2015).

The next attempt should be to grow out mixaploids and self-pollinate their flowers. Mixaploids may consist of tetraploid cells in the first and second cell layers (LI and LII) and diploid cells on the third cell layer (LIII), such that flowers derived from LII might be tetraploid (EVANS et al., 2003). Through self-pollination of tetraploid organs, it may be possible to obtain tetraploid plants in the next generation.

Induction of tetraploids through chemical soaking of two-node shoots in tissue culture yielded limited success, mainly due to long periods of growth stagnation and low percentage of tetraploid conversion. Since adjusting the concentration and exposure duration of oryzalin led to an increase in survival rate, and a tetraploid shoot was induced in culture media with supplementation of GA_3, follow-up experiments should focus on adjustments of oryzalin concentrations in combination with supplementing culture media with plant growth regulators.

Literature Cited

Acquaah, G., 2007. *Principles of Plant Genetics and Breeding*. Blackwell Pub., Malden, MA; Oxford.

Bartels, P.G. and Hilton, J.L., 1973. Comparison of Trifluralin, Oryzalin, Pronamide, Propham, and Colchicine Treatments on Microtubules. *Pest. Biochem. Physiol.* 3:462-472.

Blakeslee, A.F. and Avery, A.G., 1937. Methods of Inducing Doubling of Chromosomes in Plants by Treatment with Colchicine. *Journal Heredity* 28:393-411.

Caporali, S., Hammami, S.B.M., Moreno-Alias, I., Rapoport, H.F., Chiancone, B., Germana, M.A., and Rosati, A., 2014. Effects of Tetraploidy on Olive Floral and Fruit Biology. *Scientia Horticulturae* 179:198-203.

Chung, M.Y., Kim, C.Y., Min, J.S., Lee, D.J., Naing, A.H., Chung, J.D., and Kim, C.K., 2014. *In vitro* Induction of Tetraploids in an Interspecific Hybrid of *Calanthe (Calanthe discolor* x *Calanthe sieboldii)* through Colchicine and Oryzalin Treatments. *Plant Biotechnology Reports* 8:251-257.

Contreras, R. and Ruter, J., 2009. An Oryzalin Induced Polyploid from a Hybrid of *Hibiscus acetosella* x *H. radiatus (Malvaceae)* Exhibits Reduced Fertility and Altered Morphology. *HortScience* 44:1177.

Contreras, R.N., Ruter, J.M., and Hanna, W.W., 2009. An Oryzalin-Induced Autoallooctoploid of *Hibiscus acetosella* 'Panama Red'. *J. Am. Soc. Hortic. Sci.* 134:553-559.

Denaeghel, H., Van Laere, K., Leus, L., Van Huylenbroeck, J., and Van Labeke, M.C., 2015. Induction of Tetraploids in *Escallonia* spp., p. 453-458. In: VanHuylenbroeck, J. and Dhooghe, E. (eds.), Xxv International Eucarpia Symposium Section Ornamentals: Crossing Borders.

Dixit, V. and Chaudhary, B.R., 2014. Colchicine-Induced Tetraploidy in Garlic (*Allium sativum* L.) and Its Effect on Allicin Concentration. *J. Horticult. Sci. Biotechnol.* 89:585-591.

Evans, D., Coleman, J.O.D., and Kearns, A., 2003. Somaclonal Variation, p. 154, Plant Cell Culture. Garland Science.

Falstad, C.H., 2009. Hibiscus 'Summer Storm'. In: Office, U. S. P. T. (ed.).

Falstad, C.H., 2015. Hibiscus 'Plum Fantasy'. In: Office, U. S. P. T. (ed.).

Hebert, C.J., Touchell, D.H., Ranney, T.G., and LeBude, A.V., 2010. *In Vitro* Shoot Regeneration and Polyploid Induction of *Rhododendron* 'Fragrantissimum Improved'. *HortScience* 45:801-804.

Humphreys, M., Feuerstein, U., Vandewalle, M., and Baert, J., 2010. Ryegrasses, p. 223-224. In: Boller, B., Posselt, U. K., and Veronesi, F. (eds.), Handbook of Plant Breeding: Fodder Crops and Amenity Grasses. Springer New York.

Hurd, K.A., 2012. Hibiscus 'Midnight Marvel'. In: Office, U. S. P. T. (ed.), United States.

Kehr, A.E., 1996. Breeding for a Purpose. *American Rhododendron Society Bulliten*:131-141.

Meyer, E.M., Touchell, D.H., and Ranney, T.G., 2009. *In Vitro* Shoot Regeneration and Polyploid Induction from Leaves of *Hypericum* Species. *HortScience* 44:1957-1961.

Otto, S.P., 2007. The Evolutionary Consequences of Polyploidy. *Cell* 131:452-462.

Ranney, T.G., 2006. Polyploidy: From Evolution to New Plant Development. *Combined Proceeding of International Plant Propagators Society* 56:137-142.

Sakhanokho, H.F., Rajasekaran, K., Kelley, R.Y., and Islam-Faridi, N., 2009. Induced Polyploidy in Diploid Ornamental Ginger (*Hedychium muluense* R. M. Smith) Using Colchicine and Oryzalin. *HortScience* 44:1809-1814.

Schoellhorn, R., 2004. Hardy Hibiscus - Some Great New Hibiscus Species Are Now Available, p. 18-19, Greenhouse Product News. Great American Media Services, Michigan, USA.

Smith, M.A., 2008. Hibiscus Plant Named 'Cherry Brandy'. United Stated Patent and Trademark Office.

Tomlin, C.D.S.e., 1997. *The Pesticide Manual - World Compendium*. British Crop Protection Council, Surrey, England.

Xi-Ling, W., Jin-Xing, Z., Mao-De, Y., Zhen-Gang, L., Xiao-Yun, J., and Qi-You, L., 2011. Highly Efficient Plant Regeneration and *in vitro* Polyploid Induction Using Hypocotyl Explants from Diploid Mulberry (*Morus multicaulis* Poir.). *In Vitro Cellular & Developmental Biology - Plant* 47:434-440.

Yang, C.W., Zhao, L., Zhang, H.K., Yang, Z.Z., Wang, H., Wen, S.S., Zhang, C.Y., Rustgi, S., von Wettstein, D., and Liu, B., 2014. Evolution of Physiological Responses to Salt Stress in Hexaploid Wheat. *Proc. Natl. Acad. Sci. U. S. A.* 111:11882-11887.

Tables and Figures

Table 3.1. Two-node shoots of *Hibiscus moscheutos* were soaked in four levels of colchicine solution for three different periods of time. The number of surviving shoots per treatment was measured. After ploidy level was confirmed for each surviving shoot, the numbers of tetraploid and mixaploid shoots were recorded.

Colchicine Rate (%)	Exposure duration (h)	No. of Treated Shoots	No. of Survival	No. of Tetraploid Conversion	No. of Mixaploid Conversion
0	12	10	9	0	0
0	24	10	8	0	0
0	36	10	10	0	0
0.025	12	10	4	0	0
0.025	24	10	1	0	1
0.025	36	10	0	0	0
0.05	12	10	0	0	0
0.05	24	10	0	0	0
0.05	36	10	0	0	0
0.1	12	10	1	0	1
0.1	24	10	1	0	0
0.1	36	10	0	0	0

Table 3.2. Two-node shoots of *Hibiscus moscheutos* were soaked in four levels of oryzalin solution for three different periods of time. The number of surviving shoots per treatment was measured. After ploidy level was confirmed for each surviving shoot, the numbers of tetraploid and mixaploid shoots were recorded.

Oryzalin Rate (μM)	Exposure duration (h)	No. of Treated Shoots	No. of Survival	No. of Tetraploid Conversion	No. of Mixaploid Conversion
0	3	10	7	0	0
0	6	10	10	0	0
0	9	10	10	0	0
100	3	10	3	0	0
100	6	10	3	0	2
100	9	10	1	0	0
125	3	10	0	0	0
125	6	10	2	0	0
125	9	10	0	0	0
150	3	10	0	0	0
150	6	10	1	0	1
150	9	10	2	0	0

Table 3.3. Two-node shoots of *Hibiscus moscheutos* were cultured on GA_3-amended MS medium after being soaked in four levels of colchicine solution for three different periods of time. The number of surviving shoots per treatment was measured. After ploidy level was confirmed for each surviving shoot, the numbers of tetraploid and mixaploid shoots were recorded.

Colchicine Rate (%)	Exposure duration (h)	No. of Treated Shoots	No. of Survival	No. of Tetraploid Conversion	No. of Mixaploid Conversion
0	12	10	7	0	0
0	24	10	8	0	0
0	36	10	8	0	0
0.025	12	10	0	0	0
0.025	24	10	1	0	0
0.025	36	10	0	0	0
0.05	12	10	0	0	0
0.05	24	10	0	0	0
0.05	36	10	0	0	0
0.1	12	10	0	0	0
0.1	24	10	0	0	0
0.1	36	10	0	0	0

Table 3.4. Two-node shoots of *Hibiscus moscheutos* were cultured on GA_3-amended MS medium after being soaked in four levels of oryzalin solution for three different periods of time. The number of surviving shoots per treatment was measured. After ploidy level was confirmed for each surviving shoot, the numbers of tetraploid and mixaploid shoots were recorded.

Oryzalin Rate (μM)	Exposure duration (h)	No. of Treated Shoots	No. of Survival	No. of Tetraploid Conversion	No. of Mixaploid Conversion
0	3	10	9	0	0
0	6	10	7	0	0
0	9	10	9	0	0
100	3	10	4	0	0
100	6	10	3	0	1
100	9	10	1	0	0
125	3	10	2	0	1
125	6	10	2	0	0
125	9	10	2	0	0
150	3	10	1	0	0
150	6	10	2	1	0
150	9	10	2	0	0

Table 3.5. Two-node shoots of *Hibiscus moscheutos* were soaked in four lower levels of oryzalin solution for three different shorter periods of time. The number of surviving shoots per treatment was measured. After ploidy level was confirmed for each surviving shoot, the numbers of tetraploid and mixaploid shoots were recorded.

Oryzalin Rate (μM)	Exposure duration (h)	No. of Treated Shoots	No. of Survival	No. of Tetraploid Conversion	No. of Mixaploid Conversion
0	2	9	9	0	0
0	4	9	8	0	0
0	6	9	9	0	0
12.5	2	9	0	0	0
12.5	4	9	4	0	0
12.5	6	9	0	0	0
25	2	9	5	0	0
25	4	9	1	0	0
25	6	9	3	0	0
50	2	9	3	0	0
50	4	9	3	0	0
50	6	9	0	0	0

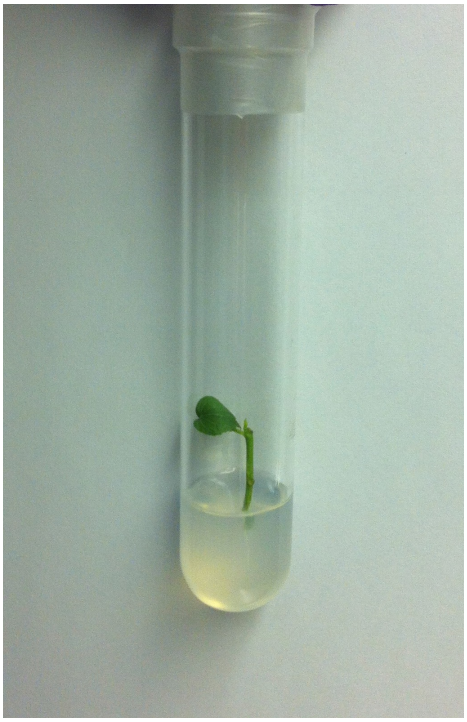

Figure 3.1. Two-node segments of *Hibiscus moscheutos*.

Figure 3.2. Leaf necrosis of *Hibiscus moscheutos*.

CHAPTER 4

EFFECT OF GLUTAMINE ON GROWTH OF *HIBISCUS MOSCHEUTOS* AXILLARY SHOOTS *IN VITRO*[3]

[3]Li, Z. and J. Ruter. To be Submitted to *Journal of Environmental Horticulture*.

Abstract

Nitrogen is the most essential element for plant growth and development. Amino acids, serving as the main organic nitrogen source in tissue culture media, provide for shoot and root elongation. Glutamine has been widely used in tissue culture for dedifferentiation and re-differentiation processes. Experiments were designed to assess the effects of glutamine in comparison to some commonly used plant growth regulators (PGRs). An initial study suggested that 10 $mg \cdot L^{-1}$ glutamine in MS basal medium was optimal for shoot elongation. At this optimal rate, glutamine showed superiority over other PGRs. No difference was found between glutamine treatments and the control in a later study. When comparing glutamine with arginine, shoots cultured on media with arginine displayed slightly greater growth. With arginine containing two extra nitrogen groups in its molecular structure, the higher percentage of nitrogen may have resulted in improved growth.

Introduction

Nitrogen supports plant growth and development in its various forms: ammonia, nitrate and nitrogen gas as inorganic sources, and amino acids as organic nitrogen supplements. Of these, both nitrogen absorbed from the atmosphere and nitrate absorbed from soil and water need to be reduced to ammonia before being transported into metabolic pathways (CANOVAS et al., 1998). Amino acids in the glutamine/glutamate family (glutamine, glutamate, proline, arginine) initiate and accelerate ammonia and nitrite entering into organic nitrogen metabolism. Under catalysis of glutamine synthetase, glutamine is reduced to glutamate, and can be further modified into proline or arginine (HORTON et al., 2006; SHAHSAVARI, 2011). These amino acids also function as nitrogen storage sites (FLAIG AND MOHR, 1992; OKUMOTO et al., 2016), as intermedia to incorporate ammonia into amino acids (HORTON et al., 2006; OKUMOTO et al., 2016), and eventually as building blocks of proteins or nucleotides, such as purine and pyrimidine (HORTON et al., 2006). Because of this, amino acids serve as an efficient nitrogen source through direct synthesis of proteins (EFZUENI ROZALI et al., 2014), and provide an efficient pathway for nitrogen assimilation during long distance metabolic transportation (OKUMOTO et al., 2016), particularly for plants in tissue culture environments (SAAD, 2012).

In Murashige & Skoog basal media, inorganic nitrogen is supplied in sufficient quantities in both the ammonia and nitrate forms (MURASHIGE AND SKOOG, 1962). Therefore, performance and growth of cultured plant tissues is thought to be limited by the nitrogen uptake and assimilation efficiency within the tissues. Supplementing tissue culture basal media with glutamine enables higher nitrogen intake due to an increase of both nitrogen sources and assimilation ability (OKUMOTO et al., 2016). Glutamine has been used in tissue culture media in both the dedifferentiation and re-differentiation processes (HABIB et al., 2015): Callus induction

(CAI et al., 2013), shoot differentiation (CHEN et al., 2015; LI et al., 2014; PERVEEN AND MANSURI, 2015), faster rooting (LIU et al., 2015; TOPPO et al., 2012) and somatic embryogenesis (YAPO et al., 2011).

Besides glutamine, other amino acids in the glutamine family, specifically proline and arginine, are also beneficial to plant tissue growth and have been used for shoot and root induction (CHEN et al., 2015; TOPPO et al., 2012). As a nitrogen source for plants, arginine ($C_6H_{14}N_4O_2$) contains 32.2% nitrogen, which is greater than the 19.2% nitrogen content in glutamine ($C_5H_{10}N_2O_3$) (WINTER et al., 2015). Hibbs et al. (1987) also stated a possible L-arginine deiminase activity that transfers the two extra imino groups from L-arginine structure into nitrite, which is easier for plants to assimilate (HORTON et al., 2006).

The goal of the present study was to investigate glutamine and arginine as organic nitrogen sources for their effects on enhancing axillary shoot multiplication and elongation. Specific objectives were to: (1) compare the effects of glutamine with those of some plant growth regulators (PGRs) conventionally used for axillary shoot production and elongation, (2) identify an optimal rate for glutamine for shoot elongation, (3) compare the effect of shoot growth and root initiation specifically between glutamine and gibberellic acid, and (4) determine whether glutamine or arginine was the preferred amino acid as soluble nitrogen source to support shoot elongation.

Materials and Methods

Plant material

A purple foliage selection of *Hibiscus moscheutos*, Hib 2014-113, from the breeding program of Dr. John Ruter, University of Georgia, GA, was used in this study (Figure 4.1).

Healthy, vigorous Hib 2014-113 *in vitro* plants were selected when they were under culture in MS medium (MURASHIGE AND SKOOG, 1962). Two-node shoots were cut from mid-stem of the plants and leaves on the lower node were removed before shoots were transferred into culture treatments.

All media was prepared using filter sterilization for any PGRs or amino acids, one day before the treatments. MS basal medium was prepared from powder mix (Sigma-Aldrich®, St. Louis, MO) and was supplemented with 30 g sucrose and gelled with 7 g agar (Fisher Scientific®, Hampton, NH). The pH of the tissue culture medium was adjusted to 5.7. MS medium was pre-autoclaved and placed in a 68 °C water bath while PGRs or amino acids were being filter sterilized. To ensure sterile conditions, pure powder of PGRs or amino acids was first completely dissolved in sterile distilled water and then the solution was sterilized through 0.2 μm pore-size filter (Whatman® sterile PVDF, Maidstone, UK) using a sterile syringe. Sterile PGRs or amino acids were then proportionally added to warm sterile MS basal media.

After shoots were transferred into tissue culture treatments, they were placed on shelves in a culture room with a constant 20 °C temperature and 16-h photoperiod under 98 $\mu mol \cdot m^{-2} \cdot s^{-1}$ florescent light (measured with Li-250A light meter, Li-Cor®, Lincoln, NE) (F40T12/DX, Philips® Lightning, Amsterdam, the Netherlands).

Study I: Comparisons of BAP, GA_3, TDZ and L-glutamine

Two-node shoots of similar length and vigor were selected. They were then transferred in four treatment groups of MS media with 1.5 $mg \cdot L^{-1}$ BAP (6-Benzylaminopurine), 0.35 $mg \cdot L^{-1}$ GA_3 (Gibberellic acid), 10 $mg \cdot L^{-1}$ L-glutamine, or 0.22 $mg \cdot L^{-1}$ TDZ (Thidiazuron). Additionally, a control group with no PGRs or glutamine for a comparative study was prepared. There were 10 replicates per treatment with one shoot per replicate. Each shoot was transferred into MS gel

media perpendicular to the surface of the gel in an 25 x 150 mm glass test tube (Pyrex®, Corning, NY). Test tubes were then capped and sealed with Parafilm tape (Parafilm M®, Neenah, WI). New shoot elongation was measured weekly for seven weeks. Leaf necrosis of each plant was visually rated at the end of the study. The rating was designed as a 0 to 3 numbering system. Shoots with no necrosis were annotated as "0", shoots with light leaf necrosis were annotated as "1", shoots with medium necrosis were annotated as "2", and shoots with severe leaf necrosis were annotated as "3".

Study II: Comparisons of different concentrations of L-glutamine

Two-node shoots of similar length and vigor were prepared for the following study. Shoots were transferred in gelled MS media supplemented with 0-, 0.01-, 0.05-, 0.1- or 0.5- $g \cdot L^{-1}$ glutamine. Each treatment group contained 10 replicates. The length of new shoot growth was measured from outside the test tubes weekly. The entire study was terminated when the first tissue-cultured plant of any treatment reached the top of test tube cap. To take measurements after this stage of growth would provide inaccurate results.

Study III: Comparisons between GA_3 and L-glutamine

Vigorous two-node shoots were used in this study. Two rates of GA_3, 2 $mg \cdot L^{-1}$ and 4 $mg \cdot L^{-1}$ and two rates of glutamine, 5 $mg \cdot L^{-1}$ and 10 $mg \cdot L^{-1}$ were added to MS basal medium. Adding an additional control group with no amino acid supplements, shoots were transferred into media of these five treatment groups in a method described in Study I. Each treatment unit contained 10 replicates. The length of new shoot growth was measured weekly for seven weeks. Binomial data was also taken on the presence or absence of roots at the termination of this study.

Study IV: Comparisons between L-glutamine and L-arginine

In this study, except for the control group, to the MS media in each treatment group was added 10 $mg \cdot L^{-1}$ glutamine, 10 $mg \cdot L^{-1}$ arginine, or a combination of both. Healthy two-node shoots were transferred into these four treatments using the method described in Study I. There were 10 replicates per treatment. The length of new shoot elongation was assessed (from outside of the test tubes) weekly until the first tissue-cultured plant reached the top of the test tube cap at week seven, at which point the study was terminated. Plants were inspected weekly to determine if rooting had occurred. The number of weeks it took, post treatment, for the first root to emerge was recorded as rooting data. Shoots that had not rooted before termination were not included in the data analysis.

Data analysis

Data for each study was collected weekly and analyzed using R statistical software (R, 2015). The statistical significance of different treatment groups was assessed in ANOVA and means comparisons were made using Tukey's honest significance test (HSD Test) (TUKEY, 1949) or Dunnett's test (DUNNETT, 1964).

Results

Study I: Comparisons of BAP, GA_3, TDZ, L-glutamine

After seven weeks of culture, some two-node shoots expanded leaves, elongated stems and grew up to the top of the test tubes. Glutamine treatments (13.9 ± 2.2 cm) and GA_3 treatments (12.0 ± 4.4 cm) displayed significantly greater shoot elongation than other treatment groups (Table 4.1). TDZ did not stimulate, but inhibited, shoot growth when overall shoot elongation was compared to the control group. BAP promoted shoot differentiation at an early

stage of culture and largely encouraged axillary shoot proliferation, but overall impact on length of shoot elongation was minor. BAP, glutamine and GA_3 performed the best in preventing leaf necrosis after seven weeks of culture (Figure 4.2). Shoots in the control group and the TDZ-treated group showed significant leaf yellowing and abscission (Table 4.1).

Study II: Comparisons of different concentrations of L-glutamine

Different concentrations of glutamine were added to the culture to allow for examination of their effect on shoot growth over the seven-week period. At week seven, shoots growing in media with 0.01 $g \cdot L^{-1}$ glutamine showed the greatest elongation at 5.8 ± 2.8 cm (Table 4.2). While there was no significant differences between the control group and 0.05 $g \cdot L^{-1}$, 0.1 $g \cdot L^{-1}$ or 0.5 $g \cdot L^{-1}$ glutamine treated groups, shoots cultured in 0.01 $g \cdot L^{-1}$ glutamine-amended medium were significantly longer than those of the control group ($p=0.003$).

Study III: Comparisons between GA_3 and L-glutamine

Two-node shoots in this study showed slow or no growth initially, but most of them resumed growth at week six or seven and developed rapidly in the last two weeks. The experiment was terminated after week seven because some of the shoots reached the top of the test tubes. The means of shoot elongation in the GA_3 treatments (1.27 ± 0.88 cm, 1.41 ± 0.96 cm, for 2 $mg \cdot L^{-1}$and 4 $mg \cdot L^{-1}$, respectively) were lower than those of other groups; however, there were no significant difference between the GA_3 treatments and glutamine treatments or the control due to great variability among replicates within treatments. Shoots treated with GA_3 did not initiate rooting at any concentration. Fifty percent of the plants in the control group developed roots, while 50 $mg \cdot L^{-1}$ glutamine promoted 90% rooting among treated plants, which was the highest of all treatments (Table 4.3).

Study IV: Comparisons between L-glutamine and L-arginine

Shoots in different treatment groups developed varying lengths of new growth at varying speeds. This study was terminated when some shoots reached the tops of sealed test tubes. Although shoots with the greatest elongation were observed in the treatment containing both glutamine and arginine, there was no significant difference among treatments ($p=0.73$) (Table 4.4). Observations from the last two weeks of this study, however, revealed a greater increase in shoot elongation for the treatment containing glutamine plus arginine ($p=0.002$) (Table 4.5 and Figure 4.3). Within the seventh week, shoots in the glutamine plus arginine treatment developed greater elongation (7.6 ± 3.1 cm) than those in other treatments, and shoots in the glutamine treatment (2.7 ± 6.1 cm) developed the least elongation among all treatments. No significant difference was found between any treatments for when the first root emerged (Table 4.4).

Discussion

Although it is evident that glutamine accelerates plant development (OKUMOTO et al., 2016), inconsistent performance was found among our experiments. Glutamine was found effective in promoting shoot elongation at a rate of 10 $mg \cdot L^{-1}$ and in preventing leaf necrosis. In repeated experiments, however, the same concentration of glutamine did not display a significant difference in shoot elongation from the control group. As a nitrogen provider, the effect of glutamine might be dependent on the demand of certain plant species. More trials and observations are needed to articulate the effect of glutamine on tissue-cultured *H. moscheutos*. Including BAP in the tissue culture media was determined to be ineffective for promoting shoot elongation, however it was very effective at inducing shoot proliferation, thus it can be used for the purpose of promoting the number of axillary shoots.

One positive result is that glutamine displayed a greater effect on shoot elongation than GA_3. GA_3 has long been recognized as an effective PGR for promoting shoot elongation, especially in tissue culture (CURTIS AND CROSS, 1954; DAVIES, 2010). Shoots cultured in 0.35 $mg \cdot L^{-1}$ GA_3 displayed significant shoot growth; while GA_3 showed degrees of inhibition on stem elongation at levels of 2 $mg \cdot L^{-1}$ and 4 $mg \cdot L^{-1}$. A general reference for an effective concentration at which to apply GA_3 was 5 $mg \cdot L^{-1}$; however, the rate can be species dependent (NICKELL, 1958). While a low concentration of GA_3 can be stimulating to stem elongation; a high concentration of GA_3 can be inhibitory on shoot elongation and root development.

Although no significant difference was found for this experiment overall, treatment with arginine, as well as a mixture of arginine and glutamine, resulted in greater shoot elongation in the last two weeks. Of all 21 major amino acids, arginine has the highest nitrogen to carbon ratio (WINTER et al., 2015). Due to its unique double-imino structure, arginine has an extra nitrogen atom per molecule, and these imino structure could be transformed into nitrite for assimilation (HIBBS et al., 1987). The extra nitrogen groups may have given arginine the potential to transform into soluble nitrogen and provide nitrogen at later stages of the study when growth was rapid.

Literature Cited

Cai, X.D., Wang, G.Y., and Cao, W.J., 2013. *In vitro* Induction and Proliferation of Callus from Immature Cotyledons and Embryos of *Juglans regia* cv. 'Xiangling'. *Not. Bot. Horti Agrobot. Cluj-Na.* 41:378-384.

Canovas, F.M., Canton, F.R., Garcia Gutierrez, A., Gallardo, F., and Crespillo, R., 1998. Molecular Physiology of Glutamine and Glutamate Biosynthesis in Developing Seedlings of Conifers. *Physiologia Plantarum* 103:287-294.

Chen, Z., Tang, J., and Li, H., 2015. Method for Culturing Peony Plant Tissue, Involves Selecting Robust Growth Peony Branches, Preparing Medium, Performing Disinfection and Vaccination Followed by Removing Section, Subculturing Shoots, Transferring to Medium, and Culturing. University of Chongqing Arts & Science (UYCH-Non-standard).

Curtis, P.J. and Cross, B.E., 1954. Gibberellic Acid - A New Metabolite from the Culture Filtrates of *Gibberellia Fujikuroi*. *Chem. Ind.*:1066-1066.

Davies, P.J., 2010. The Plant Hormones: Their Nature, Occurrence, and Functions, p. 6-7. In: Davies, P. J. (ed.), Plant Hormones: Biosynthesis, Signal Transduction, Action! Springer.

Dunnett, C.W., 1964. New Tables for Multiple Comparisons with a Control. *Biometrics* 20:482-491.

Efzueni Rozali, S., Rashid, K.A., and Mat Taha, R., 2014. Micropropagation of an Exotic Ornamental Plant, Calathea crotalifera, for Production of High Quality Plantlets. *The Scientific World Journal* 2014:12.

Flaig, H. and Mohr, H., 1992. Assimilation of Nitrate and Ammonium by the Scots Pine (Pinus sylvestris) Seedling under Conditions of High Nitrogen Supply. *Physiologia Plantarum* 84:568-576.

Habib, D., Zia, M., Bibi, Y., Abbasi, B.H., and Chaudhary, M.F., 2015. Response of Nitrogen Assimilating Enzymes during *in vitro* Culture of *Argyrolobium roseum*. *Biologia* 70:478-485.

Hibbs, J.B., Taintor, R.R., and Vavrin, Z., 1987. Macrophage Cytotoxicity: Role for L-Arginine Deiminase and Imino-Nitrogen Oxidation to Nitrate. *Science* 235:473-476.

Horton, H.R., Moran, L.A., Scrimgeour, K.G., Perry, M.D., and Rawn, J.D., 2006. Amino Acid Metabolism, p. 520-529. In: Carlson, G. (ed.), Principles of Biochemistry (Fourth Edition). Pearson Prentice Hall, New Jersey.

Li, J.Y., Fan, Z.Q., and Wu, B., 2014. Callus Induction and Plant Regeneration from Immature Cotyledon of Camellia azalea, p. 157-162. In: Zhang, Q. and Jin, X. (eds.), Vi International Symposium on the Taxonomy of Cultivated Plants. Int Soc Horticultural Science, Leuven 1.

Liu, Y., Tong, X., Hui, W.K., Liu, T., Chen, X.Y., Li, J., Zhuang, C.X., Yang, Y.S., and Liu, Z.L., 2015. Efficient Culture Protocol for Plant Regeneration from Petiole Explants of Physiologically Mature Trees of *Jatropha curcas* L. *Biotechnol. Biotechnol. Equip.* 29:479-488.

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

Nickell, L.G., 1958. Gibberellin and the Growth of Plant Tissue Cultures. *Nature* 181:499-500.

Okumoto, S., Funck, D., Trovato, M., and Forlani, G., 2016. Editorial: Amino Acids of the Glutamate Family: Functions Beyond Primary Metabolism, Frontiers in Plant Science.

Perveen, A. and Mansuri, S., 2015. Rapid Propagation of a Biodiesel Plant Cassava (Manihot esculenta Crantz) through Tissue Culture. *International Journal of Biology and Biotechnology* 12:369-372.

R, R.D.C.T., 2015. R: A Language and Environment for Statistical Computing, Version 3.2.2. R Foundation for Statistical Computing, Vienna, Austria.

Saad, A.I.M.E., A.M., 2012. Plant Tissue Culture Media, p. 31. In: Leva, D. A. (ed.), Recent Advances in Plant in vitro Culture. InTech.

Shahsavari, E., 2011. Impact of Tryptophan and Glutamine on the Tissue Culture of Upland Rice. *Plant Soil Environ.* 57:7-10.

Toppo, D.D., Singh, G., Purshottam, D.K., and Misra, P., 2012. Improved *in vitro* Rooting and Acclimatization of *Jatropha curcas* Plantlets. *Biomass Bioenerg.* 44:42-46.

Tukey, J.W., 1949. Comparing Individual Means in the Analysis of Variance. *Biometrics* 5:99-114.

Winter, G., Todd, C.D., Trovato, M., Forlani, G., and Funck, D., 2015. Physiological Implications of Arginine Metabolism in Plants. *Frontiers in Plant Science* 6:14.

Yapo, E.S., Kouakou, T.H., Kone, M., Kouadio, J.Y., Kouame, P., and Merillon, J.M., 2011. Regeneration of Pineapple (*Ananas comosus* L.) Plant through Somatic Embryogenesis. *J. Plant Biochem. Biotechnol.* 20:196-204.

Tables and Figures

Table 4.1. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium containing 1.5 mg·L^{-1} BAP, 0.35 mg·L^{-1} GA_3, 10 mg·L^{-1} L-Glutamine or 0.22 mg·L^{-1} TDZ. Overall shoot growth was measured after culturing for seven weeks. A Tukey's HSD test was performed at 95% significance level on the overall shoot elongation and foliage necrosis. Foliage necrosis was rated in a numbering system from 0 (no necrosis) to 3 (severe necrosis).

Treatment	Shoot Elongation (±SD) (cm)	Plant Necrosis * (±SD)
Control	9.5 (±3.4) bc	2.6 (±0.7) a
BAP	8.3 (±2.8) bc	1.7 (±0.5) bc
GA_3	12.0 (±4.1) ab	1.4 (±0.5) c
L-Glutamine	13.9 (±2.2) a	1.5 (±0.5) bc
TDZ	7.1 (±1.2) c	2.2 (±0.6) ab

Table 4.2. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium supplemented with 0-, 0.01-, 0.05-, 0.1-, or 0.5 $g \cdot L^{-1}$ glutamine. Length of shoot growth was measured at seven weeks before the study was terminated. Dunnett's test was performed to look at the difference between each glutamine concentration and the control group.

Glutamine Concentration ($g \cdot L^{-1}$)	**Shoot Elongation (±SD) (cm)**
0	4.2 (±1.6)
0.01	5.8 (±2.8)
0.05	4.2 (±3.6)
0.1	4.7 (±4.7)
0.5	4.4 (±3.6)

Dunnett's Test	**Significance**
0 vs 0.01 $g \cdot L^{-1}$	**
0 vs 0.05 $g \cdot L^{-1}$	NS
0 vs 0.1 $g \cdot L^{-1}$	NS
0 vs 0.5 $g \cdot L^{-1}$	NS

**- significant at $p \leqslant 0.01$, NS-not significant.

Table 4.3. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium supplemented with 10 mg·L^{-1} glutamine, 50 mg·L^{-1} glutamine, 2 mg·L^{-1} GA_3 or 4 mg·L^{-1} GA_3. Treatment groups was considered as categorical factors for a one-way ANOVA. Length of shoot elongation was measured after culturing for seven weeks. Rooting percentage of each treatment was analyzed in Tukey's HSD test.

Treatment	Shoot Elongation (±SD) (cm)	Rooting Percentage (%)
Control	4.8 (±5.1)	50 (±52.7) b
Glutamine (10 mg·L^{-1})	5.0 (±3.9)	40 (±51.6) b
Glutamine (50 mg·L^{-1})	5.7 (±5.6)	90 (±31.6) a
GA_3 (2 mg·L^{-1})	1.3 (±0.9)	0 (±0) b
GA_3 (4 mg·L^{-1})	1.4 (±1.0)	0 (±0) b
Analysis of variance		
Treatment	NS	*

*-significant at $p \leqslant 0.05$, NS-not significant.

Table 4.4. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium supplemented with 10 $mg \cdot L^{-1}$ glutamine, 10 $mg \cdot L^{-1}$ arginine, 10 $mg \cdot L^{-1}$ glutamine plus 10 $mg \cdot L^{-1}$ arginine, or in plain MS media. Overall shoot elongation was measured after seven weeks. Rooting week was the number of weeks after the initial treatment when the first root was observed on individual tissue-cultured plants. No significant treatment effect was found from Tukey's HSD test, on either shoot growth or root initiation.

Amino Acids	**Shoot Elongation (±SD) (cm)**	**Rooting Week (±SD)**
Control	11.2 (±3.7)	3.5 (±1.0)
Glutamine	11.8 (±6.1)	4.2 (±1.2)
Arginine	14.0 (±3.5)	4.0 (±0.7)
Glutamine & Arginine	14.9 (±3.1)	4.2 (±0.7)
Analysis of variance		
Treatment	NS	NS

NS-not significant

Table 4.5. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal media supplemented with 10 $mg \cdot L^{-1}$ glutamine, 10 $mg \cdot L^{-1}$ arginine, 10 $mg \cdot L^{-1}$ glutamine plus 10 $mg \cdot L^{-1}$ arginine, or in plain MS media. The study was terminated after seven weeks. Shoot elongation within the seventh week was compared among treatments.

Amino Acids	Shoot Elongation (±SD) (cm)
Control	3.6 (±3.7) bc
Glutamine	2.7 (±6.1) c
Arginine	5.1 (±3.5) ab
Glutamine & Arginine	7.6 (±3.1) a
Analysis of variance	
Treatment	**

**- significant at $p \leqslant 0.01$

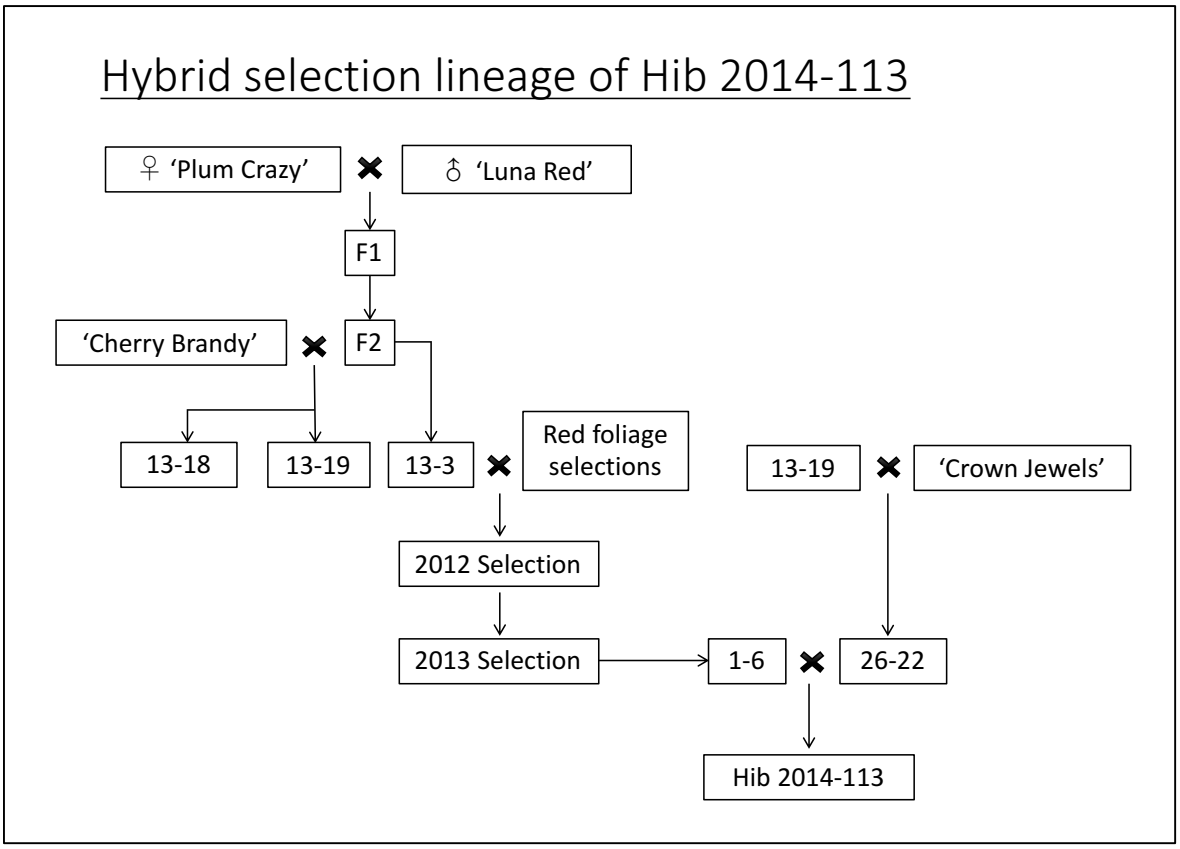

Figure 4.1. Pedigree map for *Hibiscus moscheutos* selection Hib 2014-113. Numbered names, such as "13-18", were selections during breeding process.

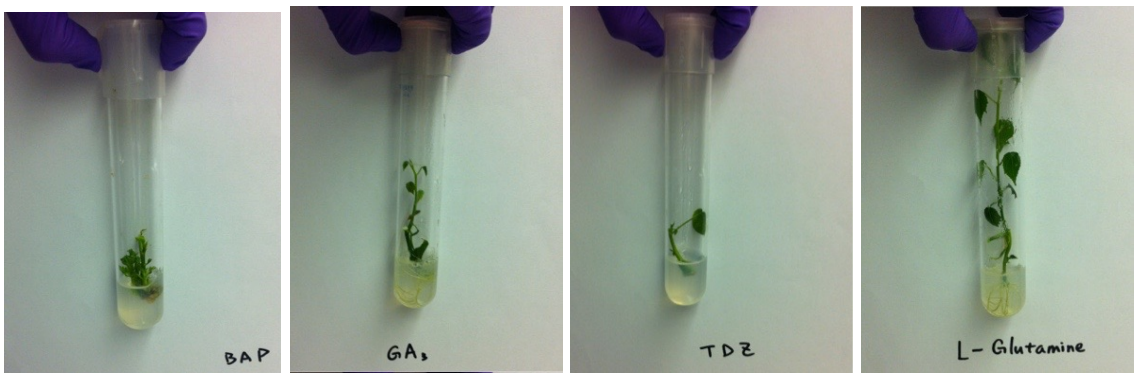

Figure 4.2. Shoot elongation of *Hibiscus moscheutos* shoot segments after 7 weeks of culture in MS basal medium amended with BAP, GA_3, TDZ or glutamine.

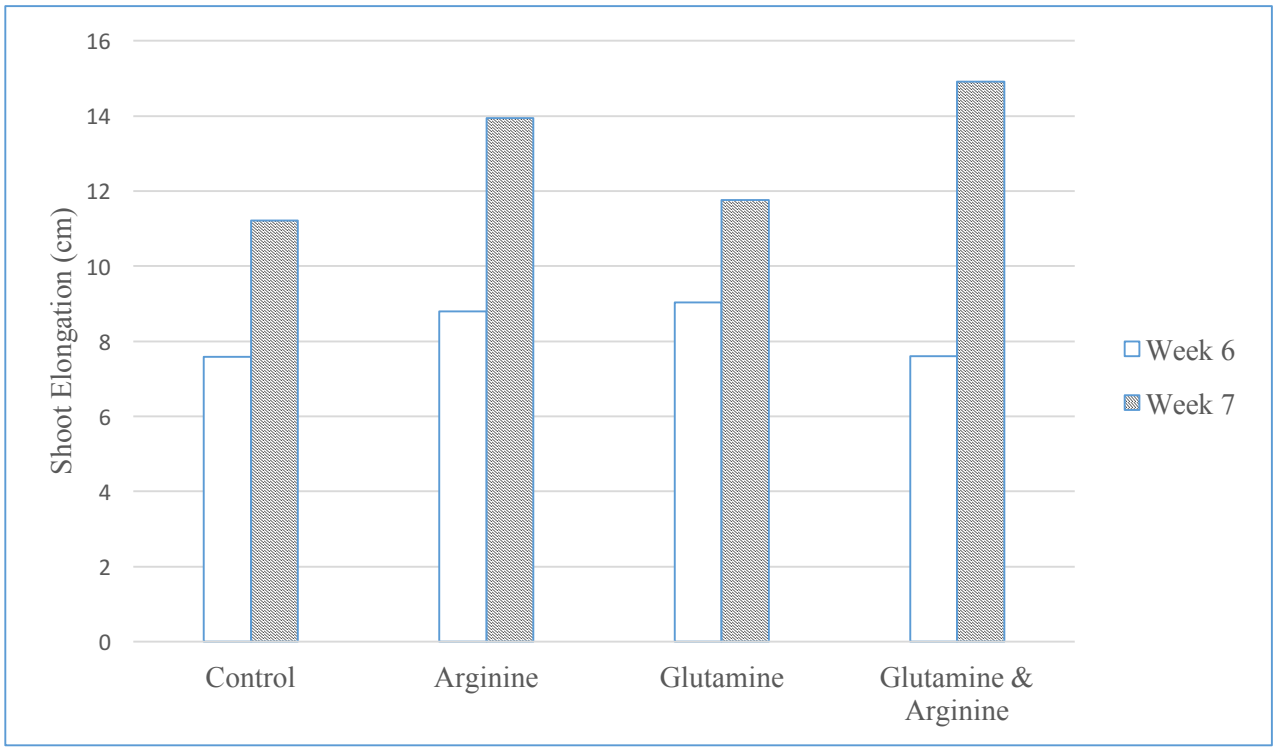

Figure 4.3. *Hibiscus moscheutos* shoots were cultured in MS medium amended with 10 $mg \cdot L^{-1}$ glutamine, 10 $mg \cdot L^{-1}$ arginine or a combination of both. Shoot elongation was observed weekly for seven weeks. This chart showed the new shoot growth in last two weeks of the study.

CHAPTER 5

CONCLUSIONS AND NEXT STEPS

This thesis presented methods and results of tissue culture and induction of polyploid *Hibiscus moscheutos*. Conclusions and further plans are discussed as follows.

An inbred cultivar 'Luna Red' was utilized in the study described in Chapter Two. In this study, soaking germinated seedlings proved to be more effective than soaking entire seeds for induction of polyploidy. Separate treatments of both colchicine and oryzalin both yielded tetraploids, and oryzalin treatments performed at a high efficiency with a 6-hour exposure duration. This newly developed protocol suggests methods to further polyploidy breeding in hibiscus. When induced tetraploids and triploids were compared with diploid lines, a decrease in some morphological traits, such as flower diameter, was observed in the tetraploids and triploids. While the "enlargement" effect was expected in polyploids, induction of polyploidy of inbred plants resulted in reduction of many morphological traits. Due to the inbred nature of the plant material, the product of induction was unstable and infertile autopolyploids, which are difficult to work with in long-term breeding projects. Breeding work for the next step should adopt diverse genetic resources in each generation. This is because of their hybrid vigor and because of the consistency of selections. With regard to cultivar improvement, breeding for hybrid triploids should be the route taken, due to the highly reduced rates of fertility and at the same time maintaining diploid traits.

A description of the induction of polyploid *H. moscheutos* in tissue culture was presented in Chapter Three. As hibiscus showed high sensitivity to chemical treatment in tissue culture,

only a few shoots survived and only one shoot was eventually converted to a tetraploid. Considering the efficiency in induction of polyploids, the seedling-soaking method is preferred. For situations with limited plant material, soaking vegetative cuttings should be attempted prior to micro-propagation.

Chapter Four described a series of experiments evaluating how glutamine affected shoot elongation in tissue culture. This chapter also compared the effects of glutamine to those of common PGRs and arginine. Glutamine displayed a positive effect on preventing leaf necrosis. However, it performed inconsistently in promoting shoot elongation and root initiation. Arginine showed slightly higher efficiency than glutamine in promoting shoot elongation. More research should be attempted using various amino acids, such as arginine and proline. Only when different amino acids are tested could we find a superior method specifically for *H. moscheutos*. A mixture of various amino acids or casein hydrolysate (CH), could be adopted in future studies. However, CH might not be a good choice for a scientific study, since the exact components and their concentrations are unknown. A general protocol can prove helpful to commercial tissue culture labs with regard to large scale production.