THE PRODUCTION OF RED-FLESHED GRAPEFRUIT/PUMMELO TETRAPLOID PARENTS TO AID THE GRAPEFRUIT IMPROVEMENT PROGRAMS

By

DIVYA KAINTH

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2010

To my late father Dr. Satvinder Singh, my mother Mrs. Surinder Kaur and my beloved sister Kavya

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Dr. Jude W. Grosser, the chairperson of my graduate committee for giving me an opportunity to work with him on such an interesting project and for giving me encouragement, moral and financial support throughout my degree program. I had a great learning experience under his constant scientific guidance. I would like to extend my gratitude to Dr. Frederick G. Gmitter, Dr. Jim H. Graham and Dr. Jose Chaparro for serving on my committee and for their help, academic support, and valuable advice and constructive criticism that helped me throughout my research experience. Deepest thanks to United States Department of Agriculture/ Cooperative State Research, Education, and Extension Service, Florida Citrus Production Research Advisory Council, New Varieties Development and Management Corporation/ Florida Department of Citrus and Barney and Harriett Greene for providing me financial support through my entire program.

I would like to acknowledge my lab mates including postdoctoral research associates, graduate students, biological scientists and OPS employees. Many thanks to Dr. Manjul Dutt for his expert advice, Gary Barthe for providing me with all the equipments and chemicals I needed for my research. Special thanks to Milica Calovic who gave me training in somatic hybridization technique as well as helped me finding solutions to the research problems I faced, from her expertise and experience. I am also very thankful to my friends in my lab Monica Vasconcellos, Julie Gmitter, Jamuna, Elaine, Pamela for their support and friendship. I am thankful to Chuck and Ralph Story for taking care of my plants in the greenhouse. I want to thank Katherine Snyder and Paul Weikel for helping me with poster preparations and other technical things. Great thanks to the entire Citrus Research and Education Center (CREC) community for

making me feel at home away from home. I am very grateful to my friends Harsimran gill, Gaurav Goyal, Sunehali Sharma, Amit Kohli, Omar, Tejdeep, Ekta Pathak, Ashish and Jagroop Gill for their support through all thick and thin. Finally, I would like to thank my family and friends in India who supported my decision to come here and learn and their unconditional love.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

THE PRODUCTION OF RED-FLESHED GRAPEFRUIT/PUMMELO TETRAPLOID PARENTS TO AID THE GRAPEFRUIT IMPROVEMENT PROGRAMS

By

Divya Kainth

December 2010

Chair: Jude W. Grosser
Major: Horticultural Sciences

Seedless and red-fleshed grapefruit cultivars have a distinct upper-hand in the citrus market and are preferred by consumers over white and seedy cultivars. Since seedlessness is the characteristic of triploids, more triploids via different diploid by tetraploid crosses need to be produced to incorporate all the desired traits. Although a large variety of citrus diploids are available, a broader tetraploid gene pool is still needed. The objective of this research was to produce grapefruit/pummelo type tetraploids for use in crosses with diploids to produce seedless triploids. To accomplish this objective, the first approach was to somatically hybridize leaf protoplasts from red fleshed pummelo parents with protoplasts from the callus of Ruby Red grapefruit by PEG (polyethylene glycol) induced fusion to obtain allotetraploids. The second approach was to produce autotetraploids by using colchicine, an anti-mitotic agent. Both *in vitro* and *in vivo* experiments were conducted. Under *in vitro* experiments, the explants from the etiolated seedlings grown under sterile conditions were treated with different colchicine concentrations - 0.01, 0.05 and 0.1% for different durations - 4, 8, 16 and 32 hours. The shoots obtained through indirect organogenesis from the explants

from each treatment were tested for their ploidy via flow cytometery. Pregerminated seeds were also treated with colchicine at 0.1, 0.2 and 0.3% for 12 and 24 hours, in another experiment. For *in vivo* experiments, mature budsticks having 4-5 buds were cleft-grafted onto vigorous rootstocks and treated with 1% colchicine. The treatment is given for 1 and 2 days. The sprouting buds were tested for their ploidy. Thirdly, 600 to 700 seeds per pummelo cultivar were planted and seedlings were selected on the basis of root morphology and screened for natural altered ploidy levels i.e. triploids or tetraploids. The seedlings obtained by these approaches were tested using flow cytometry. The confirmed tetraploids were micrografted onto vigorous rootstocks to expedite transfer to the greenhouse and subsequently to the field. The tetraploids produced in these experiments will be very valuable starting material for the programs involved in improving hybrid pummelo/grapefruit quality.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

History of Grapefruit

Grapefruit (*Citrus paradisi* Macf.) is an evergreen subtropical citrus cultivar that originated as a natural hybrid in Barbados in mid 18th century. It is a relatively new member in the citrus family. It was first described as 'The forbidden fruit' of a pyriform shape and with no winged petioles in 'The Natural History of Barbados' by Griffith Hughes in 1750. Grapefruit was also growing in most parts of Jamaica. Naturalist John Lunan first recorded it as 'grapefruit' because of its taste resembling that of grapes. Tussac on the other hand described its habit of being borne in clusters and hence the name grapefruit. The latter explanation was more logical and accepted in 1943. The Latin classification for grapefruit is *C. paradisi* Macf. It means citrus of paradise and assigned by Macfadyen (1830, 1837). Due to grapefruits resemblance to pummelo (*C. grandis L.* Osbeck) in the fruit appearance, the latter was named as a probable parent. However, there were conflicting beliefs about how grapefruit originated. It was though either to be a mutant from pummelo or a hybrid from a cross between pummelo and sweet orange (*C. sinensis* L. Osbeck). Chemical and morphological evidences provided by Scora et al.(1982), support the latter hypothesis. More recently, the analysis of its genetic profile confirmed that grapefruit originated as an interspecific hybrid as a result of chance hybridization between pummelo and sweet orange (Scora, 1975; Barret and Rhodes., 1976; Scora et al., 1982; Gmitter, 1995). This also gives a logical explanation for its size characteristics from the pummelo parent and the nucellar embryony characteristic from sweet orange. William C. Cooper, a citrus scientist (USDA, ARS, Orlando, Florida, 1975), mentions in his book, In Search of the Golden Apple, that

grapefruit was first introduced in Florida in 1823 when grapefruit seeds from the Bahama Islands were taken to Safety Harbor near Tampa in Florida. From there, it spread to the surrounding areas and eventually was found all over Florida. At that time, the grapefruit was considered mostly an ornamental novelty. In 1870, John A. MacDonald was drawn towards the cluster of lemon colored fruits on a single grapefruit tree in the Drawdy property at Blackwater, Florida. He used the seeds from the fruit of this tree as the source to establish the first grapefruit nursery in Florida. George W. Bowen established the first grapefruit grove for commercial production in Florida in 1875. By 1885, growers in Florida started shipping the fruit to New York and Philadelphia where the people were developing the taste for the fruit and the demand was starting to rise. A single grapefruit tree that repeatedly survived freezing temperatures was observed in Kennedy Ranch in southern Texas and lead to successful production of grapefruit even under relatively cooler climatic conditions prevailing there. By 1910, grapefruit acquired the status of an important commercial crop in the Rio Grande Valley of Texas, while it was still in the process of getting established as a commercial crop in California and Arizona. Grapefruit continued gaining popularity for its fruit and juice in countries like Jamaica, Israel, United States, Brazil and other South American countries. By the 1940s, grapefruit was recognized as an important part of healthy diet and was also called the ‘Breakfast fruit’. People had developed a liking for its taste, increasing the grapefruit demand and production to a great extent. At present, the United states of America is the leading producer of grapefruit in the world, followed by China, South Africa, Mexico, Syria, Israel, Turkey, India, Argentina and Cuba respectively according to FAO (Anonymous., 2009) The U.S.

also ranks first in grapefruit export and export value. The main grapefruit producing states in the U.S. are Florida, California, Texas and Arizona. Over 30% of the world grapefruit production is contributed by Florida. Around 35% of Florida's grapefruit production goes to the fresh fruit market and over 55% goes for juice processing.

Grapefruit Nutrition

Nutritionally, grapefruit is considered a very rich fruit. It is a very rich source of many nutrients and phytochemicals and an excellent source of Vitamin C that supports the immune system and helps fight against colds. The rich pink and red grapefruits get their color from the presence of the pigment 'lycopene', a carotenoid phytonutrient. Lycopene is a powerful antioxidant with a high capacity to fight oxygen free radicals that cause damage to the cells. Grapefruit also have liminoids which help to prevent tumor formation. Compounds called glucarates in the pulp helps in breast cancer prevention. Grapefruit consumption has also shown to reduce cholesterol levels due to the presence of a form of soluble fiber called pectin (Cerda et al., 1988). Being highly nutritious, low in calories, having anti-carcinogenic properties and all the above mentioned health benefits, grapefruit has found a niche in healthy diet plans.

Importance of Seedless Cultivars

Challenges to increase the demand and popularity of grapefruit have led citrus breeding programs to improve the quality and production of grapefruit-type fruits. At present, the major concern for the grapefruit breeders is to develop cultivars which would be resistant to diseases such as citrus canker and to produce cultivars which would be more attractive to more consumers. Today, the trend in consumer preference is towards seedless fruits. This has been best exemplified by the increased demand of seedless watermelons and grapes in the recent past. In citrus, seedless cultivars were

recognized in 1943 by Krug and Bacchi. Seedless fruits are of significance to the juice industry also, as the seeds in the fruit can be associated with unfavorable aromatic compounds and bitterness in the juice. Seediness can be a problem for a variety to be released commercially. In citrus, a number of seedy citrus cultivars could not attain commercial importance although they had all the other desired horticultural traits (Fatta-Del-Bosco et al., 1992). In developed countries, maturity season gaps are filled with seedy cultivars that exhibit other superior traits just because there are not enough seedless cultivars available at that time of the year. Another very desirable trait in grapefruits is its pink or red colored flesh. Out of the three flesh colors available in grapefruits, white grapefruit varieties appear to be least appealing to consumers and fetch lower prices in the commercial markets than those from pigmented grapefruits. This has led grapefruit breeders to devote more effort toward development of colored cultivars. Incorporation of the traits like seedlessness and pink/red flesh color into single cultivars would make it highly desirable in the consumer market. Seedlessness in diploid citrus is related to self incompatibility, male or female sterility, or early embryo abortion (Recupero et al. 2005).

Scientists have investigated different ways to produce triploid plants. Triploid callus lines have been produced by tissue culture techniques (Zhang, 1985), *in vitro* endosperm cultures (Gmitter et al., 1990), selection of spontaneous triploid embryos from 2x X 2x crosses (Ollitrault et al., 2010), inducing seedlessness by induced mutations via gamma irradiation (Hearne, 1984; Hensz, 1977) and by interploid hybridization, using tetraploids as the female (seed) parent (Cameron and Soost, 1969; Esen and Soost, 1972; Cameron and Burnett, 1978; Oiyama and Kobayashi, 1990).

Male or female sterility is generally associated with triploidy (Geraci et al., 1975; Esen et al., 1978). The diploid x diploid crosses with parents having genes for male/female sterility, pollen incompatibility and parthenocarpy are being made to produce seedless citrus fruits in Australia (Sykes and Lewis, 1996; Koltunow et al., 2000). More recently, triploid breeding has proven to be very successful in obtaining the seedless trait in citrus. The process involves either a cross between two diploid parents resulting from the fertilization of 2n megagametophyte (Luro et al., 2004) or via interploidal crosses between a diploid and a tetraploid parent (Esen and Soost, 1973). Elite diploid species selected on the basis of important horticultural traits such as fruit color and size, flavor, 'peelability', maturity time and other quality characteristics are included in the breeding program to produce a quality hybrid (Grosser et al., 1998; Grosser et al., 1992a).The sterility in the triploid seedless progeny is caused by the odd number of chromosomes that impair the normal meiotic division process and results in chromosomally unbalanced gametes (Reforgiato Recupero et al., 2005). In general, triploids can be obtained from crosses between three types of parent combinations – 4x X 2x, 2x X 4x and 2x X 2x. Out of these, 4x X 2x have shown to give maximum triploid recovery (Esen and Soost, 1971; Cameron and Burnett, 1969; Cameron and Burnett, 1978). This high triploid recovery rate is due to the successful fertilization between a diploid female gamete and a haploid male gamete (18n + 9n) (Cameron and Soost, 1969; Cameron and Burnett, 1978), followed by the development of triploid zygotic embryos. The hybrids resulting from 2x X 4x crosses have much poorer triploid recovery rates (Cameron and Soost, 1969; Furusato, 1957; Tachikawa, 1971; Esen et al., 1978). . Seeds from such crosses are normally smaller or shriveled. Many of the seeds from

such crosses were found to be empty, shriveled or poorly developed. An abortion rate of 92-99% was observed for triploid embryos from the 2x X 4x crosses (Esen and Soost, 1973b, 1973c). It was thought that the smaller size and higher abortion rate was due to the early termination of the pentaploid endosperm development which leads to early initiation and termination of the triploid embryo development. Later it was found that a low triploid hybrid recovery rate is due to the 3:5 embryo to endosperm ratio in the seeds which characteristic of non-viability (Esen and Soost, 1973a). The ratio for normal diploid seeds is 2:3. A ratio below this impairs the embryo vialbility (Esen and Soost, 1972a). This problem has been overcome by the application of embryo rescue techniques, and at present triploid recovery from 2x X 4x crosses is quite efficient (Viloria et al., 2005).

Problems in the Grapefruit Market

There are some other factors in grapefruit that limits fruit consumption. Grapefruit contains a flavanoid called naringin, which is the cause of its distinctive bitterness. The bitter taste in the fruit or juice from grapefruit is not necessarily liked by everyone. Despite the cholesterol lowering effects of naringins (Silva et al., 2001), some people especially children do not consume the juice because of the presence of the bitter taste it imparts to the juice, which they would happily consume otherwise. This factor reduces the consumer market to some extent.

Another problem in grapefruit market is its interaction with some drugs when taken together. It is called ‘the grapefruit juice effect’ and it is the result of the presence of furancoumarins like bergamottin and dihydroxybergamottin, as well as naringin. These compounds make some of the pharmaceutical drugs more potent by increasing their bio-availability when they are combined with grapefruit juice. These interactive drugs

include the immunosuppressant cyclosporine and calcium channel blocker drugs, such as felodipine, nifedipine and verapamil (Bailey et al. 1994). Other drugs whose bioavailability is enhanced by grapefruit juice are the antihistamine terfenadine, the hormone estradiol and the antiviral agent saquinavir. The furanocoumarins and naringin interfere with the normal detoxification and metabolism processes in the intestines and liver, which hampers the body's ability to breakdown these drugs and eliminate them. These compounds cause inactivation of enzyme –cytochrome P450 3A4 in the small intestine that is responsible for the metabolism of the statin drugs (Veronese et al. 2003). Another mechanism occurs simultaneously which involves inhibition of P-glycoprotein which is carrier molecule transporting statin drugs back to the gut. Both these mechanisms cause accumulation of these drugs which may result into severe muscle damage or paralysis or even death in certain instances. The drug interactions and its deleterious effects further narrow the consumer market. Improved grapefruit-like cultivars having a lower level of these compounds should have more consumer appeal, especially to the elderly population.

Introduction of Pummelos in Breeding Programs

Considering all the desirable traits, breeders have to face some challenges in breeding cultivars to meet all the consumer expectations. Grapefruits have a comparatively narrow gene pool since it is a group of related cultivars that have arisen through mutation from a single hybrid genotype. This gives little latitude for the breeders to find desirable traits and incorporate them in the commercial cultivars in order to make them more improved and marketable. Grapefruit offers less variation that can be exploited to aid grapefruit improvement programs, as compared to the other citrus types.

Pummelo (*C. grandis*), an ancestor of the grapefruit, can be utilized for its much larger gene pool and is thus involved in the breeding of pummelo x grapefruit hybrids. Cultivars ‘Melogold’ (Soost and Cameron, 1985), Oroblanco (Soost and Cameron, 1980) and ‘Wheeny’ grapefruit are some of the examples of successful pummelo-grapefruit hybrids. The red fleshed pummelo selections/cultivars can serve as excellent breeding parents to produce red fleshed hybrids. Pummelos, being true species, are a good source for adding variation for incorporation into the grapefruit gene pool. Pummelos are monoembryonic and produce zygotic seedlings each of a different genotype, which allows expression to a high degree of phenotypic variations. Grapefruit is polyembryonic and almost all the seedlings are identical to the mother genotype, limiting its variation potential. Many traits like fruit quality, resistance to various pathological and environmental stresses, monoembryony, etc. can be transferred from pummelos to commercial grapefruit cultivars via sexual hybridization. Pummelo being monoembryonic facilitates the breeding process. Usually, in polyembryonic cultivars the hybrid produced by the cross gets suppressed by numbers of vigorous nucellar embryos surrounding it. In general, in order for it to survive, the embryo has to be rescued under sterile condition which requires skill and is a very labor intensive technique (Rangan et al 1978), or biochemical techniques have to be used to distinguish zygotic embryos from nucellar ones (Geraci et al., 1981). However, it depends on percentage of zygotic embryos which vary with genotype. On the contrary, when a monoembryonic tetraploid is used as a seed/female parent in the interploidal crosses, it eliminates the need of embryo rescue, saving time and labor. Each zygotic

embryo gives rise to a single triploid seedling without having to deal with any suppressing effects of nucellar seedlings.

Another advantage of using pummelos is that in general, they have less naringin content in comparison to grapefruits and are thus taste sweeter and less bitter than the latter. A hybrid with one pummelo parent would help dilute the naringin content and help in development of sweeter and more appealing cultivars. Similarly, the lesser furanocoumarin content in pummelos make them ideal to be included as parents in breeding programs as that would help lower the non-desirable furanocoumarins, widening the consumer market for grapefruits. Thus, producing tetraploid parents of such red-fleshed pummelo/grapefruit types would aid the grapefruit improvement program tremendously by providing superior tetraploid breeding parents for use in interploid crosses.

History of Tetraploids in Citrus

Breeders have used the following methods to produce tetraploids:

Somatic Hybridization

Conventional plant breeding in higher plants like citrus is limited to the crosses between phylogenetically related plant species. This is due to problems like sexual incompatibility, heterozygosity, male or female sterility, polyembryony, differences in ploidy levels etc (Grosser and Gmitter, 1990). The prolonged juvenile period makes cultivar development much more time consuming. To overcome these impediments, a technique involving fusion of somatic cells to produce hybrids, known as somatic hybridization, has been developed. Many of these impediments can be overcome to a great extent using this technique (Grosser and Gmitter, 1990). The somatic hybridization is a nonconventional breeding technology which involves fusion of

protoplasts derived from two cultivars which may be distantly to closely related at intraspecific, interspecific, intergeneric, and interfamily levels. The fused protoplasts form heterokayons which undergo several cell divisions and colonize to form an embryo, ultimately growing into a hybrid plant.

Somatic hybridization has diversified the tetraploid gene pool available for hybridization with diploid cultivars. These somatic hybrids when used as parents in interploidal crosses have produced many triploids. The first somatic hybrids, using somatic hybridization were produced from *Nicotinia glauca* and *N. langsdorffii* (Carlson et al., 1972). Since then, a number of hybrids have been produced in various crops via somatic hybridization (Grosser et al., 2000; Johnson and Veilleux, 2001; Orczyk et al., 2003). This technique did not prove to be very successful in most crops but in citrus, steps from protoplast isolation to plant regeneration have become routine (Vardi et al., 1982; Grosser, 1994a; Grosser and Gmitter 1990 & 2005; Grosser et al. 2000).

The allotetraploids produced from protoplast fusions, when used as breeding parents in interploid crosses, produce seedless triploids (Grosser and Gmitter, 2005). In citrus, the first somatic hybridization was done between *C. sinensis* and *Poncirus trifoliata* (Ohgawara et al., 1985).This technique has been widely used in citrus, where its most important application is to produce tetraploid breeding parents and increase genetic diversity in the parental gene pool. During the past two decades, over 300 intergeneric and interspecific somatic hybrids have been produced worldwide (Guo and Deng, 2001; Grosser et al., 2000). Out of these, more than 200 somatic hybrids have been developed at the Citrus Research and Education Center (CREC) in Lake Alfred, FL. At the CREC, a primary objective of these somatic hybrids is to serve as parents for

use in interploidal crosses to produce triploid seedless cultivars. Thousands of triploids have been produced and are now in trials at the CREC.

The increase in ploidy level from diploid to tetraploid has proven beneficial in some cases with respect to the horticultural traits of the somatic hybrids. Some of the somatic hybrids produced and evaluated were found to have improved horticultural performance in respect to pest, disease, nematode resistance and other abiotic stresses (Grosser et al., 1996a; Ollitrault et al., 1998a). Some of the tetraploid hybrids can be directly used as rootstocks as they have good potential for tree size control (Grosser et al., 1995; Grosser and Chandler, 2003), better horticultural performance or improved disease resistance (Louzada et al., 1992).

This technique has been successfully used to develop germplasm from sexually incompatible or difficult to hybridize citrus relatives to expand the citrus germplasm (Grosser et al., 1994; Grosser et al., 1996b; Motomura et al., 1997). A somatic hybrid from the fusion of *C. sinensis* cv. Hamlin (sweet orange) protoplasts isolated from an embryogenic suspension culture with *Severinia disticha* (Philippine box orange) protoplasts isolated from epicotyl-derived callus was the first example of a somatic hybrid produced between sexually incompatible woody species (Grosser et al., 1987).

In seedless scion breeding, the hybrid allotetraploids produced by protoplast fusion can be used as males or females in interploid crosses. The crosses where diploids are used as female parent have some limitations. In early work, the number of triploid hybrids produced was often very low and sometimes triploids remain undetected. It was difficult to isolate them from the nucellar seedlings in polyembyonic cultivars (Esen et al., 1978). The difficulty in recovery of the triploid hybrids due to

embryo abortion was also reported (Esen et al., 1979; Okudai et al., 1981). In such cases, the immature triploid embryos have to be rescued 12 – 15 weeks after pollination and cultured *in vitro* (Satarrantio and Resupero, 1981; Oiyama and Kobayashi, 1990). An alternative and easier way of producing triploid hybrids is using tetraploid hybrids as the female parents in the interploid crosses (Cameron and Burnett, 1978; Esen et al., 1979). Such crosses have high seed set compared to the reciprocal crosses, and triploids obtained are generally more vigorous (Esen and Soost., 1972; Cameron and Burnett., 1978). Another way to produce triploids involving somatic hybridization is by fusing protoplasts isolated from a haploid and a diploid parent (Ollitrault et al., 1998b, 2000a).

Somatic hybridization has been used for the production of hybrids which are better adapted to climatic conditions or have improved disease resistance. Mandarin + pummelo hybrids with potential tolerance/resistance to sting nematode have been produced (Grosser et al., 2007). Mandarin and sweet orange with pummelo fusions have been made to produce hybrids with citrus tristeza virus resistance in efforts to replace sour orange rootstock (Grosser et al., 2007). Somatic hybrids resulting from the fusion of ‘Cleopatra’ mandarin (*C. reticulata* Blanco) with ‘Rangpur’ lime (*C. limonia* L. Osbeck) and with sour orange have potential tolerance against blight and citrus tristeza virus (CTV), as they had combined complimentary characteristics from both parental sources (Mendes et al., 2001).

Spontaneous Altered Ploidy

Polyploidy has a lot of potential to contribute in crop improvement programs (Frost, 1925b, 1926). Polyploidy in citrus such as triploids, tetraploids, pentaploids, hexaploids and octaploids can occur spontaneously in nature. Most cultivars of citrus were found as

spontaneous seedlings. Tetraploid triphasia desert lime (Esen and Soost., 1972), *Clausena excavata* (Froelicher et al., 2000), tetraploid Hongkong wild kumquat (Longley, 1925) and triploid Tahiti lime (Bachi, 1940) are some of the excellent examples of spontaneously occurring polyploidy genotypes.

Naturally occurring polyploids which arise from nucellar embryos are considered to be somatic in origin (Barret and Hutchinson, 1978). Somatic tetraploids occur very commonly in nucellar seedling progenies (Iwamasa, 1966). Such polyploids were classified as autopolyploids (Esen and Soost, 1972b). However in order to avoid the problem in distinction between autopolyploids and allopolyploids, the preferable term used for them is spontaneous polyploids. However, they are few in number, because the diploids generally are more vigorous and provide competition for endosperm nourishment in the seed. The frequencies of polyploids may also depend on certain external environmental factors including temperature and light intensity (Barrett and Hutchinson, 1978).

It is possible to detect ploidy variants on the basis of morphological characteristics for certain cultivars in the past. Tetraploid seedlings typically have thicker leaves than diploids nucellar seedlings progenies (Frost., 1925,1926). Frost found that 2.5% of the cultivated citrus species were tetraploids. Work done in Russia had similar results with 34 tetraploids found from an apomitic population of 1379 seedlings (2.5%) in two cultivated citrus species and *P. trifoliata* (Lapin, 1937). Lapin also reported that maximum percentage of triploids occurring naturally in citrus was observed in the cultivar Kaghzi (15.45%), followed by Foster (14%), Kinnow (11.33%), Musambi (9.33%), and the lowest percentage in cv. Feutrell's Early. These results were later

confirmed for some of these cultivars (Usman et al., 2006). Two spontaneous tetraploids have been obtained in Japan in the *Citrus* species observed (Nakamura, 1942). Tetraploids among nucellar seedlings of *C. aurantium* L. and *C. limon* were reported in Italy (Russo and Torrisi, 1951). In Japan, 7 tetraploids out of 4,196 plants (0.2%) were derived from a citrus hybrid and 2 other citrus species, on the basis of thicker roots and fewer lateral roots in comparison to diploids (Furusato, 1953a). Furusato noticed underdeveloped trees growing on *P. trifoliata* rootstocks in Japanese citrus groves. He determined that 0.7% of the rootstocks to be tetraploid. Later, spontaneous tetraploid seedling percentage among *P. trifoliata* seedlings was determined to be 1.5% (Iwasa and Shiraishi, 1957). Occurrence of tetraploids has also been reported from nucellar seedlings of *Citrus*, *Poncirus* and other hybrids by Barrett and Hutchinson in 1978. Development of zygotic embryos in polyembryonic cultivars is the cause of spontaneous triploid seedlings (Geraci et al., 1975; Wakana et al., 1982). Therefore the frequency of triploids is much less in polyembryonic cultivars as compared to monoebryonic cultivars (Geraci et al., 1977).

Different ploidy levels in a plant would produce distinct morphological characteristics which can be used to identify these polyploids. In many plant species, increased ploidy generally causes reduction in the growth rate (Lindstorm, 1936). In *Citrus*, Frost first reported (1925) distinct appearance and reduced growth as characteristic of a citrus tetraploid. Tetraploids in citrus have distinctive vegetative morphology which can be used to identify them (Frost, 1938; Frost and Krug, 1942, Furusato, 1953a; Tachikawa, 1971; Barrett and Hutchinson, 1978), as well as reproductive morphology (Tachikawa, 1971; Barret and Hutchinson, 1978). Tetraploids

have lower fruit yields and number of seeds per fruit. Shriveled or underdeveloped seeds are found to have a higher ploidy level comparatively in sweet orange and tangor cultivars (Wakana et al., 1982). The underdeveloped characteristic of these seeds may be due to intrinsic capability of the ovule due to polyploidy, number of pollinations or delayed pollination which results in shortage of endosperm required for growth and development of the embryo (Usman et al., 2006). Roots of citrus autotetraploids are found to be shorter and stouter with fewer lateral roots (Furusato, 1953a; Barrett and Hutchinson, 1978). Tetraploids were recently obtained from ‘Hudson’ grapefruit by planting a large population of seeds. The selected seedlings were screened via flow cytometry to confirm the tetraploids (Grosser et al., personal communication). Selection of the seedlings was based on the thicker root morphology, thicker leaves and dark green color and slower growth rate. The quality of fruit from autotetraploids is generally poorer in comparison to the diploid counterparts and is usually not used for commercial purposes. Tetraploids produce a smaller tree size and vigour than diploids (Frost, 1938; Frost and Krug, 1942; Barret and Hutchinson, 1978; Lee, 1988). Also, tetraploids used as rootstocks impart dwarfing to the normal scion cultivars grafted on them (Russo and Torrisi, 1951; Furusato, 1953b). Variability in the vigor between diploid and tetraploid rootstocks was noticed by Mukherjee and Cameron (1958). The dwarfing effect of these tetraploid rootstocks is beneficial for the growers as dwarf trees lower the cost of pruning and harvesting.

Colchicine Induced Polyploidy

Since there is a limited number of tetraploids available in citrus, representing a minor section of the total gene pool available (Barrett, 1974), different methods have been employed to induce increased ploidy levels, ultimately aiming towards triploidy.

Doubling the chromosome number of the plant itself has been a very successful approach to produce autotetraploids. Autotetraploids serve as important breeding parents in inteploidal crosses. However, the process to obtain autotetraploids from the respective diploids has been quite challenging. Various techniques and chemicals have been tried over the past, each attempting to improve the ease, safety and efficiency of the method. Treatment of plant parts with compounds which have potential to cause chromosome doubling, like colchicine, trifluralin, oryzalin, 2,4-dichlorophenoxyacetic acid and amiprophosmethyl (AMP) have resulted in elevated levels of ploidy in various plant species in the past. These compounds inhibit the spindle formation during mitosis and interfere with normal polar segregation of sister chromatids to form a restitution nucleus (Blakeslee and Avery, 1937). As a result, duplicate chromosomes stay in one cell, doubling the ploidy of the cell.

Colchicine is the most commonly used chemical for doubling the chromosomes in plants. However, other chemicals have also been tried but fail to produce tetraploids with the same efficiency as colchicine. Colchicine is an alkaloid derived from meadow saffron (*Colchicum autumnale* L.). It has been used successfully for doubling of chromosomes in fruit crops like banana (Hamill et al., 1992), cherry (James et al., 1987), grapes (Notsuka et al., 2000) and blueberry (Lyrene and Perry, 1982). Some success has been seen with oryzalin on lily, nerine and apples (Van Tuyl et al., 1992; Bouvier et al., 1994). In citrus, oryzalin has been used in efforts to produce autotetraploids by treating the embyogenic callus of the citrus cultivars such as 'Umatilla' and 'Dweet' tangors (*C. reticulata* Blanco x *C. sinensis*), 'Caffin' Clementine (*C. clementina* Hort ex Tan.) and 'Wheeny' grapefruit (*C. paradisi* Macf) (Wu and

Mooney., 2002). Unfortunately, oryzalin did not produce any autotetraploids because it is more toxic than colchicine, and probably should be used at a lesser concentration. However, colchicine was successfully used to produce autotetraploids in the same cultivars. Nucellar callus cultures of lemon cultivars were successfully induced to polyploidy when treated with 2,4-dichlorophenoxyacetic acid (Vardi., 1982). The use of chemicals other than colchicine for inducing polyploidy has remained limited because of their comparatively lower efficiency.

There are two important factors that determine the effectiveness of these mutagenic agents. These factors are the concentrations in which they are applied and the duration for which the plant part to be mutated is exposed to the chemicals. Excessive concentration and exposure causes the death or necrosis of the exposed tissue due to the toxic effect. Initially, lower concentrations with longer exposure periods in vitro were tried to produce autotetraploids (Gmitter et al., 1990; Gmitter and Ling, 1991). More recently, autotetraploids have been produced in sweet orange (*Citrus sinensis*) at a higher frequency using higher concentrations with shorter exposure periods (Zhang et al., 2007). Zhang suggested an efficient method to produce a high frequency of autotetraploids by first determining the peak period of division in callus in terms of days after subculturing, and then administering the treatment at that peak period.

Colchicine treatments have been tried on various plant parts. In citrus, Frost and Lapin (1937) have obtained spontaneous tetraploids from several varieties. Two tetraploid citrus clones were obtained by colchicine treatments (Tachikawa et al., 1961). Citrus embryogenic callus has been a very common and successful target to initiate chromosome doubling in the cells, followed by regeneration of plants with doubled

chromosome number (Wu and Mooney, 2002; Zhang et al., 2007). Treatments on cell suspension cultures have regenerated complete tetraploid plants in ‘Ponkan’ mandarin (Dutt et al., 2010). Cell suspension cultures have increased chances of producing stable, nonchimeric tetraploid plants as the embryos originate from single cells (Stewart et al., 1958). Callus or cell suspensions regenerate complete autotetraploids more efficiently as the subsequent plant regeneration is from single cell and a single mutated cell is able to produce a complete tetraploid plant. Treatment of callus from monoembryonic cultivars with colchicine has produced autotetraploid tangor plants (Wu and Mooney, 2002). However, the frequency of autotetraploids obtained was quite low, and in general, it is extremely difficult to produce embryogenic callus from monoembryonic genotypes. Non-chimeric autotetraploids have been produced successfully via treatment of underdeveloped ovules from the immature fruits of 'Orlando' tangelo and 'Valencia' sweet orange with colchicine (Gmitter and Ling, 1991).

Polyploids have also been recovered by treating the axillary buds and shoot tips with colchicine under sterile conditions followed by an in vitro micrografting technique (Juarez et al., 2004; Yahata et al., 2005; Oiyama, 1992; Oiyama and Okudai, 1986). However, treating the buds or shoot tips often produce plants composed of tissues with varying chromosome numbers that are called cytochimeras (Barrett, 1974; Jaskani et al., 1996). These chimeras are mostly unstable and sterile, and do not have any application in breeding programs. Many of these chimeras revert back to their original ploidy level because of their instability. These chimeras can be of different types and can be classified on the basis of the histogenic layers (LI, LII or LIII) mutated. A chimera can be periclinal (one mutated layer), mericlinal (part of the apical dome mutated) and

sectorial (section of the apical meristem mutated). The cause of these chimeras from the treated bud is basically the fact that the buds are muticellular. In muticellular tissue, colchicine may not be able to affect all the cells and the unaffected cells remain diploid. When such type of source is used as explants for colchicine treatments, a large proportion of chimeric tetraploids are produced (Kadota and Niimi, 2002).

Treatment of shoot tips *in vitro* to induce tetraploids is very common in monoembryonic citrus cultivars as production of callus lines, used for treatment otherwise, is very difficult. Stable tetraploid plants have been produced in the monoembryonic cultivar ‘Clementine’ by in vitro shoot tip grafting (Juarez et al., 2004).

Efforts have been made to produce tetraploids from monoembryonic cultivars by techniques *ex vitro*. Barrett (1974) tried treatment of axillary buds of monoembryonic diploid clones with 1.0% colchicine solution *ex vitro*. He could not produce a complete and stable tetraploid but he induced eight types of cytochimeras based on the three histogenic layer mutations. He used large plants for his experiment; however, in large, broadly differentiated meristems, it is very difficult to affect the entire tissue to produce stable tetraploids (Sanford, 1983). Later, smaller explants – young axillary buds were used for colchicine treatments which were then micrografted onto the vigorous rootstocks to produce complete autotetraploid plants (Oiyama and Okudai, 1986). There are no reports of artificially producted autotetraploid plants from monoembryonic pummelos. New autotetraploid pummelo cultivars are needed which can be hybridized with grapefruit cultivars to increase genetic diversity for grapefruit cultivar improvement.

Objectives

The overall objective of this research project is the generation of red fleshed grapefruit/pummelo tetraploid parents with various fruit quality or disease resistance

characteristics to facilitate the grapefruit improvement breeding program. In order to achieve this objective various approaches were applied which are as follows.

- Somatic hybridization to produce allotetraploids.
- Screening for naturally occurring triploids and tetraploids.
- Induction of tetraploidy by the mutagen ‘colchicine’- *in vitro* (pregerminated seeds, stem segments) and *in vivo* (axillary buds).

CHAPTER 2
INDUCTION OF AUTOTETRAPLOIDS IN PUMMELO (CITRUS GRANDIS) THROUGH COLCHICINE TREATMENT OF MERISTEMATICALLY ACTIVE SEEDS IN VITRO

Introduction

Grapefruit (*Citrus paradisi*), which originated in Barbados, is cultivated worldwide and is among the most popular fresh citrus cultivars. The United States followed by China and South Africa are the biggest producers. Grapefruit's growing demand and popularity exerts pressure on breeders to come up with improved cultivars with traits to meet consumer's expectations such as dark red flesh color and seedlessness.

Excessive number of seeds in citrus makes it unappealing to the consumers and unacceptable for local and international markets. Breeders worldwide are increasing efforts to come up with quality seedless cultivars. Since, triploidy is associated with sterility, seedlessness has been successfully achieved in the past in many cultivars through ploidy manipulations. Several approaches from traditional hybridization to molecular biotechnology have been applied in order to induce seedless fruits in citrus. These include selection of spontaneous triploids from a natural population (Geraci et al., 1975; Wakana et al., 1981) or those from 2x X 2x crosses (Esen and Soost, 1971; Esen et al.,1978), somatic variation (Deng et al., 1985), endosperm culture (Wang and Chang, 1978; Gmitter et al., 1990; Chen et al., 1991), somatic hybridization between a diploid and a haploid (Kobayashi et al., 1997); genetic transformation (Koltunow et al., 1998), and interploidal hybridization between a diploid and a tetraploid parent (Esen and Soost, 1972). Out of these, the interploidy hybridization has been the most effective and is commonly used approach to produce seedless triploids. However, scarcity of tetraploids in the citrus gene pool has given rise to the need to induce tetraploidy in breeding lines that could be used as parental material for the interploidal crosses.

Crosses where the tetraploid is used as the female parent to produce triploids are more efficient because of the normal sexual fertilization between a diploid female gamete and a haploid male gamete (18 + 9) (Esen and Soost, 1972; Soost and Cameron, 1975; Cameron and Burnett, 1978). In such crosses, when tetraploid polyembryonic cultivars are used as a female parent, there arises a need to rescue the embryo via tissue culture techniques. This is to avoid the suppression and subsequent abortion of the zygotic hybrid embryo by the more vigorous nucellar seedlings. Though the identification and selection of the zygotic seedling is possible through morphological, isozyme, histochemical, cytological and molecular techniques, it becomes a costly, labor intensive and time consuming process. Use of a tetraploid monoembryonic female parent instead eliminates the need for embryo rescue making the process much simpler and efficient.

Considering the present market demands, scarcity of superior tetraploid parents in grapefruit and factors slowing triploid breeding, pummelos are beginning to be included as germplasm sources in grapefruit breeding programs. Pummelo, being the ancestor of grapefruit and monoembryonic, has a comparatively greater diversity in its gene pool which makes it an ideal candidate to contribute towards grapefruit improvement. Pummelo's lower naringin and furanocoumarin content could help balance out higher contents present in grapefruits. Use of such red fleshed tetraploid pummelo types as a female parent in the interploidal crosses could produce hybrids which are more appealing and consumer friendly.

Colchicine, an alkaloid obtained from meadow saffron (*Colchicum autumnale*), is a mitotic inhibitor (Blakeslee and Avery, 1937) and is commonly used to induce tetraploidy

in breeding lines in *Citrus*. Barrett (1974) attempted to produce autotetraploids in monoembryonic cultivars by treating the axillary buds with colchicine *in vivo*. However he was not able to produce any stable tetraploid plants. The most probable explanation for this was that he used bigger plants and it is difficult to double the chromosomes in large and broadly differentiated meristems (Sanford, 1983). Stable autotetraploids in monoembryonic cultivars have been produced by cochicine treatment of the axillary buds (Oiyama and Okudi, 1986), shoot tips (Juarez et al., 2004) and somatic embryogenic callus (Wu and Mooney, 2002). Lehrer et al (2008) induced tetraploidy by treating germinated seeds of Japanese barberry (*Berberis thunbergii var. atropurpurea*) with colchicine and oryzalin *in vivo*. This technique has also been used to produce tetraploids in ornamental plants *Syringa spp*. L. (*lilac*) (Fiala 1988), *Rhododendron spp*. L. (*rhododendron*) (Leach, 1961), *Iris spp.* L. (*iris*) (McEwen, 1990). However, there is no report of tetraploid induction from seed treatment in *Citrus*. Also, there has not been any report on tetraploid induction in pummelo cultivars to date. This study reports an efficient method for induction of tetraploids by treatment of germinating seeds from elite pummelo selections with colchicine *in vitro*. It compares the effect of different colchicine concentrations and exposure durations of seedling growth and developmen as well as tetraploid induction.

Materials and Methods

Plant Materials

Seeds were extracted in 2008-2009 from the fruits of red fleshed pummelo selections 5-1-99-2, C2-5-12 and UKP-1 located at the Citrus Research and Education Center (CREC, Lake Alfred). All selections were open-pollinated seedlings of ‘Hirado Buntan’ pink pummelo selected for high fruit quality. The extracted seeds were washed

under running water 3-4 times and were treated with 0.6% (v/v) sodium hydroxide for 8 minutes to remove slime. The seeds were dried, peeled and sterilized using 10% Clorox bleach before they were placed on seed germination medium under sterile conditions. The seeds were placed on this medium for 12-14 days to germinate and for hypocotyl, about 5-8 mm in length, to emerge out of the cotyledons. At this point, the seeds were undergoing high meristematic activity and were ready for treatment.

Colchicine Treatments

The experiment design was a two way factorial consisting of 3 colchicine concentrations and 2 exposure periods. There were three replications of each treatment. The Colchicine stock solution was prepared by dissolving colchicine in dimethylsulfoxide (DMSO) at a final concentration of 1g/ml. This solution was filter sterilized. Per treatment, 15 pre-germinated seeds were placed in conical Falcon tubes containing 10 ml of liquid seed germination media with final colchicine concentrations of 1, 2 and 3g/L. Seeds immersed in liquid seed germination media without colchicine were used as controls. The seeds exposed to different concentrations of colchicine were exposed for periods of 12 or 24 hours each. Flasks were put on the rotary shaker at 30 rpm for the respective exposure periods to facilitate the contact and penetration of colchicine in the meristems, which were covered by the cotyledons. The rotary shaker was contained in a dark chamber maintained at 25 ± 2 °C. After the each treatment's respective exposure time, the seeds were taken out and placed on solid seed germination media and placed under dark conditions to facilitate seedling elongation. The seedlings were transferred to rooting media supplemented with naphthalene acetic acid (NAA) after about 2 weeks, and were placed under 16 h light / 8 h dark conditions

for further growth. Emergent seedlings were analyzed for their ploidy via flow cytometry at a stage when the seedling had at least 3 fully expanded leaves. The seedlings confirmed to be tetraploid were micrografted on to vigorous trifoliate rootstock seedlings. The micrografted tetraploids were put under shade for 10-14 days prior to moving them to the greenhouse with set points of 21-17°C day/night temperatures.

Ploidy Analysis

Ploidy was analyzed using a tabletop Ploidy Analyser flow cytometer (Partec GmbH, Germany). This technique makes it possible to analyze 150-200 genotypes per day. Flow cytometry works by estimating the volume and florescence of isolated nuclei. The ploidy is presented in form of a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample.

The protocol is a series of steps starting with excision of a 0.2 – 0.3 cm^2 piece of fully expanded leaf tissue and placed in a 60X15mm plastic Petri dish. Each sample was chopped with a sharp razor blade after adding few drops of Nuclei Extraction Buffer. After chopping, 6-7 more drops of Nuclei Extraction Buffer were added and the sample was filtered through a 50µm filter into a 3.5 mL (55mmX12mm) Sarstedt tube. The fluorescent dye – 4',6-Diaminidino-2-phenylindole (DAPI) was added drop by drop through the filter to infiltrate the remaining cells, until the half of the tube was filled. Each sample was incubated for 10-15 seconds at room temperature before running it on the flow cytometer. The sample moves as a very narrow, laminar flowing sample stream through the flow cuvette. When the cells labeled with fluorescent dye pass through the measuring area one after the other, they get illuminated by the excitation light of a defined wavelength. The light activates the fluorescent molecules so that they emit light

back. The fluorescent light intensity is proportional to the DNA content in labled cells depicts the respective number of chromosomes and hence the ploidy level of the sample.

Results and Discussion

The colchicine treatment induced tetraploidy in seedlings from all three pummelo selections. However, the frequency of tetraploids varied among treatments. Some treatments also produced cytogenic chimeric plants having tetraploid and diploid nuclei in varying proportions of cells. Chimeric plants have been recovered in similar *in vitro* studies conducted by Wu and Mooney (2002); Kodota and Niimi (2002). Data was assessed by calculating the survival rate of the seedlings and tetraploid induction efficiency (TIE) for each treatment. Tetraploid induction efficiency was computed by the formula given below by Bouvier et al (1994).

TIE = % Seedling survival X % tetraploid seedlings

The most important factors that determine the tetraploid induction efficiency are colchicine concentration and the exposure period for which seeds were exposed to colchicine. Higher colchicine concentrations and longer duration period hampers seedling growth, causes hyperploidy, browning, necrosis in the meristematic tissue and death of the seedling (Sanford, 1983). In this study, all colchicine treatments greatly decreased the growth rate of the treated seedlings from all three selections. Five weeks after the treatment, the control counterparts grew up to a height ranging from 5 to16 cm long whereas all the surviving treated seedlings remained stunted with a height no more than 2.5 cm. Average heights of the seedlings at x days after colchicine treatments were 12.4, 13.5 and 12.8 cm for selections 5-1-99-2, UKP1 and C2-5-12 ,respectively (Table 2-1).

Table 2-1. Average heights of seedlings recovered from treatments with various colchicine concentrations and exposure periods.

Treatment	5-1-99-2	UKP-1	C2-5-12
Control	12.4 ± 2.6	13.5 ± 2.21	12.8 ± 2.67
0.1% X 12 hours	1.2 ± 0.63	1.6 ± 0.5	1.5 ± 0.4
0.1% X 24 hours	1.2 ± 0.56	1.3 ± 0.51	1 ± 0.95
0.2% X 12 hours	0.9 ± 0.5	1.4 ± 0.35	1.2 ± 0.38
0.2% X 24 hours	1.1 ± 0.56	1.3 ± 0.6	1.1 ± 0.4
0.3% X 12 hours	0.5 ± 0.1	1.8 ± 0.64	X
0.3% X 24 hours	1.1 ± 0	13.5 ± 2.21	1.6 ± 0

Mean separation (in columns) by Duncan's multiple range test, 95% level

High mortality was observed in all the treatments. Overall seedling survival including all treatments was highest for selection 5-1-22-9 which had 30% of seedling survival rate followed by C2-5-12 having 25.6% and UKP1 with 21.1% (Table 2-2). The difference in the survival rates among different selections may be attributed to genotype effect or just random variation. The control treatments without any colchicine exposure had 93 to 100% seedling survival rates. Higher mortality rate in the treated seedlings was due to the toxicity of cochicine. This explains decreasing seedling survival when cochicine concentrations were increased or when seeds were given longer exposures to the chemical. At the lowest concentration of cochicine (0.1%), the surviving seedling percentage was around 50%. The survival rate dropped to 0 to 20% at 0.3% colchicine concentration. The seedling survival rate dropped to almost half when the exposure period was increased from 12 hours to 24 hours for every concentration. These results indicate that the seedling survival rate is inversely proportional to the concentration and exposure periods of colchicine.

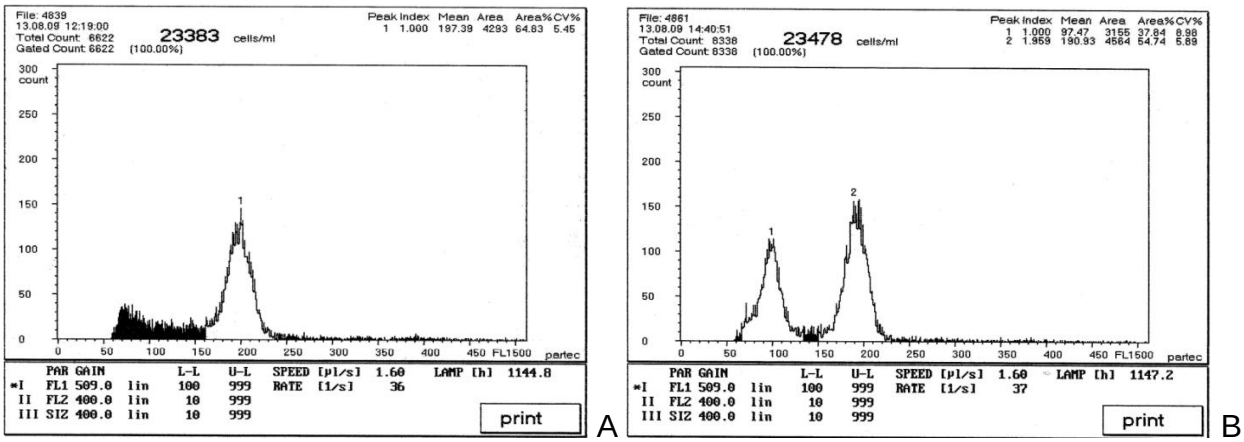


Figure 2-1. Flow cytometry histograms representing seedlings from selection UKP-1. A) tetraploid profile. B) mixoploid profile.

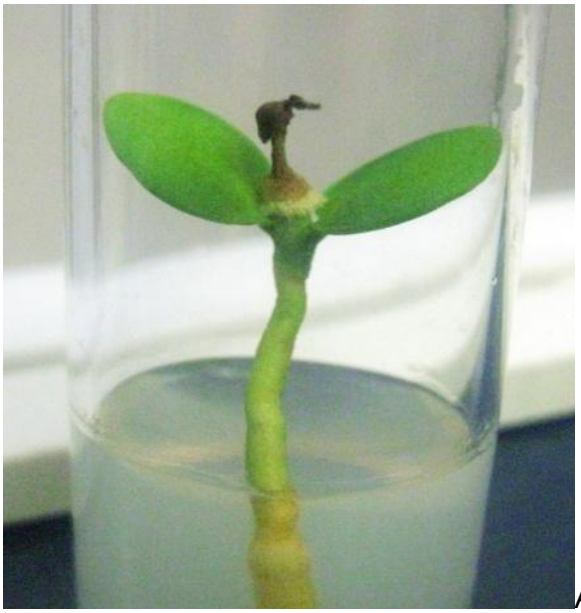
Figure 2-1 shows the histograms obtained from the ploidy analyzer for a non-chimeric tetraploid and chimeric samples. The most effective concentration at which the tetraploids were regenerated was 0.1%. This in agreement with Oiyama and Okudai (1986), who reported that 0.1% of colchicine was the best concentration for tetraploid induction in shoot tips in citrus. Most of the tetraploids produced were derived from the treatments with 0.1% colchicine concentration. Concentration of 0.2% at 12 hours of exposure period also produced 2 stable tetraploids, 1 each for selections 5-1-99-2 and C2-5-12, respectively. Table 2-2 lists number of tetraploids and mixoploids obtained from each treatment as well as the corresponding tetraploid induction efficiencies. There were a total of 5 tetraploids regenerated from the 3 selections. Different selections generated varying numbers of tetraploids and mixoploids. Three tetraploids were produced in selection UKP1, two in 5-1-99-2 and one in C2-5-12. There were also three mixoploids obtained from UKP1, one from 5-1-99-2 and two from C2-5-12. Mixoploids are commonly found when the targeted tissue is muticellular. In such cases, a few cells are mutagenized and the others remain diploid. When these partially mutated

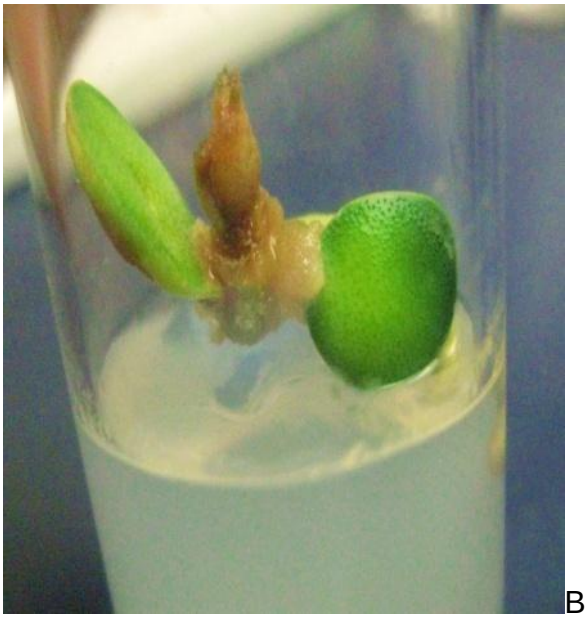
meristems differentiate to form plant organs, a mixture of tetraploid and diploid tissue is observed.

Table 2-2. Survival rate, ploidy and tetraploid induction efficiency from treatments with various colchicine concentrations and exposure periods from selections 5-1-99-2, UKP1 and C2-5-12 of pummelo (*Citrus grandis*)

Treatment	Seedling survival (%)	Mixoploid Number	Tetraploid Number	Tetraploid Induction Efficiency
5-1-99-2				
Control	93	-	-	-
0.1% X 12 hours	53	1	1	35
0.1% X 24 hours	47	0	0	0
0.2% X 12 hours	27	0	1	18
0.2% X 24 hours	20	0	0	0
0.3% X 12 hours	20	0	0	0
0.3% X 24 hours	7	0	0	0
UKP-1				
Control	100	-	-	-
0.1% X 12 hours	50	2	2	67
0.1% X 24 hours	44	1	0	0
0.2% X 12 hours	31	0	1	21
0.2% X 24 hours	19	1	0	0
0.3% X 12 hours	13	0	0	0
0.3% X 24 hours	6	0	0	0
C2-5-12				
Control	100	-	-	-
0.1% X 12 hours	53	1	0	0
0.1% X 24 hours	40	0	1	27
0.2% X 12 hours	33	1	0	0
0.2% X 24 hours	20	0	0	0
0.3% X 12 hours	0	0	0	0
0.3% X 24 hours	7	0	0	0

Tetraploid induction efficiency is a good measure to find out the most effective treatment as it takes into account both seedling survival rate and number of tetraploids produced. The highest TIE of 67% was obtained from treatment with 0.1% concentration of colchicine and 12 hour exposure period in selection UKP1.


A


B


C

Figure 2-2. Dried and successfully recovered seedlings. A) Browning of the epicotyl before the leaves emerged. B) Necrosis of the seedling and subsequent death after the emergence of the leaves. C) A stable tetraploid confirmed by flow cytometry micrografted onto a vigorous rootstock.

Treatments with higher concentrations – all with 0.3% and most with 0.2% and some of 0.1% were lethal. The seedlings showed necrosis with subsequent death even before the new flush emerged (Figure 2-2. A). Some of the surviving seedlings from

concentrations of 0.2 and 0.3% showed mixed hyperploidy. Such plants were unstable and ceased to grow after a certain point of time. One tetraploid obtained from selection UKP1 and a mixoploid from 5-1-99-2 reverted back to diploid after 8 weeks. The reversions of the tetraploids into diploids show that they were not stable over time. The tetraploids produced need to be tested at multiple time intervals to confirm their stability. The stable tetraploid plants confirmed by flow cytometry were micrografted onto vigorous trifoliate rootstocks for further growth and were transferred to the greenhouse (Figure 2-2C).

Conclusion

The tetraploids from elite monoembryonic pummelo selections selected for their red flesh and superior quality may be of significant value in grapefruit triploid breeding programs. In this study, a method to induce tetraploidy in seedlings by treating pre-germinated seeds with colchicine at various concentrations and exposure periods is described. Stable tetraploids were successfully produced in all three selections and were confirmed by flow cytometry. This method facilitates treatment of large number of seeds at the same time considering safety concerns when working with colchicine as opposed to the shoot tip grafting where individual shoot tips that have to be treated separately.These tetraploids are potential female parents in interploidy crosses for triploid breeding and will be used to produce red fleshed seedless pummelo/grapefruit types.

CHAPTER 3
SELECTION OF ALTERED PLOIDY LEVELS FROM NATURAL POPULATIONS OF MONOEMBRYONIC PUMMELOS (CITRUS GRANDIS)

Introduction

Seedlessness is one of the most important commercial traits in citrus cultivars. Seedlessness is associated with triploidy. Triploid breeding programs attempt to incorporate seedlessness trait into commercial cultivars by natural and artificial ploidy manipulations. Seedless cultivars have been successfully produced by crosses between diploid and tetraploid parents. These days, seedlessness is becoming popular and seedless cultivars have been produced in many fruit crops like grapes, watermelon, banana and citrus. In citrus, because grapefruit is very popular for its taste and nutritional benefits, there is a demand for improved seedless cultivars for fresh fruit in commercial grapefruit markets. Even in the juice industry, seedy cultivars are not desirable as they are associated with unfavorable aroma and bitterness. However, production of triploids has been limited by the scarcity of suitable tetraploid parents in the grapefruit gene pool, which could be used as parents in interploidal crosses.

Polyploids can occur spontaneously in nature. The ‘Hong Kong’ kumquat is the first naturally occurring tetraploid to be discovered (Longley, 1925). Since then many tetraploids have been selected and identified from natural populations in different citrus species (Frost, 1943; Lapin. 1937; Nakamura, 1942; Russi and Torrisi, 1951). However the frequency of naturally occurring tetraploids varies with the cultivar and species. In general, tetraploidy ranges from <1 to 6%. Spontaneous tetraploids have also been found in citrus relatives including *Poncirus trifoliata* (Lapin, 1937). In grapefruits, tetraploids were recently obtained by selecting seedlings of ‘Hudson’ grapefruit on the basis of thicker roots, thicker and darker leaves, and slower growth rate, followed by

confirmation of tetraploidy by flow cytometry (Grosser et al, personal communication). In polyembryonic genotypes, tetraploidization seems to occur more frequently as a result of chromosome doubling in the nucellar tissue (Cameron and Frost, 1968). There has not been any report of naturally occurring tetraploids in monoembryonic cultivars. Monoembryonic tetraploids are very significant in interploidal crosses when used as female parents, as they eliminate the need to rescue the hybrid embryo, saving time and labor. Pummelo, being monoembryonic and an ancestor of grapefruit is of great interest to grapefruit breeders who are trying to incorporate the diversity from pummelos into improved grapefruit-type hybrids.

Triploids occur with comparatively higher frequencies in citrus cultivars. Triploids with frequencies of 15.45%, 14%, 11.33% and 9.33% were found in Kaghzi, Foster, Kinnow and Musambi respectively (Lapin, 1937; Usman et al., 2006). The smaller and abnormal seeds in monoembryonic cultivars selected from 2x X 2x crosses have higher probability for triploidy (Esen and Soost, 1977; Wakana et al., 1981; Soost, 1987). The triploidy is such seeds originate from 2n megagametophytes fertilized by 1n (Esen and Soost, 1971, 1973; Geraci et al., 1975).

Although the tetraploids and triploids can be identified with cytogenic, biochemical and molecular techniques, the screening of large populations is difficult, time consuming, expensive and inefficient. Selection on the basis of ploidy characteristics like thicker roots and leaves, darker color, and slow growth rate reduces the number of plants to be screened. This avoids screening of major portion of population with low probability of being tetraploid. Screening of the selected seedlings via flow cytometry is

an efficient way to confirm specific ploidy level. In this study, the natural populations of five pummelo selections were screened for triploids and tetraploids.

Materials and Methods

Plant Materials

Five pummelo selections 5-1-99-2, HBJL-9, HBJL-7, 8-1-99-2 and 5-1-99-3 located at the Citrus Research and Education Center (CREC in Lake Alfred, FL). All are from open-pollinated seedlings of ‘Hirado Buntan’ pink pummelo (except 8-1-99-2 which is from ‘Ling Ping Yau seedling’ pummelo) selected on the basis of their superior quality and red flesh color. The seeds were extracted from the mature fruit from these selections in 2008-2009. The extracted seeds were washed under running water 3-4 times and were treated with 0.6% (v/v) sodium hydroxide for 8 minutes to remove pectin and parts of flesh attached to the seeds. The seeds from each selection were dried and stored separately at 8ºC until used.

Methodology

The study was divided into 2 experiments. The first experiment involved finding natural triploid embryos in smaller seeds. Small or abnormal seeds were selected from each selection. From selections 5-1-99-2, HBJL-9, HBJL-7, 8-1-99-2 and 5-1-99-3; the number of smaller/abnormal seeds selected were 11, 21, 26 and 17 respectively. The seeds were dried, peeled and sterilized using 10% Clorox bleach and 1-2 drops of Tween 20 for 8 minutes followed by 3-4 rinses with sterile water. The sterile seeds were dissected aseptically under a stereomicroscope with 100 X magnification to extract the embryo. Excised embryos were cultured on EME nutrient medium (Grosser and Gmitter, 1990) and incubated at 25 ± 2ºC under continuous light conditions.

Figure 3-1. Pummelo seedlings planted in 11” x 22” flat trays placed in the greenhouse

In the second experiment, the objective was to identify tetraploids from natural populations based on morphological markers. The normal seeds extracted from each selection, were planted in 11” x 22” flat trays filled with commercial potting mix (Figure 3-1). The trays were placed in a greenhouse at 28ºC and 80% relative humidity. Irrigation was given three times a week or as required. After, 8-10 weeks of growth of seedlings in the greenhouse, the seedlings were pulled out and were screened for potential tetraploids. The selection of the seedlings was done on the basis of thicker roots, slow growth rate and relatively dark color of the leaves. The seedlings were uprooted and roots were washed with running tap water prior to examination. For selection 5-1-99-2, 117 out of 470 seedlings were chosed for screening via flow

cytometry; for HBJL-9, 129 out of 837; for HBJL-7, 143 out of 682; for 8-1-99-2, 92 out of 422; and for 5-1-99-3, 130 out of 725.

Ploidy Analysis

Ploidy level of the selected seedlings was analyzed using a flow cytometer. This technique makes it possible to analyze 150-200 genotypes per day. Flow cytometry works by estimating the volume and florescence of isolated nuclei. The ploidy is represented by a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample.

The protocol is a series of steps starting with excision of a 0.05 – 0.1 cm^2 piece of fully expanded leaf tissue. Samples of 3-4 leaf pieces were pooled in a 50mm plastic Petri dish and chopped with a sharp razor blade after adding few drops of nuclei extraction buffer. After chopping, 6-7 more drops of nuclei extraction buffer were added and the sample was filtered through a 50µm filter into a 3.5 mL (55mmX12mm) Sarstedt tube. The nuclei staining buffer was added drop by drop through the filter to infiltrate the remaining cells, until half of the tube was filled. Each sample was incubated for 10-15 seconds at room temperature before running it through the flow cytometer. Observation of two different peaks from a pooled sample represented those samples with altered ploidy.

Results and Discussion

In the first experiment, embryos rescued from the smaller seeds of all the selections gave all diploid plants as confirmed by flow cytometry. Very few embryos were found in abnormal and shriveled seeds. In the shriveled and abnormal seeds, the embryos if present were brown in color and smaller as compared to the normal ones. These embryos when put on the EME nutrient media, failed to germinate and ultimately

died. Smaller seeds of monoembryonic cultivars like ‘Temple’, ‘Wilking’ and ‘Fortune’ showed high rates of diploid megagametophytes which resulted in formation of triploid embryos (Esen and Soost, 1977; Wakana et al., 1981; Soost, 1987). However, these studies were done with tangors and mandarins. There are no reports of such study in Pummelo. Difference in species of citrus probably is the reason these results differ from the previous findings. The rates of 2n gametophytes production varies with species and is probably lower for pummelo.

In the second screening experiment, no tetraploids were found in any of the seedling populations. However, a triploid seedling was found from selection HBJL-7. The described morphological characteristics are not specific to tetraploid identification, but potentially increase the efficiency of identifying actual tetraploids following flow cytometry analysis.

In polyembryonic genotypes, tetraploidization occurs more frequently as a result of doubling of chromosomes in the nucellar tissue. The same seed can contain a diploid seedling from a zygotic embryo and one or more tetraploid seedlings from the nucellar tissue surrounding that embryo. There is a low probability that a zygotic embryo will produce a monoembryonic genotypes, since no nucellar tissue is present, the chances of tetraploidization of the zygotic embryo is extremely low. For this to happen, an unreduced megagametophyte would need to be fertilized by diploid pollen. Rates of unreduced megagametophytes vary with genotype. Even if they are present at high rates, it would be an extremely unlikely that diploid pollen from a tetraploid or a diploid would be able to fertilize an unreduced gametophyte considering the competition the diploid pollen has with haploid pollen. Also, under natural conditions, tetraploid plants

which must act as the pollen donors in this case are not growing commercially as scion cultivars. Thus, the availability of such 2n tetraploid pollen would be very limited.

The triploid found from the cultivar HBJL-7 is likely the result of haploid pollen from a diploid plant fertilizing an unreduced megagametophyte. The frequency of such events would depend on the rates at which unreduced gametophytes are produced in a genotype.

Conclusion

The monoembryonic pummelo can rarely produce naturally occurring triploids. However, the frequency varies with genotype. Monoembryony limits the occurrence of tetraploids in the natural populations in absence of a triploid block. Hence, Screening for tetraploids in natural populations is fruitful only for polyembryonic genotypes in citrus.

CHAPTER 4
PRODUCTION OF COLCHICINE INDUCED AUTOTETRAPLOIDS IN PUMMELO (*CITRUS GRANDIS*) VIA INDIRECT ORGANOGENESIS

Introduction

Grapefruit (*Citrus paradisi*) is an important crop in Florida and is grown for fresh fruit and juice. Florida contributes almost 30% to the world grapefruit production. Grapefruit is believed to have originated in Barbados as an accidental cross between a pummelo (*C. grandis*) and a sweet orange (*C. sinensis*) (Scora, 1975; Barrett and Rhodes., 1976; Scora et al., 1982; Gmitter, 1995). The increasing competition and popularity of grapefruit in international markets have stimulated grapefruit breeders to develop new varieties which can meet consumer's demands.

Grapefruit vary in flesh color with white, pink and red cultivars being available. The pink and red fleshed varieties are most popular and earn higher prices in the markets compared to the white ones. Another highly desired trait in commercial grapefruit cultivars these days is seedlessness. Seedless varieties of fruits like banana, watermelon, grapes, plantain are available in the market and sell more than their seedy counterparts. Seedlessness has gained importance in citrus, in the recent past. Seediness is causing problems in the acceptance of the fruit in the local or international markets and can even act as a barrier towards release of a variety. Breeders worldwide are trying to generate seedless cultivars with improved quality and disease resistance.

Seedlessness has been achieved in the past through approaches ranging from traditional hybridization to biotechnology. The seedlessness has been long associated with triploidy. Seedless triploids have been resulted from selection of spontaneously occurring triploids in natural populations (Geraci et al., 1975; Wakana et al., 1981), from somaclonal variation (Deng et al., 1985), from diploid X diploid crosses (Esen and

Soost, 1971; Geraci et al., 1977; Geraci, 1978), somatic hybridization between a haploid and a diploid (Kobayshi et al., 1997), from endosperm culture (Wang and Chang, 1978; Gmitter et al., 1990; Chen et al., 1991), from genetic transformation (Koltunow et al., 1998), and from interploidal hybridization between a tetraploid and a diploid (Esen and Soost, 1972). Interploidal hybridization is the most common and efficient way for breeders to generate triploid cultivars. However, triploid breeding programs always face a shortage of quality tetraploids for use as parents in such crosses. Crosses where tetraploids are used as female parents are more efficient and have much higher triploid recovery than the reciprocal cross. This is due to normal fertilization between female diploid and male haploid gametes (2n + 1) (Esen and Soost, 1972; Soost and Cameron, 1975; Cameron and Burnett, 1978). However, when the tetraploid female parent is a polyembryonic cultivar, the hybrid embryo needs to be rescued under sterile conditions and has to be grown *in vitro*. This is performed to avoid the suppression of the zygotic hybrid triploid embryo by the dominating nucellar embryo/s that are present. This technique is not cost or labor effective and lowers the effectiveness of triploid breeding programs. On the other hand, use of a monoembryonic cultivar as a female parent in such crosses eliminates the need of embryo rescue and is more efficient in terms of ease, labor and cost inputs.

One approach to overcome the limitation polyembryony imparts, is to utilize the pummelo gene pool in grapefruit breeding. Pummelo makes an ideal candidate to be included in grapefruit breeding programs as it is one of the ancestors of grapefruit and is a true species and presents much more genetic diversity. It is monoembryonic and would eliminate the need of embryo rescue when used in interploidal crosses as a

female parent. A large range of red fleshed pummelo selections is available in the CREC germplasm that can be used as potential parents. Pummelos have lower quantities of undesirable compounds like naringin and furanocoumarins. Thus, when used in crosses, it should be possible to generate desirable hybrids with reduced levels of these compounds.

Colchicine is an alkaloid obtained from *Colchicum autumnale* which acts as a mitotic inhibitor (Blakeslee and Avery, 1937) and induces tetraploidy in the target cells by interfering with spindle formation at metaphase. Colchicine is commonly used to induce tetraploidy in breeding lines in *Citrus*. Early attempts were performed by Barrett (1978) in monoembryonic cultivars to generate autotetraploids by treating the axillary buds with colchicine *ex vitro.* However, his technique did not produce a non-chimeric tetraploid plant. Later, autotetraploids were produced in monoembryonic cultivars from colchicine treatment of axillary buds *in vitro* (Oiyama and Okudi, 1986), somatic embryogenic callus (Wu and Mooney, 2002) and shoot tips (Juarez et al., 2004). However, there are no reports of tetraploid induction in pummelo at present. This study reports an efficient method to induce tetraploids by *in vitro* treatment of cut stem explants from pummelo selections with colchicine followed by shoot induction via indirect organogenesis. The effect of different colchicine concentrations and exposure durations were compared for efficiency of indirect organogenesis and tetraploid induction.

Materials and Methods

Plant Materials

Pummelo selections cybrid Hurado Buntan (C-HBP), 5-1-99-3, HBJL-7 and HBJL-5 selected for this study on the basis of their red flesh and other quality characteristics.

These selections are all derived from ‘Hirado Buntan’ pink pummelo and are located at the Citrus Research and Education Center (CREC). The seeds were extracted from the mature fruit from each selection in 2008-2009. The seeds were treated with sodium hydroxide for 4-5 minutes for slime removal and rinsed 3-4 times with water. The seeds were dried and refrigerated at 4ºC until used.

For the experiment, the seeds from each selection were peeled and sterilized separately with 10% Clorox bleach and 2 drops of Tween-20. Individual seed was put into 15 cm long test tubes containing 15 ml of solid MS medium consisting of MS salts and vitamins (Murashige and Skoog 1962) supplemented with 30 g/l sucrose and7 g/l agar, pH 5.8. These were then placed in the dark for about 4 weeks. The etiolated conditions were provided to facilitate and hasten stem elongation and suppress lateral branching. At about 4 weeks, when the seedlings attained a height of about 12-15 cm, they were put under continuous light conditions for 4-5 days to allow greening and hardening of the stems. At this time, the seedlings were ready to be treated.

Colchicine Treatments

After hardening of the seedlings, the leaves were removed and the stem was cut into about 1cm long explants by making a diagonal cut at both ends. For each selection, colchicine treatments were applied separately. The experiment was designed in a 4 x 4 two way factorial design with 4 colchicine concentrations including the control and 4 exposure periods. Five replications were performed for each treatment.

A Colchicine stock solution was prepared by dissolving the reagent in dimethylsufoxide (DMSO) in sterile water to a concentration of 1g/ml. This solution was filter sterilized and stored at -20ºC. The cut explants were incubated in conical Falcon tubes containing 10 ml of liquid DBA-3 medium. Colchicine dissolved in DMSO was

added to each flask at concentrations of 0.01, 0.05 and 0.1%. DBA-3 shoot induction medium contains the cytokinin growth regulator 6-benzylaminopurine (BAP) which stimulates cell division and hence increases the frequency of dividing cells for the colchicine to act on. The untreated control treatment consisted of explants immersed in DBA-3 media without colchicine. Each treatment at different concentrations was performed for exposure periods: 4, 8, 16 and 32 hours. These flasks were placed on the rotary shaker at 30 rpm under conditions of 25 ± 2ºC and darkness for the respective treatment duration to facilitate contact and penetration of colchicine in the cells at the cut end.

After the explants were exposed for the requisite treatment, they were placed in 100x20mm Petri dishes on solid DBA-3 medium and were incubated in the dark for 2 weeks to induce callus from the cut ends. The explants were then moved from dark to the continuous light conditions to facilitate shoot induction via indirect organogenesis. The explants with emerging shoots from the cut ends were transferred to RMB+ medium supplemented with gibberellic acid (GA3) which promotes shoot elongation. When shoots were about 1-2 cm long they, individual elongated shoots were moved to the rooting medium for further hardening and growth. Emergent shoots were analyzed for their ploidy level through a flow cytometer at a stage when the shoots had about 3 expanded leaves. The tetraploid and mixoploid shoots confirmed by flow cytometry were propagated by micrografting technique onto vigorous rootstocks. The micrografted mixoploids and tetraploids were put under shade for 10-14 days after which they were moved to the greenhouse with 21-17°C day night temperatures for acclimatization.

Ploidy Analysis

Ploidy was analyzed using a tabletop Ploidy Analyser flow cytometer (Partec GmbH, Germany). This technique makes it possible to analyze 150-200 genotypes per day. Flow cytometry works by estimating the volume and florescence of isolated nuclei. The ploidy is presented in form of a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample.

The protocol is a series of steps starting with excision of a 0.2 – 0.3 cm^2 piece of fully expanded leaf tissue and placed in a 50mm plastic Petri dish. The sample was chopped with a sharp razor blade after adding few drops of Nuclei Extraction Buffer. After chopping, 6-7 more drops of Nuclei Extraction Buffer were added and the sample was filtered through a 50µm filter into a 3.5 mL (55mmX12mm) Sarstedt tube. The staining buffer – 4',6-Diaminidino-2-phenylindole (DAPI) was added drop by drop through the filter to infiltrate the remaining cells, until the half of the tube was filled. Each sample was incubated for 10-15 seconds at room temperature before running it on the flow cytometry.

Results and Discussion

Colchicine had a negative effect on the number of mutated shoots at all concentrations and all exposure periods. This is attributed to the toxic nature of colchicine. The colchicine concentration and the exposure time of the explants were the key factors affecting indirect organogenesis as well as generation of tetraploids. These two factors have been recognized previously as two main parameters affecting tetraploidization by Sanford (1983). Colchicine treatments had also affected indirect organogenesis adversely. The explants from treatments with higher concentrations of colchicine or longer exposure periods did not produce as much callus at the cut ends as

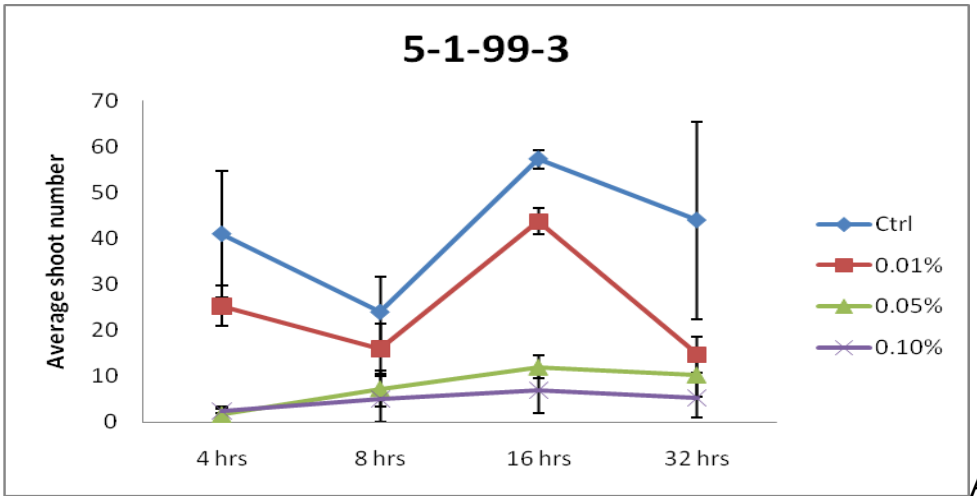
compared to the control and the lower concentration and duration treatments. Callus production at the cut ends is a healing response of the tissue to the injured cells at the cut. Colchicine seemed to impair the ability of the stem tissue to heal by inhibiting callus production at the injury sites.

A   B

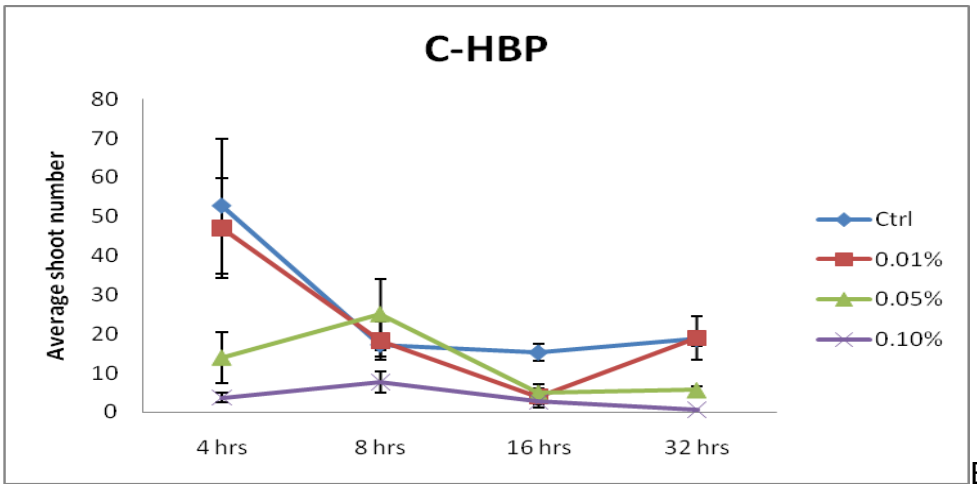
Figure 4-1. Indirect organogenesis. A) Cut explants placed on DBA3 shoot induction medium to induce shoots via indirect organogenesis. B) Closer look of the shoots emerging from the callus produced from the cut ends of the stem pieces.

The rate of shoot induction via indirect organogenesis decreased progressively with the increase in the concentration and duration of the colchicine treatments. Treatments with higher concentrations of colchicine or longer exposure periods had significantly fewer shoots as compared to those from control treatments or less severe treatments (Figure 4-2). The decrease in shoot induction is probably due to decreased callus production in the severe treatments. Reduced callus production provides less active surface area for shoot formation. Also, colchicine toxicity caused mortality of the cells which resulted in less embryo formation and subsequent shoot induction. In all the

selections, the treatments with concentration of 0.01% and 4 hours of exposure duration produced the maximum average number of shoots ranging from about 25 to 50. The highest number of shoots for this treatment was produced for selection C-HBP. Lowest numbers of shoots were produced from treatments with highest colchicine concentration of 0.1% and maximum exposure of 32 hours. This treatment produced 1-5 shoots emerging from the explants in all the selections except in selection HBJL-5 where it totally suppressed shoot induction.



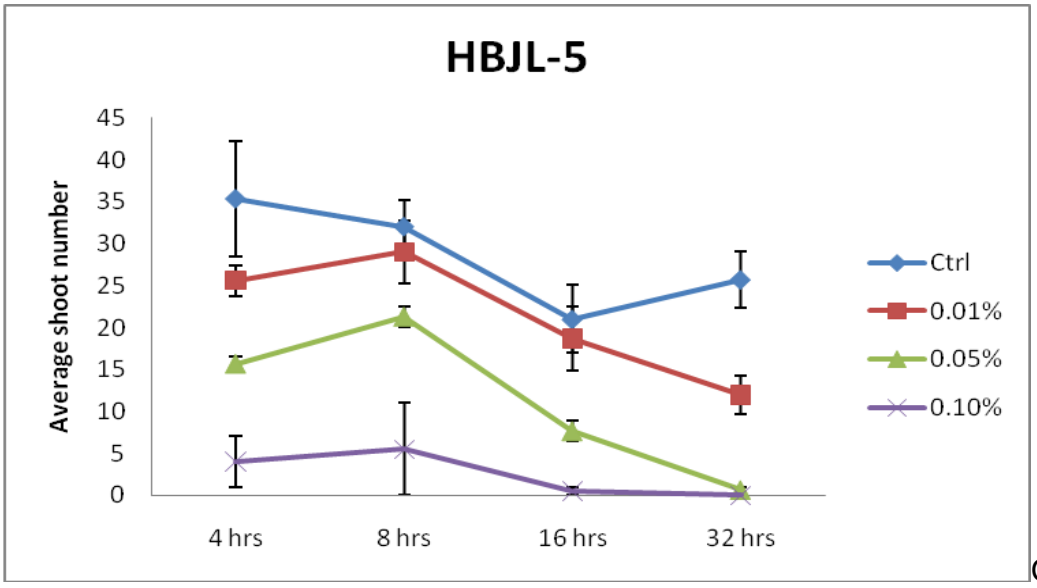
A



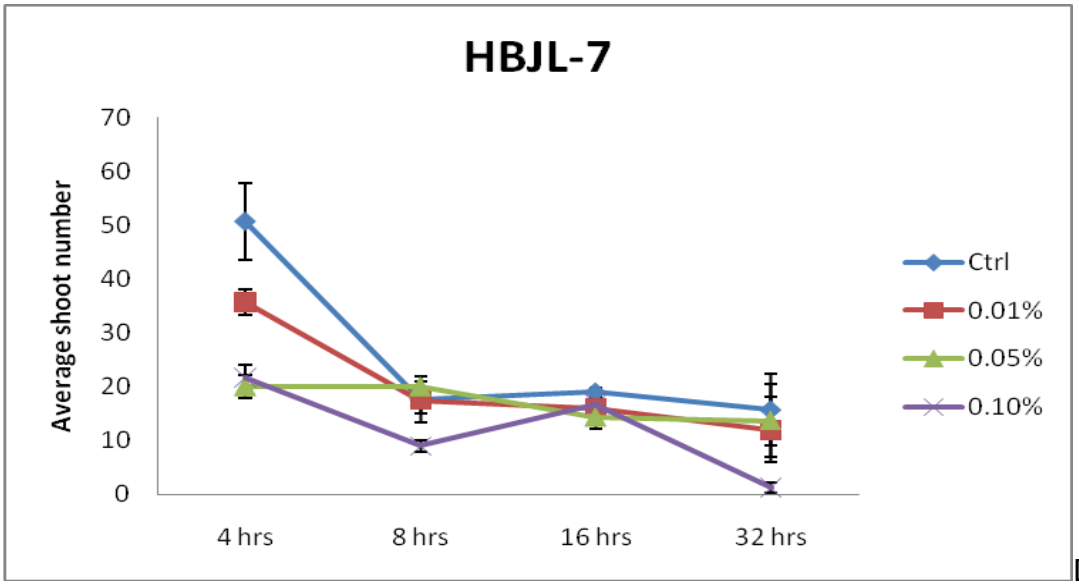
B

Figure 4-2. Average Number of shoots produced through indirect organogenesis from colchicine treatments in pummelo seedlings from selections. A) 5-1-99-3. B)

C-HBP. C) HBJL-7. D) HBJL-5 (mean separations at each hour by calculating standard error of mean at 5% level).



C



D

Figure 4-2. Continued

Indirect organogenesis from the cut ends of the explants allowed for induction of polyploidy in the target cells more efficiently. Colchicine concentrations of 0.05% and 0.1% induced maximum tetraploidy (Table 4-1). Out of the 19 total tetraploids produced

from the 4 pummelo selections, 10 were produced from the treatment with a concentration of 0.1% and 6 were produced from treatment with a concentration of 0.05%.

Table 4-1. Effect of *in vitro* application of colchicine to the indirect organogenesis of seedlings from different pummelo selections and ploidy level of the regenerated shoots.

Pummelo Selection	Conc.	Exposure Period	Avg # of shoots	Tetraploids	Mixoploids
C-HBP	0.01%	4	50	0	0
C-HBP	0.01%	8	19.6	0	0
C-HBP	0.01%	16	3.4	0	0
C-HBP	0.01%	32	20.4	0	0
C-HBP	0.05%	4	14.25	1	0
C-HBP	0.05%	8	20.4	0	1
C-HBP	0.05%	16	2.75	2	1
C-HBP	0.05%	32	8.8	0	0
C-HBP	0.10%	4	3.8	1	0
C-HBP	0.10%	8	6	0	0
C-HBP	0.10%	16	2	0	0
C-HBP	0.10%	32	0.6	2	0
5-1-99-3	0.01%	4	25.33	0	0
5-1-99-3	0.01%	8	16	0	0
5-1-99-3	0.01%	16	43.67	3	0
5-1-99-3	0.01%	32	14.67	0	0
5-1-99-3	0.05%	4	1.67	0	0
5-1-99-3	0.05%	8	7.33	0	0
5-1-99-3	0.05%	16	12	0	0
5-1-99-3	0.05%	32	10.3	1	2
5-1-99-3	0.10%	4	1.25	4	1
5-1-99-3	0.10%	8	5	2	0
5-1-99-3	0.10%	16	7	0	0
5-1-99-3	0.10%	32	4	1	1
HBJL-5	0.01%	4	25.67	0	0
HBJL-5	0.01%	8	15.67	0	1
HBJL-5	0.01%	16	2.67	0	0
HBJL-5	0.01%	32	29	0	0
HBJL-5	0.05%	4	21.33	0	0
HBJL-5	0.05%	8	4	1	0
HBJL-5	0.05%	16	21	1	1
HBJL-5	0.05%	32	7.67	0	0
HBJL-5	0.10%	4	0.33	0	0
HBJL-5	0.10%	8	12	0	0
HBJL-5	0.10%	16	0.67	0	0
HBJL-5	0.10%	32	0	0	0

Though few tetraploids were produced from treatments with the lowest concentration of 0.01%, these treatments were more effective at longer durations. Apparently, colchicine applied at 0.1% is most efficient for tetraploid induction by this technique. This colchicine concentration was also recognized as optimal for treatment of citrus shoot tips for tetraploid induction by Oiyama and Okudai (1986).

The number of tetraploids and mixoploids produced in the 4 selections varied. Selections 5-1-99-3, C-HBP, HBJL-7 and HBJL-5 produced 11, 6, 0 and 2 tetraploids and 4, 2, 1 and 2 mixoploids respectively. HBJL-7 failed to produce any non-chimeric tetraploid plants. The differential response of the selections in the number of mutated plants can be explained by the variation in the susceptibility of the genotypes to colchicine. Such genotypic effects leading to variable results have been reported previously (Aleza et al., 2009; Ganga and Chezhiyan, 2002; Stanys et al., 2006). Figure 4-3 displays the histogram obtained from the ploidy analyzer via Flow cytometry for a tetraploid sample showing a single tetraploid peak, whereas Figure 4-4 displays a histogram for a mixoploid sample showing two peaks depicting the presence of two ploidy levels in the same sample. The tetraploids were propagated by micrografting to vigorous trifoliate rootstock seedlings. Mixoploids were discarded as they are unstable, often sterile and of little use for the breeding process.

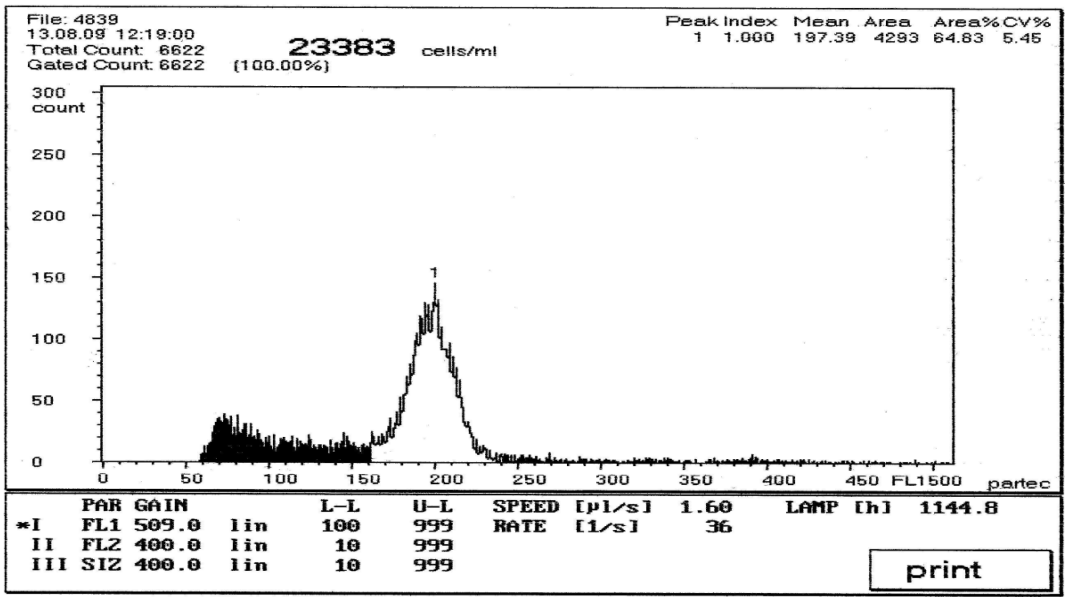


Figure 4-3. Peak obtained by flow cytometry from a tetraploid shoot of C-HBP produced by colchicine treatment in vitro.

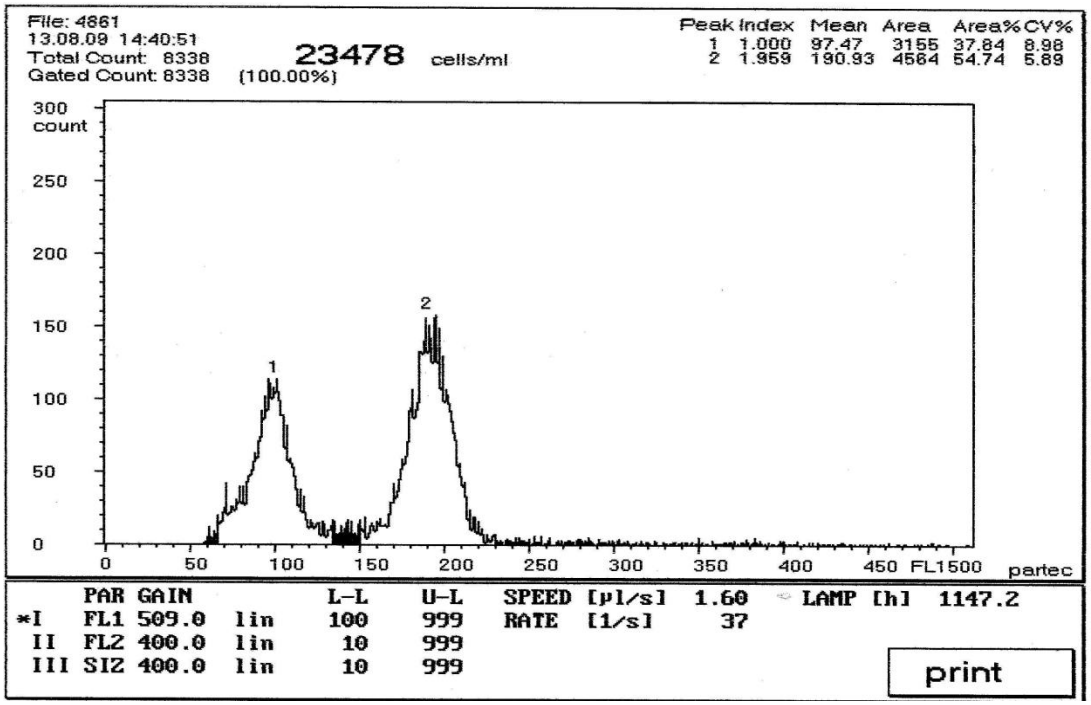


Figure 4-4. Diploid and tetraploid peaks obtained by flow cytometry from a mixoploid shoot of C-HBP produced by colchicine treatment in vitro.

Figure 4-5. Browning of pummelo seedling stem-piece explants caused by higher colchicine concentration and duration.

The explants from the treatments with higher concentrations and longer exposure periods showed browning as well as necrosis (Figure 4-5). Browning prevented the explants from producing callus and subsequent shoots. Such explants turned from light brown to dark brown in color and eventually died.

Conclusion

The results from this study demonstrated successful production of non-chimeric tetraloid plants from monoembryonic pummelo selections selected for their red flesh and superior quality. In this study, a method to induce tetraploidy in the non-apomictic seedlings by treating cut stem sections with colchicine at various concentrations and exposure periods is described. The shoot induction was through indirect organogenesis i.e. from the callus produced from the cut ends of the treated explants. Stable tetraploids were successfully produced in 3 out of 4 selections and were confirmed by flow cytometry. The tetraploids obtained could be of significant value as parents in grapefruit triploid breeding programs.

CHAPTER 5
COLCHICINE INDUCED POLYPLOIDY *IN VIVO* IN PUMMELO (*CITRUS GRANDIS L. OSBECK*)

Introduction

Considering the increasing popularity and trend of seedless fruits in fruit market, breeding programs are increasing efforts to produce seedless fruit. In seedless grapefruits (*Citrus paradise Macf.*), the most appealing characteristic for commercial markets is flesh color. Today, there is a need of grapefruit varieties that are seedless and red fleshed with their other quality characteristics intact.

Triploid breeding is one of the most efficient ways to produce seedless cultivars. This process can involve an interploidal cross between a tetraploid and a diploid parent. Since most citrus cultivars are diploid, there are very few tetraploids available to be used for such interploidal crosses. Production of superior tetraploids is first step in the process of breeding triploid lines.

Grapefruit has a narrow gene pool since it is a group of related cultivars that have arisen through mutation from a single hybrid genotype. Inclusion of pummelos (*C. grandis L. Osbeck)* in grapefruit breeding programs is being considered as an approach to increase the genetic diversity of grapefruit since it is one of the proposed ancestors of grapefruit. Pummelos being monoembryonic, when used as female parent in interploidal crosses, eliminate the need to rescue hybrid embryos, with savings in cost and labor inputs. Pummelos have lower levels of undesirable chemicals such as furanocoumarins and naringin than in grapefruits. Their inclusion as parents in crosses should help reduce these compounds in the subsequent triploid hybrids. Numerous pummelo selections with dark red flesh and superior quality are available in the CREC germplasm, which can be used for tetraploid induction.

The most commonly used method to produce tetraploids is by treatment with colchicine. Colchicine is a mitotic inhibitor that interferes with spindle formation during metaphase and hinders chromosome separation. Colchcine has been used in a number of ways and on various plant parts to induce tetraploidy. Complete autotetraploids in monoembryonic cultivars have been produced via the *in vitro* application of colchicine to the axillary buds (Oiyama and Okudi, 1986), shoot tips (Juarez et al., 2004) and somatic embryogenic callus (Wu and Mooney, 2002). Barrett (1974) attempted to induce tetraploidy using *in vivo* techniques but failed to produce a complete stable tetraploid. There has been no report of tetraploid production in mature citrus tissue using colchicine. However, tetraploids from mature tissue and the technique involved would be highly significant for breeding programs as it reduces the total time taken for triploid production by 4-5 years, and such a tetraploid should be true to type.

Materials and Methods

Plant Materials

Pummelo selections used for this study were 5-1-99-5, UKP-1 and C-2-5-12. All these selections are derived from ‘Hirado Buntan’ pink pummelo and are located at the Citrus Research and Education Center (CREC in Lake Alfred, FL). These selections were chosen on the basis of their dark pink to red flesh color and other quality characteristics.

Materials and Methods

Fresh budwood from each pummelo selection was harvested from the 6-10 year old trees in the field early in the morning. Leaves and thorns were removed using clippers and the budwood was washed 3-4 times with soap and rinsed with water. The ideal size of the budwood for this experiment is from stems 0.3-0.5 cm in diameter. The

stems were cut into bud-sticks each containing 4-5 buds. The bud-sticks were immediately stored in on ice until used. Otherwise, bud-sticks can be stored under refrigeration for 1-2 weeks. However, for this experiment, fresh budwood was preferred and the bud-sticks were grafted on the same day they were harvested from the field. These bud-sticks were further prepared by placing an absorbent cotton ball on each bud followed by tight wrapping of bud-stick with Nescofilm®, keeping the lower cut end unwrapped. The bud-sticks were then cleft grafted onto the experimental rootstock 'Orange-1' (a complex allotetraploid hybrid from 'Nova'+'Hirado Buntan zygotic x Clepatra+Argentine trifoliate orange). In the cleft grafting technique the rootstocks were prepared by making a horizontal cut perpendicular to the main axis of the stem to be grafted. Then, a vertical slit of about 1-2 cm was made down the axis of the stock. Scion was prepared by making two 1-2 cm opposing smooth cuts tapering towards the lower end of the bud-stick. The freshly prepared bud-sticks were inserted in the split created in the rootstock. The scion was not inserted to the center axis but to one side to allow cambium of the rootstock and the scion to be in good contact. Finally, the graft union was wrapped tightly with Nescofilm®.

Colchicine Treatments

The experiment was designed as a 1 X 2 two way factorial design where two factors were considered. The treatments consisted of one colchicine concentration of 0.1% and two exposure durations of 24 hours and 48 hours. The colchicine stock solution was prepared by dissolving it in dimethylsulfoxide (DMSO) to a concentration of 1g/ml. This solution was filter sterilized. The final concentration of 0.1% was prepared using distilled water. This solution was sucked into a surgical disposable syringe with a needle attached to it (Figure 5-1). In the greenhouse, the colchicine application was

applied by injecting this solution on cotton balls placed upon each bud through the Nescofilm®. For control treatments, distilled water was injected into the cotton balls in a similar manner. Considering the hazardous nature of colchicine, the application was done very cautiously by covering the applicator body with protective clothing, gloves and a face shield. Colchicine was applied twice a day to keep the cotton moist in contact with the bud. Application was done once in the morning at around 9 a.m. and second time in the evening at around 5 pm. for the respective treatment durations. After the specified treatment duration, Nescofilm® was removed with the help of a sharp razor blade and the cotton balls were discarded. However, Nescofilm® at the graft union was not removed. The bud-sticks were sprayed with the anti-transpirant Cloudcover® to avoid drying and desiccation of the bud-sticks immediately after unwrapping, and before any of the buds sprouted. The grafted plants were irrigated twice a week or as needed. Observations were made for number of buds sprouting from each treatment. The sprouted buds were tested for their ploidy to determine the number of mixoploids and tetraploids obtained from each treatment.

Figure 5-1. Application of 0.1% colchicine to the cotton balls placed on the grafted seedling pummelo buds with a surgical syringe and a needle.

Ploidy Analysis

Ploidy level of the sprouting shoots from the treated buds was determined by flow cytometery using a Partec Flow cytometer, GmbH Minster, Germany. Flow cytometry works by estimating the volume and florescence of isolated nuclei. The ploidy is presented in form of a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample. Each sample was taken by cutting a small piece of tissue of about 0.2 – 0.3 cm^2 from each sprouted leaf from each shoot. For this study, the samples were not pooled and ploidy was determined for each individual sample. The tissue was put in a 50mm plastic petri dish and chopped with a sharp razor blade after adding few drops of nuclei extraction buffer. After chopping, 6-7 more drops of nuclei extraction buffer were added and the sample was filtered through a 50µm filter into a 3.5 mL (55mmX12mm) Sarstedt tube. The staining buffer was added drop by drop through the filter to infiltrate the remaining cells, until the half of the tube was filled. Each sample was incubated for 10-15 seconds at room temperature before running it on flow cytometry. The amount of staining buffer absorbed by the tissue was directly proportional to DNA content of the sample.

Results and Discussion

The sprouting of the shoots from the buds was delayed by colchicine in all 3 selections for all treatments. Buds in control treatments started sprouting in about 4-7 days after removal of the cotton balls and Nescofilm® wrap. In contrast, for the treated buds, shoots did not emerge for about 3-4 weeks after being exposed to colchicine. The rate of sprouting and growth was slower for the treated buds than controls.

Colchcine seemed to affect the rate and frequency of sprouting of the treated buds. Each graft had 4 buds treated with colchicine. The average number of buds that sprouted from each graft for selections 5-1-99-2, C2-5-12 and UKP-1 was 1.77, 2, 1.69, respectively, for the treatment with 24 hours of exposure and was 1.54, 2.06 and 1.62, respectively, for the treatment with exposure period of 48 hours. Bud sprouts from control treatments were significantly higher than those from any of the colchicine treatments for all the three pummelo selections (Table 5-1). The buds that did not sprout became necrotic. Initially, the meristem died and then the entire bud collapsed. There was no difference between the effects of exposure duration on the number of buds that sprouted.

Table 5-1. Number of buds sprouts from the colchicine treatment for pummelo selections 5-1-99-2, C-2-5-12 and UKP-1

Cultivar	Treatment	Avg number of sprouts per graft (24 hrs)	Avg number of sprouts per graft (48 hrs)
5-1-99-2	Control	4 ± 0	4 ± 0
5-1-99-2	0.1%	1.77 ± 1.05	1.54 ± 0.84
C2-5-12	Control	4 ± 0	3.67 ± 0.47
C2-5-12	0.1%	2 ± 0.88	2.06 ± 0.73
UKP1	Control	4 ± 0	3.67 ± 0.47
UKP1	0.1%	1.69 ± 0.72	3.67 ± 0.92

Figure 5-2. Colchicine treated grafts. A) sprouting of the shoots from colchicine treated buds. B) Bud-sticks with cotton balls on the buds grafted onto the rootstocks.

None of the treatments induced complete tetraploidy in any of the selections. Barrett (1974) did a similar study where he tried to induce tetraploidy in vivo by treating the axillary buds from mature tissue of monoembryonic cultivar with 0.1% colchicine but did not produce any complete tetraploid shoots from the bud sprouts. He induced many cytogenic chimeras in the three histogenic layers. Although a different technique was used in the present study, tetraploidy was not induced. Use of larger tissues is a limiting factor and the most probable reason for lack of success (Sanford., 1983). Larger plants as opposed to plants grown *in-vitro* have broadly differentiated meristematic tissue making it difficult to mutagenize the entire tissue by applying colchicine on the surface only.

The chimeras induced in all the three selections were only from the treatments which had exposure duration of 2 days. The percentage of chimeras produced by colchicine at 0.1% concentration and 48 hours of exposure period are shown in Table 5-2 for the 3 pummelo selections.

Table 5-2. Percentages of cytochimeras produced from the buds treated by colchicine

Cultivar	Cytocjimeras at 0.1% 24 hrs	Cytochimeras at 0.1% 48 hrs
5-1-99-2	0	26.30
C2-5-12	0	12.5
UKP1	0	18.75

The differential response for chimeras of the pummelo selections is most probably due to genotypic susceptibility to colchicine. The cytochimeras produced varied in their number of diploid cells and tetraploid cells. Chimeras which had higher tetraploid proportion confirmed by flow cytometry were from the lower buds which did not sprout until upper buds were clipped to remove apical dominance. The axillary buds on these shoots were allowed to sprout and give further shoots. One of such chimera from selection 5-1-99-5 produced a shoot which was tetraploid. However, this shoot was not stable over time at this ploidy level and subsequently reverted back to diploid.

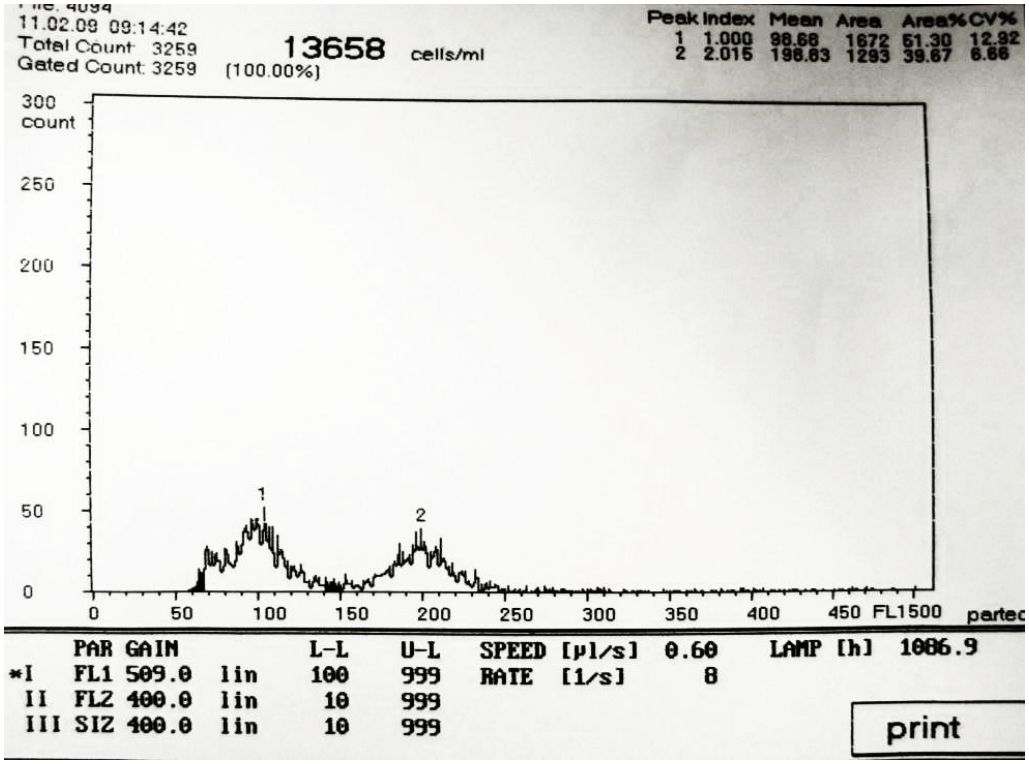


Figure 5-3. Flow cytometry histograms representing budsprouts with mixoploid profile (diploid and tetraploid cells) in selection 5-1-99-5.

Conclusion

In this study, tetraploidy induction in the mature tissue of pummelo selections was studied. Colchicine treatments did not result in the production of a complete tetraploid plant but instead induced cytochimeras. Apparently it is very difficult to induce tetraploidy in mature and broadly differentiated tissues. Improved techniques should be tried in future for similar studies.

CHAPTER 6
SOMATIC HYBRIDIZATION OF GRAPEFRUIT + PUMMELO TO PRODUCE GRAPEFRUIT/PUMMELO TYPE ALLOTETRAPLOIDS

Introduction

Seedless fruits have gained great commercial importance in local and international markets over the recent past. The trend has continued for citrus as well. Higher demand for seedless citrus varieties has led to increased efforts towards the breeding of seedless triploids. Improvement of red grapefruit (*Citrus paradisi* Macf.), being widely produced in the U.S., has also been included in triploid breeding efforts. Red or pink flesh color is another desirable trait popular in commercial grapefruit cultivars.

A commonly used way to produce triploid cultivars is through interploidal crosses between tetraploid and diploid parents (Esen and Soost, 1973). However, the challenge in using this method is limited by availability of tetraploid parents in grapefruit. Grapefruit has a narrow gene pool since it is a group of related cultivars that have arisen through mutation from a single hybrid genotype. In general, most citrus cultivars are diploid, but there are a few rare naturally occurring triploids and tetraploids. Most breeding programs induce tetraploidy in established lines to produce superior tetraploid parents to be used for interploidal crosses. Another challenge in grapefruits is its polyembryony characteristic, that complicates interploidal crosses by requiring embryo rescue for triploid embryo recovery, adding extra cost and labor inputs. Pummelo (*Citrus grandis* L. Osbeck), being an ancestor of grapefruit, should be a good parent to facilitate the production of improved grapefruit/pummelo-like triploid varieties. When used as a female parent in interploidal crosses, pummelos have an advantage that no embryo rescue is required because of their monoembryony.

Somatic Hybridization offers a unique way of producing tetraploids where two diploid genotypes can be combined into one allotetraploid. This technique can also circumvent biological problems including nucellar polyembryony and pollen/ovule sterility, faced during conventional breeding processes. Several tetraploid somatic hybrids produced in the past few decades (Guo and Deng, 2001; Grosser et al., 2000) have been successfully used as breeding parents to produce seedless triploids (Grosser *et a*l., 1998). Some of the reports where protoplast fusion has successfully produced new allotetraploid somatic hybrids involving pummelo include Ananthakrishnan et al. (2006) and Grosser et al. (2007). Somatic hybrids produced using grapefruit embryogenic suspension cultures were reviewed in Grosser et al. (2000), including 'Pink Marsh' grapefruit + 'Murcott tangor and 'Pink Marsh' + LB8-9 'Sugar Bell' tangelo.

Materials and Methods

Protoplast Isolation, Fusion, and Culture

Suspension cultures of 'Ruby Red' grapefruit from the friable callus line initiated in Texas (kindly provided by E. S. Louzada, Weslaco Citrus Center, Texas A&M) were maintained in citrus callus collection of Citrus Research and Education Center (CREC, in Lake Alfred, FL). The callus lines were maintained for several years and stability of the embryogenic potential was not determined. However, since this was the only red fleshed grapefruit callus line available, the protoplast fusion experiments were carried out using this line. Suspension cultures were established and maintained in H+H medium on a subculture cycle of 2 weeks, and the protoplasts were isolated during the period from 4 to 12 days (Grosser and Gmitter, 1990). Grafted plants of pummelo selections 8-1-99-2, 5-1-99-2, UKP-1, 5-1-99-5, were maintained in the greenhouse.

They were placed in the shade for few days before healthy and fully expanded leaves were taken for protoplast isolation.

Suspension cultures of ‘Ruby Red’ grapefruit were incubated in 0.6M BH3 protoplast culture medium and enzyme solution according to Grosser and Gmitter (1990). Leaves from the pummelo selections were sterilized by immersion in 20% Chlorox bleach with 2 drops of Tween Twenty surfactant for 15 minutes. They were rinsed with sterile distilled water 3-4 times and feathered with a sharp scalpel before overnight incubation in 0.6M BH3 protoplast culture medium and enzyme solution. Protoplasts from both suspension cultures and leaves were passed through 45 µm stainless steel mesh screen and centrifuged thereafter on a 25% sucrose/ 13% mannitol gradient (Grosser and Gmitter, 1990).

Protoplast fusion was performed in 60 X 15-mm polystyrene petri dishes using the standard method described by Grosser and Gmitter (1990). PEG (40%polyethylene glycol) was used to induce fusion of the suspension culture-derived and the leaf-derived protoplasts to form heterokaryons. After fusion, protoplasts were initially cultured in a 1:1 (v:v) mixture of 0.6M BH3 and 0.6M EME protoplast culture media. The petri dishes were sealed with Nescofilm and placed under low light conditions. Osmoticum reduction and transfer to solid EME-mannitol medium for somatic embryo induction were performed according to Grosser and Gmitter (1990).

Callus Recovery and Attempted Induction of Somatic Embryogenesis

Although the somatic fusion experiments were conducted numerous times, micro-calli recovery was inconsistent and successful somatic embryo induction from recovered calli was always unsuccessful. This indicated that the ‘Ruby Red’ grapefruit callus line had lost its totipotency. Several additional experiments were conducted

(troubleshooting) in efforts to improve micro-calli recovery and to achieve somatic embryo induction as necessary for somatic hybrid plant regeneration, and are described as follows.

Modification of Protoplast Culture Conditions

Following fusion, the protoplasts were routinely cultured in a 1:1 (v:v) solution of 0.6M BH3 and 0.6M EME, in a volume of approximately 2 ml per 60x15mm petri dish. After unsuccessful somatic embryo induction, the quantity of this protoplast culture media used in later experiments was reduced to half in an effort to improve gas exchange. Subsequent experiments also tested the culture of protoplasts following fusion directly in either 0.6M BH3 or 0.6M EME protoplast culture media separately.

Use of in vitro plants

Protoplast isolation and fusion was tried with *in vitro* grown pummelo selections in contrast to those maintained in the greenhouse. This eliminated the step of leaf sterilization prior to protoplast isolation and reduced the possibility of contamination in the leaf protoplast preparations.

Nurse culture

Following fusion, unfused protoplasts isolated from vigorous and totipotent embryogenic suspension cultures of W. ‘Murcott’ tangor was added to the fusion protoplast cultures in the 1:1 (v:v) 0.6M BH3 and 0.6M EME protoplast culture media, in efforts to stimulate growth and embryogenesis.

Treatments of ‘Ruby Red’ callus prior to suspension culture initiation

PVP (polyvinylpyrrolidone) at 1g/l was added to the DOG embryogenic callus induction medium (Grosser and Gmitter, 1990) on which the callus lines were cultured prior to suspension culture initiation in H+H medium (Grosser and Gmitter, 1990). In

another experiment, neutralized activated charcoal charcoal at 1g/l was added to DOG media (Grosser and Gmitter, 1990) on which the callus lines were cultured prior to suspension culture initiation. This was done in an effort to improve the health of the callus and to remove potential impurities.

Results and Discussion

Numerous somatic hybridization experiments were conducted in efforts to combine protoplasts isolated from suspension cultures of 'Ruby Red' grapefruit and leaf protoplasts isolated from various pummelo selections; however, no somatic embryos or plants were recovered following protoplast culture. The callus line from which the suspension cultures were established had been maintained for many years, and had probably lost it's totipotency. Suspension cells exhibited a light yellow/brown color in contrast to the white color of normal embryogenic callus. The growth rate of the 'Ruby Red' line was also much slower than typical embryogenic callus lines.

Initially, the protoplast isolation and protoplast fusion process was normal, and the fused heterokaryons were visible when observed using an inverted microscope. The protoplasts underwent normal mitotic division 2-3 days following fusion. However, later cell division seemed to be arrested and microcalli often started to shrink. A light brown layer of some unknown compound was formed on top of the protoplast culture media. It appeared that the protoplasts from 'Ruby Red' grapefruit suspension cultures were releasing some unknown brown exudates, which might have been inhibitory to the growth of the regenerating protoplast-derived microcalli.

In efforts to improve callus recovery following fusion, various approaches were tested as described above. Reducing the volume of protoplast culture in the petri dishes to increase protoplast density and improve gas exchange did not improve callus

recovery. Protoplasts cultured in 0.6M BH3 or 0.6M EME separately (rather than the usual mix) also failed to improve microcalli growth. The brown exudates were still produced on the surface of the liquid media. Experiments to determine if the greenhouse source of pummelo leaf protoplasts was the problem, by using in vitro grown pummelo leaves as an alternative, also failed to improve the results, and the brown exudate was still observed. Finally, the use of unfused W. 'Murcott' tangor nurse protopalsts isolated from a totipotent vigorous suspension culture were added to cultures following fusion still did not significantly improve the results. The idea was to provide nurse cells for the better growth of the fused protoplasts. However, the exudate from the 'Ruby Red' protoplasts seemed to inhibit the growth of protoplasts from both the fusion protoplasts and the nurse protoplasts. All protoplasts shrank and died eventually.

Since, it was clear that the protoplast-derived microcalli growth was inhibited by unknown compounds from 'Ruby Red' suspension culture; PVP and activated charcoal were added to the DOG media (separately) on which callus lines were maintained prior to suspension culture initiation. PVP and activated charcoal adsorb excessive phenolic as well as non-phenolic compounds released in the media by plant cells and can prevent these compounds from affecting cell growth. However, addition of these compounds did have any health or growth promoting effect on the callus. The 'Ruby Red' callus remained slow growing, non-embyogenic, and continued to exhibit the light yellow/brown color (Figure 6-1).

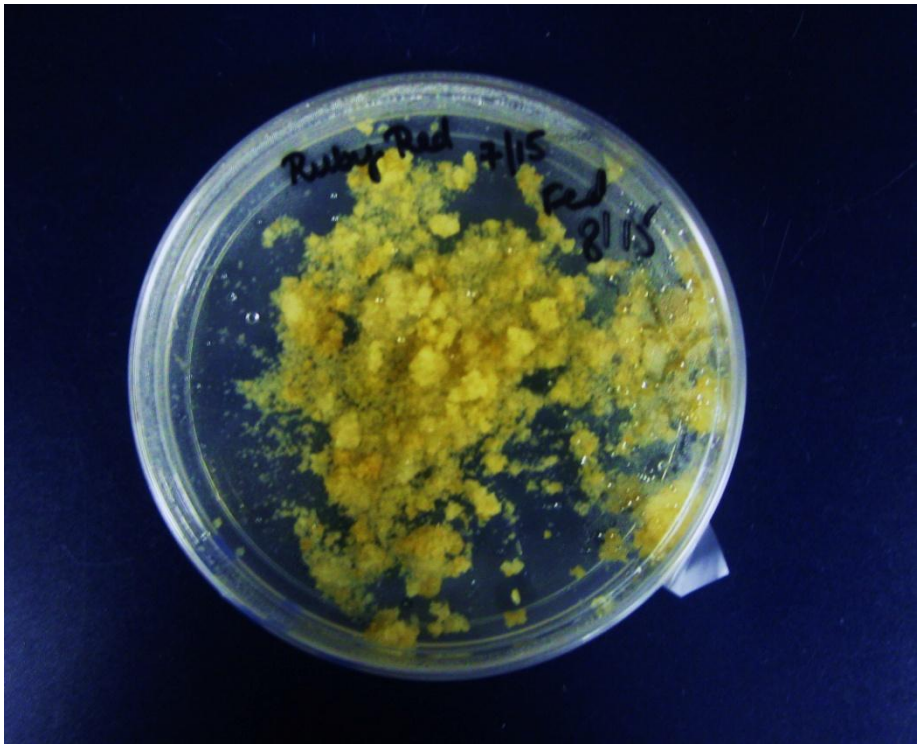


Figure 6-1. ‘Ruby Red’ callus after being sub-cultured for 6 weeks on DOG media containing 1g/l PVP.

It is extremely difficult to recover somatic embryos from callus lines which have been maintained for several years, probably due to the build-up of cytological mutations. Gmitter et al (1991) treated cultures exhibiting low embryogenic capacity with colchicine in order to induce tetraploidy. They also observed that colchicine treatment recovered the embryogenic capacity of the callus. However, colchicine was not tested to improve the totipotency of the ‘Ruby Red’ callus because it is a mutagen and could have altered the ploidy level of the callus line. Conducting somatic hybridization with protoplasts of varying ploidy levels would probably not enhance the results.

Future efforts to obtain somatic hybrids between grapefruit and pummelo, as necessary to achieve the goals of this project, would be to start with a new freshly initiated totipotent ‘Ruby Red’ or other suitable grapefruit callus line. However, the time

period available for this study could not accommodate this process, as embryogenic callus induction and habituation is a long process, especially with grapefruit (J.W. Grosser, personal communication).

CHAPTER 7
SUMMARY AND CONCLUSIONS

The successful production of tetraploid pummelo plants from diploid counterparts was achieved through mutating the ploidy with the mitotic inhibitor - colchicine. Stable tetraploid plants were recovered from zygotic seedlings from several high quality red pummelo selections. From numerous somatic hybridization experiments conducted to generate allotetraploid hybrids combining grapefruit and pummelo, none were successful. Although the somatic hybridization via protoplast fusion technique is well established and the experiments were well planned, the failure was attributed to the unavailability of totipotent friable embryogenic callus lines from a red fleshed grapefruit. Various methods were tried to improve the existing non-totipotent callus line from 'Ruby Red' grapefruit, but all were unsuccessful. For future attempts at somatic hybridization, it will be more beneficial to utilize a freshly initiated totitpotent embryogenic callus line of grapefruit for suspension culture initiation.

Treating pummelo seedling stem segments with colchicine successfully induced tetraploidy in the tissue of induced adventitious shoots. Regeneration of complete and stable tetraploid plants from this technique proved to be superior, as the shoots emerge from a single cell. A mutated tetraploid single cell could produce a non-chimeric tetraploid plant. Colchicine at 0.1% was observed to be the optimum concentration for this technique. This technique produced 16 tetraploid plants which were confirmed to be stable 6 months after micrografting.Treatment of pre-germinated seeds with colchicine also successfully resulted in induced tetraploidy. However, this technique was more likely to produce mixoploids shoots, as the target tissue in this case is a multi-celled meristem. Higher concentrations of colchicine were tried in this experiment, which

caused higher mortality. The ideal colchicine concentration for inducing tetraploids was however the same – 0.1%. This technique produced 3 tetraploid plants which were confirmed to be stable 6 months after micrografting. It should also be pointed out that treating the pregerminated seeds is much less laborious than treating the in vitro stem segments with respect to setting up the experiment as well as screening of the treated seedlings/shoots.

In the *in vivo* experiment, tetraploidy induction in the mature tissue of pummelo selections was studied. Colchicine treatments did not result in the production of any stable tetraploid plants, but induced cytochimeras instead. Apparently it is very difficult to induce tetraploidy in mature and broadly differentiated tissues. Improved techniques should be tried in future for similar studies.

The occurrence of natural triploids in monoembryonic pummelos was apparently very low and varies with genotype, as only one triploid plant was recovered from pummelo HBJL-7. Monoembryony limits the occurrence of tetraploids in the natural seedling populations in absence of a triploid block. The selection of tetraploids in a natural population of seedling plants is not practical in monoembryonic pummelo. Hence, screening for tetraploids in natural seedling populations is fruitful only for polyembryonic genotypes that produce nucellar seedlings.

The final conclusion is that the overall project can be considered a success, because more than 20 stable tetraploid plants were recovered from zygotic seedlings of the various parents. These autotetraploid pummelos have been successfully micrografted to vigorous rootstocks and can now be grown in the field for subsequent use as parents in the CREC triploid breeding program. Efforts to top-work these new

autotetralpoid pummelos to existing mature field trees, to expedite their flowering and fruiting, are underway.

APPENDIX
CITRUS PROTOPLAST MEDIA AND SOLUTIONS

Table A-1. Composition of the EME medium.

Component	mg/L
NH_4NO_3	1,650
KNO_3	1,900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.63
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Thiamine.HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5

50 g/L sucrose was added for 0.146 M EME and 205.38 g/L sucrose for 0.6 M EME. For 1500 EME malt extract was added at 1500 mg/L and sucrose at 50 g/L. Solid medium contains 8 g/L agar

Table A-2. Composition of sucrose and mannitol solutions (CPW salts).

Component	mg/L
$MgSO_4.7H_2O$	250
KNO_3	100
KH_2PO_4	27.20
KI	0.16
$CuSO_4.5H_2O$	0.00025
$CaCl_2.2H_2O$	150

Table A-3. Composition of 0.6 m BH3 nutrient medium.

Component	mg/L
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KCl	500
KI	0.63
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Glutamine	3,100
Thiamine.HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Casein hydrolysate	250
Nicotinic acid	1
Mannitol	81,990
Sucrose	51,350 (85,560 for 0.7 M)
Coconut water	20 mL
Fructose	250
Ribose	250
Xylose	250
Mannose	250
Rhamanose	250
Cellobiose	250
Galactose	250
Glucose	250
Sodium pyruvate	20
Citric acid	40
Malic acid40	40
Fumaric acid	40
Vitamin B12	0.02
Calcium pantothene	1
Ascorbic acid	2
Choline chloride	1
p-aminobezoic acid	0.02
Folic acid	0.40

Table A-3. Continued

Component	mg/L
Riboflavin	0.20
Biotin	0.01
Vitamin A (retinol)	0.01
Vitamin D3 (cholecalciferol)	0.01

Table A-4. Composition of protoplast transformation solutions.

40% polyethylene glycol (PEG) (MW = 8000)
0.3 M Glucose
66 mM $CaCl_2$
pH = 6

II- Solutions A and B

Solution A		Solution B	
Component	g/100 mL	Component	g/100 mL
Glucose (0.4 M)	7.20	Glycine (0.3 M)	2.2
CaCl2 (66 mM)		0.97	
DMSO		10 mL	
pH 6.0		pH 10.5	

Table A-5. Composition of DBA3 medium.

Component	mg/L
NH_4NO_3	1,485
KNO_3	1,710
KH_2PO_4	153
$MgSO_4.7H_2O$	333
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	21.40
$ZnSO_4.7H_2O$	7.70
H_3BO_3	5.58
KI	0.567
$Na_2MoO_4.2H_2O$	0.225
$CuSO_4.5H_2O$	0.0225
$CoCl_2.6H_2O$	0.0225
Thiamine.HCl	9
Pyridoxine.HCl	9
Myo-inositol	90
Nicotinic acid	4.5
Coconut water	20 mL
Malt extract	1,500
2,4 D	0.01
DAP	3
Sucrose	25,000
Agar	8,000

Table A-6. Composition of the enzyme solution used for citrus protoplast isolation.

Mannitol	0.7 M
CaCl2	12.0 mM
MES1 (buffer)	6.0 mM
NaH2PO4	1.4 mM
Onozuka RS cellulose	1%
Macerase or macerozyme	1%
Pectolyase Y-23	0.2%
pH = 5.6	
Filter sterilize (Nalgene®, 0.2 µm)	

LIST OF REFERENCES

Aleza, P., Juarez, J., Cuenca, J., Ollitrault, P., and Navarro, L., 2010. Recovery of citrus triploid hybrids by embryo rescue and flow cytometry from 2x * 2x sexual hybridisation and its application to extensive breeding programs. *Plant Cell Reports* 29:1023-1034.

Aleza, P., Juarez, J., Ollitrault, P., and Navarro, L., 2009. Production of tetraploid plants of non apomictic citrus genotypes. *Plant Cell Reports* 28:1837-1846.

Ananthakrishnan, G., M. Calovic, and J.W. Grosser. 2006. Production of additional allotetraploid somatic hybrids combining mandarins and sweet orange with pre-selected pummelos as potential candidates to replace sour orange rootstock. *In Vitro Cellular & Developmental Biology-Plant.* 42**:**367-371.

Anonymous., 2009. Faostat: Agriculture data. avialable at http://apps.fao.org, last accessed June 20, 2010.

Bachi, O., 1940. Observaceous Citologicalem Citrus. I.Numero de cromosomas de algunas especies y variedades. *Journal of Agronomy* (Piracicaba) 3:249-258.

Bailey, D.G., Arnold, J.M.O., and Spence, J.D., 1994. Grapefruit juice and drugs: how significant is the interaction. *Clinical Pharmacokinetics* 26:91-98.

Barrett, H.C., 1974. Colchicine-induced polyploidy in citrus. *Botanical Gazette* 135:29-41.

Barrett, H.C. and Hutchison, D.J., 1978. Spontaneous tetraploidy in apomictic seedlings of Citrus. *Economic Botany* 32:27-45.

Barrett, H.C. and Rhodes, A.M., 1976. A numerical taxonomic study of affinity relationships in cultivated Citrus and its close relatives. *Systematic Botany* 1:105-136.

Blakeslee, A.F. and Avery, A.G., 1937. Methods of inducing doubling of chromosomes in plants. By treatment with colchicine. *Journal of Heredity* 28:393-412.

Bouvier, L., Fillon, F.R., and Lespinasse, Y., 1994. Oryzalin as an efficient agent for chromosome doubling of haploid apple shoots in vitro. *Plant Breeding* 113:343-346.

Cameron, J.W. and Burnett, R.H., 1978. Use of sexual tetraploid seed parents for production of triploid Citrus hybrids. *HortScience* 13:167-169.

Carlson, P.S., Smith, H.H., and Dearing, R.D., 1972. Parasexual interspecific plant hybridization. *Proceedings of the National Academy of Sciences, of the United States of America* 69:2292-2294.

Cerda, J.J., Robbins, F.L., Burgin, C.W., Baumgartner, T.G., and Rice, R.W., 1988. The effects of grapefruit pectin on patients at risk for coronary heart disease without altering diet or lifestyle. *Clinical Cardiology* 11:589-594.

Chandler, J.L., Viloria, Z., and Grosser, J.W. 2000. Acid citrus fruit cultivar improvement via interploid hybridization. *Proceedings of the Florida State Horticultural Society*. 113**:**124-126).

Chen, R.Z., Li, G.G., and Zhang, L.Y., 1991. Callus induction and triploid plant regeneration from endosperm of 'Hongjiang' sweet orange. *Acta Botanica Sinica* 33:848-854.

Deng, X.X., Liu, G.B., and Zhang, W.C., 1985. Studies on the chromosome variation in the callus of citrus. *China Citrus* 3:4-6.

Dutt, M., Vasconcellos, M., Song, K.J., Gmitter, F.G., Jr., and Grosser, J.W., 2010. In vitro production of autotetraploid Ponkan mandarin (*Citrus reticulata* Blanco) using cell suspension cultures. *Euphytica* 173:235-242.

Eapen, S., Rangan, T.S., Chadha, M.S., and Heble, M.R., 1978. Biosynthetic and cytological studies in tissue cultures and regenerated plants of haploid *Atropa belladonna*. *Canadian Journal of Botany* 56:2781-2784.

Esen, A. and Soost, R.K., 1971. Unexpected triploids in Citrus: their origin, identification, and possible use. *Journal of Heredity* 62:329-333.

Esen, A. and Soost, R.K., 1972a. Aneuploidy in citrus. *American Journal of Botany* 59:473-477.

Esen, A. and Soost, R.K., 1972b. Tetraploid progenies from 2x X 4x crosses of Citrus and their origin. *Journal of the American Society for Horticultural Science* 97:410-414.

Esen, A. and Soost, R.K., 1973a. Precocious development and germination of spontaneous triploid seeds in Citrus. *Journal of Heredity* 64:147-154.

Esen, A. and Soost, R.K., 1973b. Seed development in Citrus with special reference to 2X X 4X crosses. *American Journal of Botany* 60:448-462.

Esen, A., Soost, R.K., and Geraci, G., 1978a. Seed set, size, and development after 4x X 2x and 4x X 4x crosses in Citrus. *Euphytica* 27:283-294.

Esen, A., Soost, R.K., and Geraci, G., 1978b. Seed set, size, and development after 4x X 2x and 4x X 4x crosses in Citrus. *Euphytica* 27:283-294.

Esen, A., Soost, R.K., and Geraci, G., 1979. Genetic evidence for the origin of diploid megagametophytes in Citrus. *Journal of Heredity* 70:5-8.

Fatta Del Bosco, S., Matrango, G., and Geraci, G., 1992. Micro and macro sporogenesis of two triploid hybrids citrus. *Proceedings of International Society of Citriculture* 1:122-124.

Fiala, J.L, 1988. Lilacs: The Genus Syringa. Timber Press, Portland, Oregon.

Froelicher, Y. and Ollitrault, P. 2000. Effects of the hormonal balance on *Clausena excavata* androgenesis. *Proceedings of the First International Symposium on Citrus Biotechnology. Acta Horticulture* 535:139-146.

Frost, H.B., 1925. Tetraploidy in Citrus. *Proceedings of National Academy of Sciences, Washington* 11:535-537.

Frost, H.B., 1926. Polyembryony, heterozygosis, and chimeras in Citrus. *Hilgardia* I:365-402.

Frost, H.B., 1938. The genetics and cytology of citrus. *Current Science* (special number):24-27.

Frost, H.B. and Krug, C.A., 1942. Diploid-tetraploid periclinal chimeras as bud variants in Citrus. *Genetics* 27:619-634.

Furusato, K., 1953. Tetraploidy in citrus. *Annual Report of the National Institute of Genetics*, Japan 3:51-52.

Furusato, K., Ohta, and Ota, Y., 1958. Notes on seedless citrus species. *Annual Report of the National Institute of Genetics, Japan 1957.*:44 p.

Ganga, M. and Chezhiyan, N., 2002. Influence of the antimitotic agents colchicine and oryzalin on in vitro regeneration and chromosome doubling of diploid bananas (Musa spp.). *Journal of Horticultural Science and Biotechnology* 77:572-575.

Geraci, G., Esen, A., and Soost, R.K., 1975. Triploid progenies from 2x X 2x crosses of Citrus cultivars. *Journal of Heredity* 66:177-178.

Geraci, G., Radogna, F., and Pasquale, F.d., 1981. Use of the coagulation and browning tests to separate zygotic seedlings from nucellar seedlings in the progenies of some Citrus selections. *Tecnica Agricola, Italy* 33:199-206.

Gmitter, F.G.J., 1995. Origin, evolution and breeding of the grapefruit. *Plant Breeding Reviews* 13:345-363.

Gmitter, F.G., Jr. and Ling, X.B., 1991. Embryogenesis in vitro and nonchimeric tetraploid plant recovery from undeveloped Citrus ovules treated with colchicine. *Journal of the American Society for Horticultural Science* 116:317-321.

Gmitter, F.G., Jr., Ling, X.B., Cai, C.Y., and Grosser, J.W., 1991. Colchicine-induced polyploidy in Citrus embryogenic cultures, somatic embryos, and regenerated plantlets. *Plant Science (Limerick)* 74:135-141.

Gmitter, F.G., Jr., Ling, X.B., and Deng, X.X., 1990. Induction of triploid Citrus plants from endosperm calli in vitro. *Theoretical and Applied Genetics* 80:785-790.

Grosser, J.W., 1986. Citrus cultivar improvement via somatic hybridization. *Proceedings of the Florida State Horticultural Society* 99:36-37.

Grosser, J.W. and Chandler, J.L., 2000. Somatic hybridization of high yield, cold-hardy and disease resistant parents for citrus rootstock improvement. *Journal of Horticultural Science and Biotechnology* 75:641-644.

Grosser, J.W., Chandler, J.L., and Duncan, L.W., 2007. Production of mandarin+pummelo somatic hybrid citrus rootstocks with potential for improved tolerance/resistance to sting nematode. *Scientia Horticulturae* 113:33-36.

Grosser, J.W. and Gmitter, F.G., Jr., 1990. Protoplast fusion and citrus improvement. *Plant Breeding Reviews* 8:339-374.

Grosser, J.W., Gmitter, F.G., Jr., Castle, W.S., and Chandler, J.L., 1995. Production and evaluation of citrus somatic hybrid rootstocks: progress report. *Proceedings of the Florida State Horticultural Society*:140-143.

Grosser, J.W., Jiang, J., Louzada, E.S., Chandler, J.L., and Gmitter, F.G., Jr., 1998. Somatic hybridization, an integral component of citrus cultivar improvement. II. Rootstock improvement. *HortScience* 33:1060-1061.

Grosser, J.W., Louzada, E.S., Gmitter, F.G., Jr., and Chandler, J.L., 1994. Somatic hybridization of complementary citrus rootstocks: five new hybrids. *HortScience* 29:812-813.

Grosser, J.W., Mourao-Fo, F.A.A., Gmitter, F.G., Jr., Louzada, E.S., Jiang, J., Baergen, K., Quiros, A., Cabasson, C., Schell, J.L., and Chandler, J.L., 1996. Allotetraploid hybrids between Citrus and seven related genera produced by somatic hybridization. *Theoretical and Applied Genetics* 92:577-582.

Guo, W. and Grosser, J.W., 2005. Somatic hybrid vigor in Citrus: direct evidence from protoplast fusion of an embryogenic callus line with a transgenic mesophyll parent expressing the GFP gene. *Plant Science* 168:1541-1545.

Guo, W.W. and Deng, X.X. 2001. Wide somatic hybrids of Citrus with its related genera and their potential in genetic improvement. Euphytica 118:175-183.

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.

Hasnain, R., Jaskani, M.J., Khan, M.M., and Malik, T.A., 2003. In vitro induction of polyploids in watermelon and estimation based on DNA content. *International Journal of Agriculture and Biology* 5:298-302.

Hearne, C.J., 1984. Development of seedless orange and grapefruit cultivars through seed irradiation. *Journal of the American Society of Horticultural Sciences* 109:270-273.

Hensz, R.A., 1977. Mutation breeding and development of 'Star Ruby' grapefruit. *Proceedings of International Society of Citriculture* 2:582-585.

Hughes, G., 1972. The Natural History of Barbados, reprinted by the Research Library of Colonial America, Arno Press.

Iwamasa, M., 1966. Studies on the sterility in genus Citrus with special reference to the seedlessness. *Bulletin of the Horticultural Research Station* 6:1-81.

Iwasa, S. and Shiraishi, S., 1957. Studies on the trifoliate orange. 1. Strains found in *Poncirus trifoliata Raf*. *Studies from the Institute of Horticulture, Kyoto* 8:59-63.

James, D.J., MacKenzie, K.A.D., and Malhotra, S.B., 1987. The induction of hexaploidy in cherry rootstocks using in vitro regeneration techniques. *Theoretical and Applied Genetics* 73:589-594.

Jaskani, M.J., Hassan, S., Bashir, M.A., and Khan, I.A., 1996. Morphologicaldescriptions of citrus colchiploids. *Proceedings of International Society of Citriculture* 8:37.

Johnson, A.A.T. and Veilleux, R.E., 2001. Somatic hybridization and applications in plant breeding. *Plant Breeding Reviews* 20:167-225.

Kadota, M. and Niimi, Y., 2002. In vitro induction of tetraploid plants from a diploid Japanese pear cultivar (*Pyrus pyrifolia* N. cv. Hosui). *Plant Cell Reports* 21:282-286.

Kawase, K., Yahata, M., Nakagawa, S., Haraguchi, K., and Kunitake, H., 2005. Selection of autotetraploid and its morphological characteristics in Meiwa kumquat (*Fortunella crassifolia* Swingle). *Horticultural Research (Japan)* 4:141-146.

Kobayashi, S., Ohgawara, T., Saito, W., Nakamura, Y., and Omura, M., 1997. Production of triploid somatic hybrids in citrus. *Journal of the Japanese Society for Horticultural Sciences* 66:453-458.

Koltunow, A.M., Brennan, P., Bond, J.E., and Barker, S.J., 1998. Evaluation of genes to reduce seed size in Arabidopsis and tobacco and their application to Citrus. *Molecular Breeding* 4:235-251.

Koltunow, A.M., Vivian-Smith, A., and Sykes, S.R. 2000. Molecular and conventional breeding strategies for seedless Citrus. *Molecular Breeding* 3:235-251.

Krug, C.A. and Bacchi, O., 1943. Triploid varieties of citrus. *Journal of Heredity* 34:277-283.

Lapin, W.K., 1937. Investigations on polyploidy in citrus. *Wks All-Union Sci. Res. Inst. Humid Subtropics* 1:3-68.

Leach, D.G., 1961. Rhododendrons of the World. Scribner's, New York.

Lee, L.S., 1988. Citrus polyploidy - origins and potential for cultivar improvement. *Australian Journal of Agricultural Research* 39:735-747.

Lehrer, J.M., Brand, M.H., and Lubell, J.D., 2008. Induction of tetraploidy in meristematically active seeds of Japanese barberry (*Berberis thunbergii* var*. atropurpurea*) through exposure to colchicine and oryzalin. *Scientia Horticulturae* 119:67-71.

Lindstrom, E.W., 1936. Genetics of polyploidy. *Botanical Review* 2:197-215.

Longley, A.E., 1925. Polycarpy, polyspory and polyploidyin Citrus and Citrus relatives. *Journal of the Washington Academy of Sciences* 15:347-357.

Louzada, E.S., Grosser, J.W., Gmitter, F.G., Jr., Nielsen, B., Chandler, J.L., Deng, X.X., and Tusa, N., 1992. Eight new somatic hybrid citrus root stocks with potential for improved disease resistance. *HortScience* 27:1033-1036.

Lunan, J., 1814. St. Jago de la Vega Gazette, Spanish Town, Jamaica. *Hortus Jamaicensis* 2:171-173.

Luro, F., Maddy, F., Jacquemond, C., Froelicher, Y., Morillon, R., Rist, D., and Ollitrault, P. 2004. Identification and evaluation of diplogyny in clementine (*Citrus clementina*) for use in breeding. *Proceedings of the Eleventh Eucarpia Symposium on Fruit Breeding and Genetics, Angers, France* 841-847.

Lyrene, P.M. and Perry, J.L., 1982. Production and selection of blueberry polyploids in vitro. *Journal of Heredity* 73:377-378.

McEven, C., 1990. The Japanese Iris. University Press of New England.

Macfadyen, J., 1828. Some remarks on the species of genus Citrus which are cultivated from Jamaica. *Hooker's Journal of Botany and Kew Garden Miscellany* 1:295-304.

Macfadyen, J., 1937. The Flora of Jamaica. Longman, Orme, Brown, Green and Longmans, London.

Mendes, B.M.J., Mourao Filho, F.d.A.A., Farias, P.C.d.M., and Benedito, V.A., 2001. Citrus somatic hybridization with potential for improved blight and CTV resistance. *In Vitro Cellular & Developmental Biology - Plant* 37:490-495.

Moore, J.N., and Janick, J., 1983. Methods in Fruit Breeding. Purdue University Press, West Lafayettena.

Motomura, T., Hidaka, T., Akihama, T., Katagi, S., Berhow, M.A., Moriguchi, T., and Omura, M., 1997. Protoplast fusion for production of hybrid plants between Citrus and its related genera. *Journal of the Japanese Society for Horticultural Science* 65:685-692.

Mukherjee, S.K. and Cameron, J.W., 1958. Tree size and chromosome number in a triai of tetraploid trifoliate orange as a citrus rootstock. *Proceedings. American Society for Horticultural Science* 72:267-272.

Nakamura, M., 1942. Cytological studies in the genus Citrus. III. Further data on chromosome numbers. *Journal of the Horticultural Association of Japan* 13:30-40.

Navarro, L., Olivares-Fuster, O., Juarez, J., Aleza, P., Pina, J.A., Ballester-Olmos, J.F., Cervera, M., Fagoaga, C., Duran-Vila, N., and Pena, L. 2004. Applications of biotechnology to citrus improvement in Spain.

Notsuka, K., Tsuru, T., and Shiraishi, M., 2000. Induced polyploid grapes via in vitro chromosome doubling. *Journal of the Japanese Society for Horticultural Science* 69:543-551.

Ohgawara, T., Kobayashi, S., Ohgawara, E., Uchimiya, H., and Ishii, S., 1985. Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata. Theoretical and Applied Genetics* 71:1-4.

Oiyama, I. and Kobayashi, S., 1990. Polyembryony in undeveloped monoembryonic diploid seeds crossed with a Citrus tetraploid. *HortScience* 25:1276-1277.

Oiyama, I. and Okudai, N., 1986a. Production of colchicine-induced autotetraploid plants through micrografting in monoembryonic Citrus cultivars. *Japanese Journal of Breeding* 36:371-376.

Oiyama, I. and Okudai, N., 1986b. Production of colchicine-induced autotetraploid plants through micrografting in monoembryonic Citrus cultivars. *Japanese Journal of Breeding* 36:371-376.

Okudai, N., Oiyama, I., and Takahara, T., 1981. Studies on the improvement of zygotic seedling yield in polyembryonic Citrus spp. I. Differences in embryo number per seed and zygotic seedling yield among varieties and strains. *Bulletin of the Fruit Tree Research Station, D*:9-21.

Ollitrault, P., Dambier, D., Sudahono, Mademba-Sy, F., Vanel, F., Luro, F., and Aubert, B., 1998. Biotechnology for triploid mandarin breeding. *Fruits (Paris)* 53:307-317.

Ollitrault, P., Vanel, F., Froelicher, Y., and Dambier, D. 2000a. Creation of triploid citrus hybrids by electrofusion of haploid and diploid protoplasts. *Proceedings of the First International Symposium on Citrus Biotechnology, Eilat, Israe*l 191-197.

Ollitrault, P., Vanel, F., Froelicher, Y., and Dambier, D. 2000b. Creation of triploid citrus hybrids by electrofusion of haploid and diploid protoplasts. *Proceedings of the First International Symposium on Citrus Biotechnology, Eilat, Israe*l 191-197.

Orczyk, W., Przetakiewicz, J., and Nadolska-Orczyk, A., 2003. Somatic hybrids of *Solanum tuberosum* - application to genetics and breeding. *Plant Cell, Tissue and Organ Culture* 74:1-13.

Recupero, G.R., Russo, G., and Recupero, S., 2005. New promising Citrus triploid hybrids selected from crosses between monoembryonic diploid female and tetraploid male parents. *HortScience* 40:516-520.

Russo, F. and Torrisi, M., 1951. Polyploidy in Citrus species. Autopolyploids and allopolyploids. *Ann. Sper. agr.* 5:1041-1062.

Starrantino, A., and Recupero, G.R., 1981. Citrus hybrids obtained *in vitro* from 2x females and 4x male. *Proceedings of International Society of Citriculture* 1:31-2

Scora, R.W., 1975. On the history and origin of citrus. *Bulletin of the Torrey Botanical Club* 102:369-375.

Scora, R.W., Kumamoto, J., Soost, R.K., and Nauer, E.M., 1982. Contribution to the origin of the grapefruit, *Citrus paradisi* (Rutaceae). *Systematic Botany* 7:170-177.

Silva, R.R.d., Oliveira, T.T.d., Nagem, T.J., Pinto, A.d.S., Albino, L.F.T., Almeida, M.R.d., Moraes, G.H.K.d., and Pinto, J.G., 2001. Hypocholesterolemic effect of flavonoids naringin and rutin. *Archivos Latinoamericanos de Nutricion* 51:258-264.

Soost, R.K. and Cameron, J.W., 1969. Tree and fruit characters of Citrus triploids from tetraploid by diploid crosses. *Hilgardia* 39:569-579.

Soost, R.K. and Cameron, J.W., 1975. Citrus. *Advances in fruit breeding. Subtropical fruits* 507-540.

Soost, R.K. and Cameron, J.W., 1980. 'Oroblanco', a triploid pummelo-grapefruit hybrid. *HortScience* 15:667-669.

Soost, R.K. and Cameron, J.W., 1985. 'Melogold', a triploid pummelo-grapefruit hybrid. *HortScience* 20:1134-1135.

Stanys, V., Weckman, A., Staniene, G., and Duchovskis, P., 2006. In vitro induction of polyploidy in Japanese quince (*Chaenomeles japonica*). *Plant Cell, Tissue and Organ Culture* 84:263-268.

Stewart, F.C., Mapes, M.O., and Mears, K., 1958. Growth and organizeddevelopment of cultured cells. II. Organization incultures grown from freely suspended cells. *American Journal of Botany* 45:705-708.

Sykes, S.R. and Lewis, W.J., 1996. Comparing Imperial mandarin and Silverhill satsuma mandarin as seed parents in a breeding program aimed at developing new seedless citrus cultivars for Australia. *Australian Journal of Experimental Agriculture* 36:731-738.

Tachikawa, T., 1971. Investigations on citrus breeding. III. The morphological characteristics of polyploidy in citrus. *Bull. Citrus Exp. Stn. Shizuoka*:1-10.

Tachikawa, T., Tanaka, Y., and Hara, S., 1961. Investigations on citrus breeding. Part I. Studies on the breeding of triploid citrus varieties. *Bull. Citrus Exp. Stn. Shizuoka* 4:33-34.

Tusa, N., Grosser, J.W., Gmitter, F.G., Jr., and Louzada, E.S., 1992. Production of tetraploid somatic hybrid breeding parents for use in lemon cultivar improvement. *HortScience* 27:445-447.

Tussac, L.C.F.R., 1824. Flore des Antilles 3:73-74. L'auteur, Paris.

Tuyl, J.M.v., Meijer, B., and Dien, M.P.v. 1992. The use of oryzalin as an alternative for colchicine in in-vitro chromosome doubling of Lilium and Nerine. *Sixth International Symposium on Flower Bulbs, Skierniewice, Poland* 2:625-630.

Usman, M., Saeed, T., Khan, M.M., and Fatima, B., 2006. Occurrence of spontaneous polyploids in Citrus. *Zahradnictvi (Horticultural Science)* 33:124-129.

Vardi, A. 1982. Protoplast derived plants from different citrus species and cultivars.

Vardi, A., Spiegel-Roy, P., and Galun, E., 1982. Plant regeneration from Citrus protoplasts: variability in methodological requirements among cultivars and species. *Theoretical and Applied Genetics* 62:171-176.

Veronese, M.L., Gillen, L.P., Burke, J.P., Dorval, E.P., Hauck, W.W., Pequignot, E., Waldman, S.A., and Greenberg, H.E., 2003. Exposure-dependent inhibition of intestinal and hepatic CYP3A4 in vivo by grapefruit juice. *Journal of Clinical Pharmacology* 43:831-839.

Viloria, Z., Grosser, J.W., and Bracho, B., 2005. Immature embryo rescue, culture and seedling development of acid citrus fruit derived from interploid hybridization. *Plant Cell, Tissue and Organ Culture* 82:159-167.

Wakana, A., Iwamasa, M., and Uemoto, S. 1982. Seed development in relation to ploidy of zygotic embryo and endosperm in polyembryonic citrus. *Proceedings of the International Society of Citiculture* 1:35-39.

Wang, T.Y. and Chang, C.J., 1978. Triploid citrus plantlet from endosperm culture. *Scientia Sinica* 21:823-827.

Wu, J. and Mooney, P. 2002. Autotetraploid tangor plant regeneration from in vitro Citrus somatic embryogenic callus treated with colchicine. *Special Issue on the New Zealand Regional IAPTC & B Conference, Mount Ruapehu, New Zealand* 70:99-104.

Zhang, J., Zhang, M., and Deng, X., 2007. Obtaining autotetraploids in vitro at a high frequency in *Citrus sinensis*. *Plant Cell, Tissue and Organ Culture* 89:211-216.

Zhang, W.C. 1985. Citrus clonal selection, progeny testing and in vitro propagation. *United States - People' s Republic of China Citrus Symposium* 39:20-33

BIOGRAPHICAL SKETCH

Divya Kainth was born in Chandigarh, India in 1985. She graduated from Gyan Jyoti Public School in 2003. She received a bachelors of science in agriculture from Punjab Agricultural University, Ludhiana, Punjab, India, with an honors degree with a major horticulture in 2008. In January 2009, she started master's program in horticulture at University of Florida and completed her degree in December 2010.