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PRODUCTION OF TRIPLOID SEEDLING PROGENIES FROM SWEET AND SOUR CALAMONDIN (x*Citrofortunella mitis* Ingram & Moore) BY CHEMICAL APPLICATIONS

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Title Production Of Triploid Seedling Progenies From Sweet And Sour Calamondin (x*Citrofortunella mitis* Ingram & Moore) By Chemical Applications

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ABSTRACT

In the world of citrus markets and considering consumers' preferences, seedless citrus varieties have a distinct commercial advantage. Seedless cultivars (triploid cultivars) can be produced through somatic polyploidization. This study, which was conducted at the Pomology Division in Maejo University, involved treatments consisting of 0, 0.01, 0.03, 0.05, and 0.07% applications of colchicine and trifluralin to meristematic buds of sweet and sour calamondin. Statistically significant results were found in stomatal guard cell length and width in the colchicine and stomatal guard cell length from trifluralin treatments in sweet calamondin while in sour calamondin, statistically significant difference was revealed in shoot length and leaf thickness from colchicine treatment and in stomatal numbers from trifluralin treatment. However, no significant differences were found in the shoot length, leaf number, thickness and area, chlorophyll content, and stomatal number from colchicine and trifluralin treatments and stomatal guard cell width from trifluralin treatment in sweet calamondin. Leaf number and area, chlorophyll content, stomatal guard cell length and width from colchicine and trifluralin treatments, stomatal numbers from the colchicine treatment and shoots length from the trifluralin treatment in sour calamondin also revealed no siginificant difference. Furthermore, comparison between sweet and sour calamondin after chemical application of leaf thickness, chlorophyll content and stomatal number and length, revealed highly significant difference while shoot length, leaf number and area, and stomatal width showed no significant differences. In addition, 34.38% of seedlings found in embryos, showed 0.33% to be triploid seedlings, and the rest remained diploid. Two seedlings from 0.03% and one seedling from 0.05% concentration of colchicine treatment successfully produced triploid seedlings in sweet calamondin. On the other

hand, triploid seedlings were not found in sour calamondin and other concentrations in observed seedlings but vegetative and reproductive growth differences were found among all the treatments. However, colchicine application was effective in triploid seedling production but in order to determine the exact number of ploidy levels, all embryo germination should be necessary.

Keywords: colchicine, trifluralin, chromosomes, polyploidy, sweet and sour calamondin.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURE IN APPENDIX

ABBREVIATIONS

am	After meridian
Assist.	Assistant
ANOVA	Analysis of variance
cc	Cubic centimeter
cm	Centimeter
CRD	Completely Randomized Design
ddH_2O	Double distilled water
^0C	Degree Celsius
DMRT	Duncan's Multiple Range Test
e.g.	For example
et al.	And other people
etc.	Et cetera
FAO	Food and Agriculture Organization
g	Gram
g/l	Gram per liter
hr	Hour
HCL	Hydrochloric acid
i.e.	That is
l	Liter
m	Meter
M	Molar
min	Minute/minutes
mg	milligram
ml	Milli liter
mm	Milli meter
MS medium	Murashige and Skoog medium
pm	Post meridian
ppm	Parts per million
Prof.	Professor
RT	Room temperature
v	Volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Citrus is one of the most important evergreen fruit bearing trees produced all around the world. According to FAO data 2007, citrus fruits are produced in nearly 104 countries and 70% of production is centered in the Northern Hemisphere, particularly in Brazil, Mediterranean countries, the United States, and China, which altogether represent more than two thirds of world citrus production (Mouhamad, 2008), and the production was estimated at over 105 million tons in the period of 2000-2004 (Citrus fruit, 2008).

Calamondin (x*Citrofortunella mitis* Ingram & Moore) is one of the dwarf natural hybrid citrus varieties which is widely distributed in Southeast Asia and related to the family Rutaceae. The fruit looks like kumquat and its flesh is juicy, sweet and sours (Takeuchi et al., 2005). Calamondins are a polyembryonic cultivar which has seeds containing more than one embryo. The polyembryonic seeds arise through a sexual or asexual process. Sexually, it is developed due to multiple fissions of the zygotic embryo or ovule containing more than one egg while asexual embryos develop from the nucellus (nucellar) or the integuments (integumentary) adventives (Maheshwari, 1950). The basic chromosome number of the citrus, and other genera in the subfamily of Aurantioideae is x=9 (Frost, 1925). Virtually, all wild and cultivated varieties of citrus are diploids (2n=2x=18) (Krug and Bacchi, 1943) so that the calamondin has the same number of chromosomes (2n=2x=18). However, the triploid citrus cultivars contain three sets of chromosomes *i.e.,* 2n=3x=27, and spontaneously its seeds are obtained from fertilization of a endoduplicated diploid (unreduced) female gamete with a regular haploid (reduced) male gamete (Esen, 1971).

In the world's citrus markets, considering consumers' preferences, seedless citrus varieties have a distinct commercial advantage. Seedy cultivars are accepted only if seedless cultivars are not present or are much superior in fruit characteristics than seedless cultivars. Nowadays, seedy citrus fruits are becoming unacceptable in international fresh markets. In addition, seediness is considered as an obstacle in releasing newly selected high quality mandarins (Vardi, 1996). There are a number of cultivars with desirable horticultural characteristics which have not attained commercial importance because of their seediness (Fatta

et al., 1992). Moreover, presence of a large number of seeds in calamondin fruits is a major limitation in consumer acceptability even though the fruits have high quality. Therefore, production of seedless (triploid) citrus fruits could lead to an improvement in the quality of fresh fruits.

Polyploidy evolution is recognized as an ongoing process in flowering plants (Soltis et al., 1992; Masterson, 1994; Leitch and Bennett, 1997; Ramsey and Schemske, 1998; Soltis and Soltis, 1999, Wendel, 2000). There have been various aspects of polyploidy in plants such as autopolyploid, genomic allopolyploid, segmental allopolyploid. Since angiosperms first appeared, genome doubling through autopolyploidy or allopolyploidy has been continuing in nature and most angiosperms are considered to have 'paleopolyploid' (genome duplications which occurred at least several million years ago) genomes revealed by comparative mapping (Reinisch et al., 1994; Lagercrantz and Lydiate, 1996; Shoemaker et al., 1996; Gaut and Doebley, 1997; Gomez et al., 1998; Lagercrantz, 1998; Muravenko et al., 1998; Sossey-Alaouni et al., 1998; Brubaker et al., 1999). There are many examples of polyploids in most of the horticultural crops such as citrus, banana, apple, grapes, strawberry and potatoes (Hilu, 1993). Moreover, the occurrence of spontaneous triploids among the progeny of certain diploid cultivars has been reported by several workers (Toolapong, 1994). And also tetraploids, hexaploids and octaploids are found spontaneously in citrus (Fatima, 2004). Additionally, the 'Tahiti' lime (*Citrus aurantifolia* Swing.) has been recognized as spontaneous triploid cultivars (Krug and Bacchi, 1943). It has been commercialized due to it being entirely seedless (Pauline et al., 1997).

Conventional breeding is one of the most important methodologies to produce improved cultivars. But varietal improvement has some difficulties in citrus due to the complex reproductive features of the plant (Swingle and Reece, 1967; Soost and Cameron, 1975) including polyembryony, a long reproductive cycle, sterility (juvenility), incompatibility and endogamy depression (Grosser and Gmitter, 1990). Nevertheless, there are several artificial methods to produce polyploids plants such as interspecific hybridization, embryo rescue technique, endosperm culture, irradiation, and somatic doubling by anti-mitotic agents (colchicine, trifluralin etc.).

Seedless fruits are derived from polyploidy breeding in which there is a combination of two or more differentiated genomes (Stebbins, 1971). Triploid *Citrus* cultivars

have great commercial potential because of their high degree of seedlessness but the frequency of naturally occurring triploids in *Citrus* is extremely low (Fatta et al., 2007). Triploid citrus (2n=3x=27) are possible between the crosses of diploid (2n=2x=18) to diploid (2n=2x=18) and diploid (2n=2x=18) to tetraploid (2n=4x=36) or vice versa. Moreover, triploids occasionally occur spontaneously in progenies from diploid by diploid crosses and can be systematically produced by controlled crossing of tetraploids with diploids (Fatta et al., 2007), although the incidence of variable seeds is low. Moreover, Usman et al., (2006) found the maximum triploid seedlings in lime Kaghzi (15.5%) followed by grapefruit Foster, mandarin Kinnow, sweet orange Musambi and mandarin Feutrell's Early (7.3%) from spontaneously underdeveloped seeds.

As mentioned before, the diploids plants before crossing with diploids need to become tetraploids. That is why the use of mitotic inhibitors (colchicine, trifluralin *etc.*) on shoot meristems has been the primary method of somatic polyploidization (tetraploid) in citrus (Barrett, 1974; Wakana et al., 2005; Yahata et al., 2005). Since the 1930s, colchicine has been widely used for polyploidization in plants (Blakeslee and Avery, 1937). Moreover, it gives the highest rate of polyploid induction in plants such as tetraploids (Adaniya and Shirai, 2001). In addition, trifluralin (anti-microtuble herbicide) has shown a greater affinity for binding to plant microtubules than animal microtubules and produces polyploidization in plants (Bayer et al., 1967; Toolapong, 2008). Furthermore, the aim of this study is to determine the optimum chemical concentrations of colchicine and trifluralin to produce triploid seedling progenies and triploid seedlings from sweet and sour calamondin.

Objectives of the study

To find optimum chemical concentrations of colchicine and trifluralin to produce triploid seedling progenies, and triploid seedlings from sweet and sour calamondin.

CHAPTER 2

LITRATURE REVIEW

Citrus and calamondin

Citrus

Genera, species and distribution

The term citrus originated from the Latin form of '*Kedros*', a Greek word denoting trees like cedar, pines and cypress. In Greek mythology citrus fruits are called hesperidies, and Linnaeus grouped all citrus species in the genus *Citrus* (Spiegel and Goldschmidt, 1996). Moreover, citrus is one of the most important evergreen fruit bearing trees produced all around the world which are reaching in germplasm resources, a great number of species and varieties (Guolu, 1987). Moreover, it is believed that all the species belonging to *Citrus* and its related genera originated in the tropical and subtropical regions of South-east Asia – north-eastern India, southern China, the Indo-Chinese peninsula, and the Malay Archipelago, and then spread to other part of the world (Webber, 1967; Chapot, 1975).

The commonly cultivated citrus fruits belong to three genera, *Citrus, Fortunella,* and *Poncirus*. They are all closely related and all belonging to the subtribe Citrinae, of the tribe Citreae, of the orange subfamily Aurantioideae, and family Rutaceae. However, the taxonomic treatment of *Citrus* is confusing and there is considerable debate over the number of species within *Citrus*, with estimates varying between 1 and 162 species, depending on the taxonomist, Swingle recognized 16, Hodgson 36 and Tanaka 162 species (Rieger, 2006). Nowadays, several hybrids among citrus species, and between *Citrus* and *Poncirus* or *Fortunella*, have been produced either naturally or through controlled breeding. Different prefixes and suffixes are used to denote the parents of such hybrids like Citrange (sweet orange x trifoliate orange), Limequat (kumquat x lime), Tangor (sweet orange x tangerine), Lemandarin (lemon x mandarin), Calamondin (x*Citrofortunella mitis*), *etc*. All citrus plants are large shrubs or small trees, reaching 5–15 m tall, with spiny shoots and alternately arranged evergreen leaves with an entire margin. The fragrant, white flowers are solitary or in short cymes, borne axillary on the current flush of

growth and also without leaves from the previous flush of growth, each flower is 2–4 cm in diameter, flowers are perfect, with five (rarely four) petals and sepals. Stamens number 20 to 25 and are arranged in a tight, columnar whorl around the gynoecium. A globular, green ovary subtends a thin style that terminates in a pronounced, donut-shaped stigma, and its position is superior and subtended by a raised nectary disc. Moreover, the fruit is a hesperidium, a specialized berry, globose to elongated, 4–30 cm long and 4–20 cm in diameter (Citrus, 2008); endocarp is the edible portion, divided into 10 to 14 segments, separated by thin septa, each containing up to eight seeds, but usually only one.

Calamondin

The calamondin [x*Citrofortunella mitis* (Blanco) J. W. Ingram & H. E. Moore] is one of the natural citrus hybrid varieties. They are usually referred to as *C. mitis* or *C. microcarpa* or sometimes referred to as *C. madurensis*, but this is incorrect as *C. madurensis* actually refers to *F. japonica* (Swingle, 1943; Wijnands, 1984). Ingram and Moore (1975) and Wijnands (1984) recognized the hybrid nature of this taxon with the coinages x*Citrofortunella mitis* and x*Citrofortunella microcarpa*, respectively. Additionally, its alternate common names are: calamondin orange (China), calamansi or Bangkok calamansi, kalamondin (Philippines), limau kesturi, limau chuit (Malayan), ma-nao-wan (Thailand).

The native place of calamondin is believed to be China and thought to have been taken in early times to Indonesia and the Philippines; also it is widely grown in India and throughout southern Asia and Malaysia. Moreover, it is a common ornamental dooryard tree in Hawaii, the Bahamas, some islands of the West Indies, and parts of Central America and also it is much utilized for greenhouse research on the various aspects of flowering and fruiting in citrus at the Agricultural Experiment Station of the University of Florida in Gainesville. The calamondin tree, ranging from 2.0 to 7.5 m high, is erect, often quite cylindrical, densely branched beginning close to the ground, slightly thorny, develops an extraordinarily deep taproot and grows well in tropical climates up to an elevation of 1,000 meters above sea level (Patena et al., 2005). The evergreen leaves are alternate, aromatic, broad-oval, dark-green, glossy on the upper surface, yellowish-green beneath, 4.0 to 7.5 cm long, faintly toothed at the apex, with short, narrowly-

winged petioles. The richly and sweetly fragrant flowers, having 5 elliptic-oblong, pure-white petals, are about 2.5 cm wide and borne singly or in 2's or 3's terminally or in the leaf axils near the branch tips. The fruits are round or oblate and nearly 4.5 cm wide, with very aromatic, orange-red peel, glossy and dotted with numerous small oil glands; tender, thin, sweet and edible. The pulp, in 6 to 10 segments, is very juicy, acidic and sweet, and has up to 5 seeds.

It is a cold, hardy and moderately drought tolerant plant. The trees are easily grown in a wide range of soils from clay-loam to limestone or sand. Calamondins are polyembryonic with 3 to 5 embryos each (Morton and Miami, 1987). It can be easily grown from seeds and also by vegetative method. Its flower is self-fertile and usually does not require cross pollination. The calamondin plant is primarily grown as a house plant because of its bushy, normally dwarfed, nearly thorn less and year round bearing nature, and often called a 'miniature orange'. So, calamondins are widely grown as an ornamental tree. It has beautiful creamy and green variegated foliage and pretty orange coloured fruit in contrast to green leaves. Fragrant flowers appear in spring to summer and a heavy set of fruit follows, ripening in time. The ripe fruit holds well on the plant adding to its decorative show. The variegation makes it more desirable for container culture and the fruit contrasts especially well against the beautiful bi-coloured leaves and thick foliage. However, only the 'Peter' calamondin variety shows the variegated nature.

Furthermore, the calamondin flesh has been used as a garnish for fish and chicken dishes in Southeast Asia (Takeuchi et al., 2005). Its halves or quarters may be served with iced tea, seafood and meats, to be squeezed for the sour juice, which is primarily valued for making sour and sweet beverages, and is preserved into sweet pickles or marmalade. The fruits juice of calamondin can also be applied to the scalp after shampooing. It eliminates itching and promotes hair growth. Rubbing calamondin juice on insect bites banishes the itching and irritation. It is taken orally as a cough remedy and antiphlogistic. When slightly diluted and drunk warm it serves as a laxative.

Zygotic and nucellar embryony

In general, citrus are cross pollinated plants, and the most common varieties are apomitic and polyembryonic seeds. Polyembryony is the phenomenon of multiple embryos in one seed which is developed from a single fertilized egg. Moreover, it is associated with nucellar embryony because it frequently results in polyembryonic seeds from which multiple seedlings germinate. Asexual reproduction in seeds is an important characteristic in citrus. It strongly influences the method and result of breeding. Most citrus species can produce asexual progeny, which arise from somatic tissue of the nucellus or the development of embryos from the maternal tissue which surrounds the embryo sac (Kobayashi et al., 1997).

Nucellar embryony shows a major problem in citrus breeding because of difficult to distinguish nucellar and zygotic seedlings at an early stage. The nucellar initial cells occur in all portions of the nucellus, but many migrate to the micropylar end so in many varieties nearly all nucellar embryos are found at the micropylar end (Wakana and Uemoto, 1988). However, the developmental competition between the zygotic and nucellar embryos, as well as the genotype of the zygote, will affect maturation, in seeds with mature nucellar embryos, immature zygotic and nucellar embryos are often present but do not germinate (Ueno et al., 1967; Esen and Soost, 1977; Koltunow et al., 1995). Additionally, the number of embryos per seed varies greatly even on one tree. The average number differs greatly according to variety, and there is no general consistency within many of the species in which polyembryony is present (Frost and Soost, 1968). Moreover, this variation has been suggested to be controlled by minor genes, pollen source and environmental conditions (Frost, 1926; Parlevliet and Cameron, 1959; Khan and Roose, 1988). Most citrus cultivars have more than 90% nucellar seedlings (Roose and Kupper, 1992); however, nucellar embryony does not prevent normal sexual reproduction (Esen and Soost, 1977; Wilms et al., 1983). Virtually all evidence indicates that the initiation of nucellar embryos in nature requires not only pollination but probably also the fertilization of the egg or the polar nuclei. Fertilization of the egg cell in citrus has been reported to occur from 2 to 3 days or more than a week after pollination, and cell division of the zygote may commence soon after.

However, the pollen parent can sometimes influence the proportions of zygotic seedlings. Thus rough lemon, when self pollinated, can produce almost exclusively nucellar

seedlings. Additional embryos in a seed are not always of nucellar origin. Cultivars which are typically mono-embryonic occasionally produce two or more embryos per seed. Cultivars that produce nucellar seedlings also occasionally produce zygote twins, but typically mono-embryonic cultivars have not been shown to produce nucellar seedlings under natural conditions. The attribute of nucellar embryony seems to be inherited in a fairly simple fashion. Most citrus and related species are highly heterozygous and thus produce widely variant sexual (zygotic) progeny. The percentage of nucellar seedlings ranges from zero for zygotic species like Pummelo [*Citrus grandis* (L.) Osbeck] and cultivar such as Clemantine mandarin and Temple orange to virtually 100% for 'Dancy' and 'Kara' mandarins. The tendency to produce nucellar compared with zygotic embryos is inherited simply in a 1:1 ratio in some instances, but deviates from this ratio in other crosses depending on the species involved (Cameron and Soost, 1979).

Polyploidy

Polyploids may simply be defined as plants combining three or more basic genomes [A genome is the total DNA in one basic set of chromosomes (x)] of the taxonomic group in which they belong. Or it belongs to either duplication of a single genome (autopolyploidy) or from the combination of two or more differentiated genomes (allopolyploidy) (Stebbins, 1971; Grant, 1981). Furthermore, polyploidy is an active and ongoing process in the plant kingdom (Stebbins, 1950; Soltis et al., 1992; Masterson, 1994; Leitch and Bennett, 1997; Ramsey and Schemske, 1998; Soltis and Soltis, 1999; Wendel, 2000).

Polyploidy has played an important role in applied plant breeding. Allopolyploids result from increase of a chromosome through hybridization while autopolyploids emerge from chromosome doubling of the same genome. Therefore, the genetic and genomic attributes of polyploids may have both biochemical and ecological benefits that contribute to the success of polyploids in nature (Soltis and Soltis, 1999). The several allopolyploids wheat, cotton, *Brassica napus* L., *Arabidopsis suecica* Fr. soybean and tobacco, have become experimental systems for addressing questions in younger allopolyploids. Even more recent are allopolyploids in *Tragopogon, Spartina* and *Senecio* that were formed within the past 200 years (Sebastian, 2000; Abbott and Lowe, 2004; Ainouche et al., 2004; Soltis et al., 2004). It provides genome buffering,

increased allelic diversity and heterozygosity, and novel phenotypic variation to be generated (Udall and Wendel, 2006). Additionally, it is widespread in the plant kingdom, common in many genera and numerous species comprise diploid and polypolid plant types (Sebastian, 2000). Moreover, at least 50% of all angiosperm species are polyploid (Hia and Bennett, 1997; Hancock, 2004).

Diploids are the general rule in citrus (Krug and Bacchi, 1943) and the gametic (n) chromosome number is 9 (Frost, 1925). *Fortunella, Poncirus*, *etc.* are regularly diploid. Triploids, tetraploids and other higher ploidy plants occasionally occur spontaneously as zygotic seedlings in citrus (Frost, 1925; 1935; Longley, 1925; Luss 1935; Lapin, 1937; Krug, 1943; Cameron and Frost, 1968; Esen and Soost, 1971; Cameron and Burnett, 1978). The triploids are significantly important due to seedlessness among polyploids and the frequency was as high as 5% among the seedling progeny (Frost, 1925, 1935; Lapin, 1937). Moreover, triploids have been reported among progeny of openly pollinated 'Lisbon' lemon, 'Eureka' lemon, 'Rubey' sweet orange and 'Imperial' grapefruit suggesting unexpected triploids to be common among progenies of citrus cultivars (Esen and Soost, 1971). Only 'Tahiti' lime attained commercial significance among naturally occurring triplods due to their low seed contents and seedlessness (Vardi and Spiegel-Roy, 1978; Vardi, 1996).

The triploids may arise from unions of 2x or unreduced gametes with x gametes among diploids (Froelicher et al., 2000). The result of triploid embryos from the union of the usual haploid egg with diploid male gametes from the tetraploid pollen parent or an unreduced egg with haploid gametes from diploid pollen parents or diploid egg (tetraploid plant) with haploid gametes from diploid pollen parents. It contains three set of chromosomes, *i.e.*, 2n=3x=27. The embryo and endosperm are initiated and appear to develop normally, but in most cases the endosperm fails before seed maturity and the embryo does not survive. In these crosses (2x X 2n = 4x), the triploids result from the production of diploid gametes by the seed parents in low frequencies.

The most spontaneous tetraploids have been obtained as nucellar seedlings in the course of breeding and varietal studies. They have occurred in frequencies ranging from under 1-6% in all majors group of cultivars, including orange, lemon and grapefruit (Cameron and Frost, 1968). Moreover, Usman et al. (2006) recovered seedlings from underdeveloped spontaneously

occurred seeds and found that maximum triploid plants in lime Kaghzi (15.5%) followed by Grapefruit Foster, Mandarin Kinnow, Sweet orange (Musambi) and Mandarin Feutrell's Early (7.3%). Lime also produced the highest percentage of tetraploids (9.1%) followed by Mandarin, Kinnow and Grapefruit Foster, while the minimum number of tetraploids was found in Mandarin Feutrell's Early. Spontaneous tetraploids have been obtained from other cultivars and individuals that produce nucellar seedlings in sufficient quantities. They often occur in seeds which produce diploid seedlings and seeds which mainly produce diploids, indicating that doubling occurs in the ovule or ovary (Cameron and Soost, 1979). Additionally, the effect of tetraploidy in citrus can be rather accurately evaluated in nucellar sections. Except for possible bud variation, such tetraploids differ genetically from sister nucellar diploids only in chromosome number. Furthermore, morphologically, the polyploids tend to be larger than diploids, with thicker leaves and a robust plant structure, and they may respond differently to environmental conditions (Ray, 2002). Additionally, the character of citrus tetraploid shows that these forms have little economic value in themselves. However, they are of interest as breeding material, since they can be used in crosses with diploids to produce triploids.

Triploids occasionally occur spontaneously in progenies from diploid to diploid crosses. Unlike the spontaneous tetraploids, they are always of zygotic origin as high as 5% of triploids have been identified in some progenies. Triploids can also be systematically produced by a controlled crossing of tetraploids with diploids (Fatta et al., 2007), although the incidence of variable seeds is low. The triploids show considerably more than average vigor than tetraploids. They also show many genetic variability characteristics of sexual citrus progenies. They produce large flowers, fruits with a thick rind, large oil glands and large seeds, and can usually be identified as polyploidy by their thick, rounded leaves (Cameron and Soost, 1979; Wakana et al., 2005).

In citrus breeding, hybridization between diploid and tetraploid parents to produce triploid progeny is common and being widely used to develop of new seedless varieties mostly in mandarins (Williams and Roose, 2000), and also several researchers reported that it is a way to produce triploids by crosses of diploid seed parents with tetraploid parents (Esen and Soost, 1972; Oiyama and Okudai, 1988, Oiyama and Kobayashi, 1990); but the 2x X 4x crosses have a limitation to recover triploid progenies due to the problem of embryo abortion in citrus (Esen et

al., 1979; Oiyama et al., 1981). Another problem is a lower setting of fully developed seeds (Cameron and Soost, 1969; Esen and Soost, 1972), and many empty and few fully developed seeds where a small percentage of the seeds produced were triploid (Tachikawa et al., 1961; Cameron and Soost, 1969; Esen and Soost, 1972; Cameron and Burnett, 1978; Esen et al., 1979; Oiyama et al., 1981; 1991). However, result showed that 4x X 2x crosses had a higher seed set than that of reciprocal crosses, and high proportions of 3x hybrids were obtained from crosses of 4x sexual seed parents by 2x pollen parents in citrus (Cameron and Burnett, 1978).

Induction of polyploidy by chemicals

Citrus is a highly heterozygous plant and produced mostly by nucellar embryony. That is why genetic improvement of citrus through conventional plant breeding has been limited. However, mutation breeding is one of the ways to improve the genetic constituents of citrus. Natural mutations and axilliary bud sports (parts of a plant, which due to a genetic mutation clearly differ from the rest of the plant) have often been found in citrus. On the other hand, breeders use to apply physical and chemical mutagens on various *Citrus* species and varieties for somatic cell mutation. Colchicine, trifluralin *etc.* are used as chemical mutagens for inducing somatic chromosomes to double in order to obtain artificial autopolyploids, from their diploid ancestral species. The mutagenesis technique offers the opportunity of obtaining seedless polyploidy types of the established cultivars without modification of their main qualities (Whu et al., 1986).

Colchicine

Colchicine is an alkaloid found in the corms and seeds of the autumn crocus or meadow saffron (*Colchicum autumnale* L.). Its generic name is (S)-N-(5, 6, 7, 9-tetrahydro-1, 2, 3, 10-tetramethoxy-9-oxobenzo (a) heptalen-7-yl) acetamide ($C_{22}H_{25}NO_6$) and is used in aqueous solutions, in paste mixture with lanoline, in agar, or in glycerin solutions. Moreover, it is applied in low concentration to the growing tips and lateral buds of plants and it can be used to produce new cultivars (Harrington, 2000). It inhibits the spindle mechanism at mitosis (Autopolyploidy

and somatic polyploidy, 2008) and results in cells with double or more than double chromosome number. Additionally, it gives the highest rate of polyploid induction (Chulalaksananukul and Chimnoi, 1999) such as tetraploids (Adaniya and Shirai, 2001; Chen and Gao, 2007).

Jaskani et al. (1996) reported that treatment of citrus buds with colchicine is a useful approach for creating tetraploids while watermelon tetraploid plants were produced through treating diploid seedlings with an aqueous solution of colchicine (Kihara, 1951; Andrus et al., 1971). Triploid progenies also could be obtained in this way (Madon et al., 2005; Hongzhi et al., 2007). Hannweg (1999) induced triploid and tetraploid *in vitro* using colchicine in mandarin and oranges while Zhang et al., (1988) used the chemical mutagen colchicine *in vitro* which resulted in polyploidic mutants (Jincheng 5.25% and Daoyicheng 58.3% of 3x and 4x plants, respectively).

Aneuploids may occasionally arise after colchicine treatment because irregularities in chromosome division and separation; that is one or more chromosomes may be added or lost at the original level. However, the changes not involving loss or gain in the chromosome number may arise as a result of colchicine treatment (Franzke and Ross, 1952 Cited by Elliott, 1958). The highest chromosome doubling success rate was found in haploid cereal plants when a study was carried out at a temperature-controlled greenhouse with a hydroponic system (Ahmad and Darvey, 2001). Furthermore, tetraploids were derived from diploid cultivars (like *Citrus*) and treated with colchicine (Tachikawa, 1971; Koutoulis et al., 2005). Barrett (1974) successfully induced tetraploids from diploid citrus cultivars as well as periclinal ploidy chimeras by a colchicine treatment (1.0% solution with distilled water) on the sprouting axillary buds, and found that 25% were changed and remained unchanged at the ploidy level. In polyembryonic diploid cultivars, Gmitter and Ling (1991) recovered non-chimerical tetraploids from the undeveloped ovules cultured on the medium supplemented with 0.01 and 0.10% colchicine. Moreover, Tetraploid forms of the cultivars (acid citrus cultivars) were derived from the shoot sections treated with 0.05-0.4% colchicine solutions for 12 hr for 'Yuzu', 0.05-0.4% for 6 hours for 'Kizu' and 0.1 and 0.2% for 6 hours for 'Hanayu' (Wakana et al., 2005). Additionally, the tetraploid plants were produced by somatic hybridization between polyembryonic cultivars and between polyembryonic and monoembryonic cultivars using embryogenic callus from polyembryonic cultivars (Kobayashi and Ohgawara, 1988; Gmitter and Ling, 1991). However, the presence of a

barrier of nucellar embryony in these tetraploids, led to the use of them as pollen parents, since production of triploids is very difficult or at extremely low frequencies (Soost, 1987).

The effect of colchicine on the polyploidisation in seeds of *Colophospermum mopane* Kirk ex J. Leonard (Leguminous trees in subtropical southern Africa) was studied *in vitro* by Rubuluza et al., (2007). Seeds of *C. mopane* were imbibed in colchicine solutions of 0.05%, 0.10% and 1.00% (w/v) for 24, 48 and 96 hr before transfer to a 1/8 MS basal medium, and which resulted in 44% of the seedlings being tetraploids, one was a chimera while the remainder were diploids. Additionally, 89% of the plantlets from calli treated with colchicine were double haploids (2n=6x=42) found in 'Povan' (wheat variety) (Hassawi and Liang, 1991). Furthermore, Lehrer et al. (2008) noticed the effect of colchicine in the Japanese barberry plant (*Berberis thunbergii* var. *atropurpurea* DC.), when the plant's pre-germinated seeds were treated with an aqueous solution of colchicine (0.02%, 0.05%, 0.10%, and 0.20%). After 6 and 52 weeks the seedlings' ploidy level were determined and it was found that the most efficient colchicine concentrations were in the range from 0.05%-0.20% and produced 40% to 76% tetraploid seedlings respectively.

Yang et al. (2006) revealed that the vigorously growing globular embryos of grape vines were selected and treated by 0, 10 or 20 mg/l colchicine for 1, 2 or 3 days, the number of surviving embryos and regenerated plantlets decreased with increasing the colchicine concentration and treatment time, and found that five solid tetraploids (2n=4x=76) among 29 randomly investigated plantlets regenerated from colchicine treated somatic embryos, and remained diploid (2n=2x=38). However, only 2 plants out of 152 studied contained the tetraploid (2n=4x=80) and the rest of the plants having the normal diploid (2n=2x=40) were found in *Ilex paraguariensis* St. Hil. (Tea like beverage, popular in Argentina, Uruguay, Paraguay and southern Brazil) were treated with the anti-microtubule agent colchicine 0.10%, 0.20%, 0.50% (Rey et al., 2002). The seedlings shoot apices of *Phlox drummondii* Hook. (kind of flower, Polemoniaceae) were induced with 0.50% of colchicine and found to be autotetraploid plants, that show excessive growth, both at the cellular level as well as at the organ level (Dhillon, 2007). Moreover, in the axillary shoot buds of the haploid Pummelo treated with 0.05%, 0.10% and 0.20% colchicine and it was found that those new shoots had cytochimera of x+2x, appearing at a high frequency under all treatment conditions: 40.0% at 0.05%, 25.0% at 0.10% and 37.5% at 0.20% colchicine

treatment, respectively (Yahata et al., 2005). Furthermore, the cytochimera of 2x+4x was obtained from 20.0% and 12.50% of new shoots arising from axillary shoot buds treated with colchicine at 0.05% and 0.10%, respectively, but a complete diploid was not found under any treatment conditions while liquid colchicine was used in sugar beet (*Beta vulgaris* L.) and gave higher chromosome doubling rate and was more effective (Gurel et al., 2000).

Trifluralin

Trifluralin is a man made chemical that looks like a yellowish orange solid or crystal. Its generic name is α, α, α-trifluoro-2, 6-dinitro-N, N-dipropyl-para-toluidine [$F_3C(NO_2)_2C_6H_2N(C_3H_7)_2$] and it is insoluble in water while soluble in xylene, acetone and ethanol. The herbicidal properties of trifluralin were first reported by Alder et al. (1960), and its anti-mitotic behaviour was found in trifluralin treated root tips of onion (*Allium cepa* L. 'Yellow') (Bayer *et al.*, 1967); moreover, they reported the degree of anti-mitotic behaviour to be variable. After that, many workers have demonstrated that trifluralin causes aberrant mitosis and the obtainable soluble concentrations inhibit the microtubule mediated process in plants (Hess and Bayer, 1977). Moreover, trifluralin affected mitosis in the same manner as colchicine. Arrested metaphases, c-pairs, micronuclei, amoeboid nuclei, and polyploidy were observed at various times after treatment in onion bulbs (Lignowski and Scott, 1972).

The doubling of male bicellular cells were found in the flowers of mandarin, calamondin, and lime when different concentrations of trifluralin (0.05%, 0.10%, 0.20%, and 0.40%) were applied to the 35-40 cm length of flush twigs, and produced a correspondingly high number of flowers with male bicellular cells and also the normal and male bicellular cells appeared to be similar in size, because the central arrangement of the chromosomes is affected by trifluralin and the natural form of spindle micro-tubules failed during cell division, which resulted in the 'doubling' of the chromosome numbers (Toolapong, 2008). Furthermore, Zlesak et al. (2005) investigated the efficiency of trifluralin for polyploidization in *Rosa chinensis minima* Sims Voss seedlings, when 0.086% and 0.0086% of trifluralin solution was applied to the apical meristem of seedlings in the process of cotyledon expansion and found that the 0.086% level was the most effective treatment for polyploidization in the meristematic layer and its effectiveness

was 20.20%. While Akio (1999) found 24% of triploid and 4% of aneuploid ($2n$ = 19, 21, and 22) seedlings by fertilization between a central cell ($2n$) of diploid plants and the mitotically arrested generative cell ($2n$) of the bicellular pollen induced by trifluralin solution on maize (*Zea mays* L.).

Identification of polyploids

There are many plant phenotypic characteristics which show the plant's ploidy levels, as well as some microscopic observations which allow reasonable accuracy of ploidy determination because always chromosome counts from each plant is not possible, but the only sure ways to determine exact ploidy levels, is to count the chromosome numbers (Ray, 2002).

Morphology

The citrus leaf is unifoliately compound and pinnately reticulate in venation and the newly formed leaf primordia are cylindrical and curve over the apical dome. As the leaf primordium enlarges, it becomes erect and then gradually reflexes away from the axis. In most *Citrus* species, the petioles are winged. Grapefruit (*Citrus paradisi* Macf.) and Pummelo [*Citrus grandis* (L.) Osb.] petiole wings are large, those of sweet orange are smaller, and petioles of the lemon leaf area without wings. Leaf blades of oranges and lemons are oval to oblong in form, dark green on the upper surface and light yellow-green on the lower surface when mature, and may persist for two seasons or more. The oil glands of diploid leaf blades appear as numerous, circular bright areas and are slightly greenish yellow. However, in calamondin, the evergreen leaves (technically single leaflets) are alternate, aromatic, broad-oval, dark-green, glossy on the upper surface, yellowish-green beneath, 4-7.5 cm long, faintly toothed at the apex, with short, narrowly-winged petioles (Morton and Miami, 1987).

The growth and development of plant organs, including leaves, depend on cell division and expansion. Leaf size is increased by greater cell ploidy, but the mechanism of this effect is poorly understood. But increasing ploidy results in larger cells, thicker (dark green) and wider leaves which are less pointed, large flowers and fruits. Branches and stems will often be thicker, with shortened nodes and internodes while lower ploidy is characterized through thinner

(pale green), narrower and more pointed leaves, small flowers and flower parts, thinner branches and stems and distinct loss of vigour. Additionally, for high ploidy levels very slow and weak growth rates are found (Sanford, 1983). Tetraploid cultivars have faster leaf elongation rates than diploid cultivars, resulting in longer leaves, mainly due to their longer mature cells. The increase in cell length of the tetraploid cultivars was caused by a faster cell elongation rate, not by a longer period of cell elongation (Sugiyama, 2005). Furthermore, it has been reported that the leaves of tetraploid citrus genotypes are broader and thicker and have darker green colour as compaired to those of the sister diploid genotypes (Barrett and Hutchison, 1978; Khan et al., 1992).

Nevertheless, the physiological processes of plants and their productivity are not well understood and some cultural techniques (irrigation, fertilization, *etc.*) can improve them (Marini and Trout, 1984; Sansavini et al., 1985).

Stomatal observation

The upper epidermis of the mature leaf is composed of tabular parenchyma cells covered by a thick layer of cuticle, but stomata are lacking, while the lower epidermis is made up of tabular parenchyma cells interspersed with stomata. The structure of stomata both in leaf and stem is similar and consists of a pore and two guard cells. The mesophyll of mature leaf cells is composed of a palisade parenchyma which is tightly packed into two or three layers. But the spongy mesophyll is approximately eight layers thick and contains a large amount of intercellular space (Schneider, 1968).

Leaves are the primary photosynthetic organs of most plants and they have small pores (stomata) on their surface which allows carbon dioxide to enter the leaf and oxygen to escape to facilitate photosynthesis. Additionally, photosynthesis, transpiration, dry matter production and yield are more or less related to pore size, frequency and distribution of stomata (Miskin et al., 1972; Liang et al., 1975; 1997; Sapra et al., 1975; Ackerson and Hebert, 1980). Stomata size and number is related to the ploidy level. But, the number of stomata on leaf surfaces varies widely among different species of plants and the ploidy levels (Case, 1994). Furthermore, number of stomata decreased with increases in the chromosome numbers in coffee (Achyuta and Vishveshwara, 1960). But, van Duren et al. (1996) and Azhar et al. (2000)

demonstrated that stomata density was significantly higher in mixoploid than in diploid plants in banana. In *Scutellaria baicalensis*, the stomata of autotetraploids were larger but fewer compared to the controls (Gao et al., 2002) and for ornamental Alocasia, the stomatal length increased with the ploidy level (Thao et al., 2003). The effect of ploidy in stomatal frequencies, stomatal guard cell length, and genotypic differences in stomatal frequency were observed among cultivars of same ploidy level, however; stomatal frequency can be predicted with 83 and 87% accuracy by measuring the stomatal guard cell length in coffee (Mishra, 1997). Nevertheless, Madon et al. (2005) found no significant difference in the stomata density between polyploids and controls (2n) while plant morphology and polyploids seemed to be significantly shorter than their respective controls in oil palm. Furthermore, it is known that various physiological factors, for example, light intensity, leaf development and water content of the plant can influence the stomata density (van Duren et al., 1996).

Chlorophyll content observation

Chlorophyll is a green pigment found in most plants and is vital for photosynthesis, which allows plants to obtain energy from light. Chlorophyll molecules are specifically arranged in and around photosystems which are embedded in the thylakoid membranes of chloroplasts. In these complexes, chlorophyll serves two primary functions. The function of the vast majority of chlorophyll is to absorb light and transfer that light energy in the reaction center of the photosystems.

The chlorophyll content has also been explored as a possible means to differentiate between different levels of ploidy with varying levels of success in different species (Joseph and Randall, 1981; Mathura et al., 2006). While distinict differences in chlorophyll content were found in relation to ploidy level in some species (Warner and Edwards, 1993; Romero-Aranda et al., 1997), chlorophyll content was found to be constant in relation to ploidy in other species (Timko and Vasconcelous, 1981; Warner and Edwards, 1989). Furthermore, the tendency of chlorophyll content to increase with an increase in ploidy level is not always apparent. Ploidy determination on the basis of a change in chlorophyll content might be misleading if the nature of the change has not been predetermined. Warner and Edwards (1989) showed that chlorophyll

content remained constant in various levels of ploidy in *Atriplex confertifolia* Torr. & Frem.; diploid, tetraploid, hexaploid, octaploid and decaploid, whereas other proteins were found to increase. Moreover, similar evidence was reported in castor bean, *Ricinus communis* L., where the relative chlorophyll content was found to be similar in haploid, diploid and tetraploid plants (Timko and Vasconcelos, 1981).

Ovule Observation/ Unreduced gamete observation

In seed plants, the word "ovule" literally means "small egg". It consists of three parts, the integuments forming its outer layer, the nucellus (or megasporangium), and the megaspore derived female gametophyte (or megagametophyte) in the center. The ovule is located within the actual flower, the part of the carpel known as the ovary, which ultimately becomes the fruit.

Depending on the plant, flowers may have one or multiple ovules per ovary. The ovule is composed of diploid maternal tissue that gives rise to the haploid tissue of the female gametophyte. The maternal tissues of the ovule include the integuments and the nucellus. The next "generation" formed within the ovule are the haploid megaspore and megagametophyte, or embryo sac. After fertilization of the egg cell and formation of a zygote, the ovule contains the embryo of the next generation. The integuments are the outer cell layers of the ovule enclosing the nucellus. The integuments develop into the seed coat when the ovule matures after fertilization. The integuments do not enclose the nucellus completely but leave an opening at its apex referred to as the micropyle. The micropyle opening allows the pollen tube to enter the ovule for fertilization. Located opposite from the micropyle is the chalaza where the nucellus is joined to the integuments.

The nucellus (plural: nucelli) is the central portion of the ovule inside the integuments. It consists of diploid maternal tissue and has the function of a megasporangium. In immature ovules, it contains a megasporocyte (megaspore mother cell), which undergoes sporogenesis via meiosis. Three of the four haploid cells produced in meiosis degenerate, leaving one surviving megaspore inside the nucellus. After fertilization, the nucellus develops into the perisperm (nutritive tissue from a plant nucleus that surrounds the seed embryo) that feeds the

embryo. In some plants, the diploid tissue of the nucellus can give rise to a seed through a mechanism of asexual reproduction called nucellar embryony. The megagametophyte, also referred to as embryo sac, is much smaller and develops from the megaspore through three rounds of mitotic divisions. The cell closest to the micropyle opening of the integuments differentiates into the egg cell, with two synergid cells by its side that may be involved in the production of signals that guide the pollen tube. Three antipodal cells form on the opposite (chalazal) end of the ovule and later degenerate, serving no obvious function. The large central cell of the embryo sac contains two polar nuclei. The pollen tube releases two sperm nuclei into the ovule. In flowering plants, one sperm nucleus fuses with the egg cell into a zygote, the other fuses with the two polar nuclei of the central cell to give rise to the triploid endosperm. This double fertilization is unique to flowering plants. After fertilization, the ovule develops into a seed (Ovule, 2008).

If a polyploid arises from the union of unreduced gametes from closely related diploid parents, it will have four homologous chromosomes and is often referred to as an autotetraploid (Sanford, 1983; Ramsey and Schemske, 1998), and may lack fertility due to the presence of multiple homologous chromosomes (Stebbins, 1950; Riesberg, 2001; Ranney, 2006). Polyploids that result from unreduced gametes from different species are referred to as allopolyploids and are often fertile due to nonrandom, disomic pairing between two distinct sets of chromosomes during meiosis (Ramsey and Schemske, 2002; Ranney, 2006). Triploids that produce unreduced gametes can be utilized as bridges for the development of tetraploids by crossing them back with diploids (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). Moreover, hexaploids may also be produced by intercrossing among triploids that produce viable unreduced gametes (Ehlenfeldt and Vorsa, 1993). Frequencies of unreduced gamete production are highly variable, may differ considerably among individuals, and vary with environmental factors such as temperature (Dweikat and Lyrene, 1988; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998).

Polyploids were generated from the cross of the diploids 'Tosa-Buntan' and 'Suisho-Buntan' pummelo [*Citrus grandis* (L.) Osb.]. The chromosome compositions of diploid, triploid, and tetraploid progenies were observed and it was found that thirteen kinds of chromosome composition were manifested by 38 diploid seedlings and which indicates that the chromosome compositions of seedlings are highly variable. The number of chromosome varied

among progenies suggesting that they resulted from an unreduced female gamete at the first or second meiotic division, or an unreduced male gamete at the second meiotic division. Triploid seedlings also originated from an unreduced male gamete as well as from an unreduced female gamete by crossing between diploid 'Tosa-Buntan' and 'Suisho-Buntan' (Yang et al., 2002).

Chromosome observation

The chromosomes are an organized structure of DNA and proteins which are found in cells. They are bearers of hereditary material and vehicles of transmission from cell to cell and generation to generation. The presence of a chromosome was first demonstrated by Strasburger (1875 cited by Sen and Kar, 2005). Accurate studies of the chromosomes are possible in the mitotic metaphase and reveal chromosome length, location of primary and secondary constrictions and number of satellites of the chromosomes. Such studies have great significance in *Citrus* species because of taxonomic complexity of varieties and species. Researchers showed that the cytological analysis or chromosomes counts confirmed the exact and different ploidy levels in citrus progenies (Grosser et al., 1996).

Nucleoli observation

A nucleolus (plural nucleoli) is a small, typically round granular body composed of proteins and nucleic acids found within the nucleus. It is usually associated with a specific chromosomal site and involved in ribosomal RNA synthesis and the formation of ribosomes. Ribosomal RNA (rRNA) is transcribed and assembled within the nucleolus. The nucleolus ultra-structure can be visualized through an electron microscope.

The somatic ploidy levels (genome number) and nucleoli numbers have a very close relationship. The genome size and the number of nucleoli may be used to determine the ploidy level as an alternative to chromosome counting (Vilhar et al., 2002). However, phytohormone treatment increased both the sizes and the number of nucleoli in meristematic cells of seedling roots in all wheat species studied and it was assumed that the differences between the responses of wheat species with three different ploidy levels to different concentrations of

phytohormones were related to their effects (Fatkhutdinova et al., 2002). Moreover, Valdemar et al. (1986) suggested that the numbers of active Ag-NORs was virtually constant in all cells, plants and cultivars, although the size of NORs was different in many cases. Nevertheless, Toolapong (1999) found the sizes; numbers and frequencies of nucleoli in observed cells differentiated by types, ploidy levels, stages of cell development or cell cycle, and genotype of citrus hybrid progenies.

CHAPTER 3

MATERIALS AND METHODS

Plant materials

Twenty six sweet and seventeen sour calamondin pot plants approximately 2.5 feet high, each were obtained from the chairperson of the advisory committee and placed at the orchard of the Pomology Division of Maejo University. The plants were grown under natural daylight conditions.

Chemicals and other materials

Various types of chemicals and other materials were used in this experiment for observations of stomata, chromosome, nucleoli number, *etc.* as mentioned below:

Colchicine

Trifluralin

Acetic acid

Ethyl alcohol

Hydrochloric acid

Lacto-propionic orecin

8-hydroxyquinoline (0.002 M)

Aqueous silver nitrate

Nail polish

Cryomatrix

Freezing microtome

Fast green

Safranin

Petri dishes

Microscope

Slides

Cover slips

Experiment and experimental design

Experiment observation of triploid seedling progenies from sweet and sour calamondin.

In order to fulfill the above mentioned experiment's objectives the following chemicals were used and the experiment was conducted with completely randomized design (CRD) and with four replications.

Colchicine treatments

Treatment 1 control
Treatment 2 0.01%
Treatment 3 0.03%
Treatment 4 0.05%
Treatment 5 0.07%

Trifluralin treatments

Treatment 1 control
Treatment 2 0.01%
Treatment 3 0.03%
Treatment 4 0.05%
Treatment 5 0.07%

Replications = 4

Method of chemical preparation

Colchicine concentration preparation

98% colchicine = 980,000 ppm

980,000 ppm = 10,000 ppm x 100 g

$$V = \frac{10{,}000 \text{ ppm} \times 100}{980{,}000}$$

= 1.0204 g

=1.02 g

1.02 g colchicine diluted and added to 100 ml water = 1% stock solution of colchicine

Concentration	Stock solution of colchicine	Distilled water
0.01%	1 ml	99 ml
0.03%	3 ml	97 ml
0.05%	5 ml	95 ml
0.07%	7 ml	93 ml

Trifluralin concentration preparation

44.5% trifluralin = 445,000 ppm

445,000 ppm = 10,000 ppm x 100 ml

$$V = \frac{10{,}000 \text{ ppm} \times 100 \text{ ml}}{445{,}000}$$

= 2.247

= 2.25 ml

2.25 ml trifluralin + 97.75 ml water = 1% stock solution of trifluralin

Concentration	Stock solution of trifluralin	Distilled water
0.01%	1 ml	99 ml
0.03%	3 ml	97 ml
0.05%	5 ml	95 ml
0.07%	7 ml	93 ml

Method of experiment

Twenty six sweet and seventeen sour calamondin pot plants were used for this study. 40 healthy branches were selected of the sweet calamondin and the same number for sour calamondin, and the apex part of the selected branches was removed except in three axillary buds that were approximately 2 inches high. The cotton wool was soaked with 0.00, 0.01, 0.03, 0.05 and 0.07% of colchicine and trifluralin and was applied to the axillary buds, wrapped with a plastic sheet, tied with thread, and then covered with aluminium foil. The treatments were done from 4.00 – 5.00 pm. After six days, all the wrapping materials were removed and then the buds started to emerge slowly. Any new growth except that originating from the three treated buds on each branch was removed. All treated shoots' lengths were measured, the number of leaves was counted as well as the thickness of leaf, area of leaf, chlorophyll content, and stomatal number and size were measured from three randomly selected leaves of treated shoots.

The treated shoots initiated flower buds, and those matured flower buds were emasculated in the evening and then covered with a small paraffin paper bag. Emasculated flowers' pollen grains were put under artificial light for full blooming at least for 12 hours. The next morning from 9.0 - 10.0 am the treated emasculated flower was pollinated by untreated emasculated pollen grains and vice versa. Then again the pollinated flowers were covered with a small paraffin paper bag to control unwanted pollination. The paraffin paper bag was removed completely when the pollinated flower started to set fruit. The data related to the reproductive phase was collected from the treated shoots.

Stomatal observation

The following procedure was carried out for counting the stomata number and their size from treatments and the methodology of Case (1994) was followed.

1. Three leaves were randomly selected from each treatment.
2. A thick patch (at least one square centimetre) was painted of clear nail polish on the underside of the selected leaf surface.
3. The nail polish was allowed to dry completely.
4. After that the peel of nail polish was removed using forceps and put on a slide with 1-2 drops of distilled water and then covered with a cover slip.
5. The slide was examined under a light microscope.
6. All the stomata in one microscopic field were counted at least three times and an average number was determined and recorded in the data table.
7. A microscopic scale was used to measure the actual size of stomata and the size of the stomata was recorded in the data table.

Chromosome Observation

Fruits were obtained from treatments, seeds were extracted and the outer and inner seed coats were removed. After removing the outer and inner seed coats, the embryos were checked and germinated on moistened double thickness filter papers in petri dishes at room temperature (RT) and very small embryos which could not germinate were eliminated. Meristematic roots of well germinated embryos were examined for the ploidy level. The following steps developed by Love and Love (1975) were followed during chromosomes observation.

A. Pretreatment of roots

Actively growing roots were collected from germinating seeds which were grown in a petri dish. Furthermore, a sample root tip was cut 10 mm long and prepared for fixation by using 8-Hydroxyquinoline. Active growing meristematic root tips (specimens) were

treated with 0.002 M (0.5 g/l) aqueous solution [dissolved in double distilled water (ddH_2O) at room temperature] of 8-hydroxyquinoline for 18 hr at 18°C.

B. Fixation of roots

For fixation, the pretreated specimens were washed with distilled water, kept in a vial and the material was fixed in three parts of ethanol (95 to 100%) and one part of glacial acetic acid. The specimens were kept for 24 hours at RT.

C. Staining of chromosomes

After fixation, the specimens were taken out, washed with distilled water and placed on the glass slide. The active part (white color) of root tip was cut 2-3 mm long and 1 N HCl (1-2 drops) was put on the specimen. The specimen was kept 15-30 minutes for proper softening. Additionally, the specimen was cut in very small pieces, and one drop of 1% lacto-propionic-orcein solution was put on them and kept for 3-5 minutes.

The Lacto-Propionic-Orcein was made by adding 2 g orcein to 100 ml of a mixture of equal parts of lactic acid and propionic acid at RT. The stock solution was diluted and filtered to 45% with dH_2O.

The specimen was covered with a cover slip, and a layer of tissue paper was put on the cover slip and pressed to suck up any excess stain. The squashing technique was used to break the specimen, after that the specimen slide was placed on a microscope and chromosome numbers were counted and recorded with a photographic image.

Nucleoli observation

The following procedure was done for counting the nucleoli number from treatments and the methodology was followed according to Toolapong (1999).

1. Actively growing roots were collected from germinating seeds which were grown in a petri dish.

2. The sample root tip was cut 10 mm long and prepared for fixation; the specimens were put in a vial and the specimen was fixed with three parts of ethanol (95 to 100%) and one part of glacial acetic acid (100%).

3. The specimens were kept for 1.5 hours at 5^0 C then washed 3-4 times within an hour and the amount of distilled water was 10 cc per specimen everytime.

4. The specimens were hydrolyzed in 1N HCl: 45% acetic acid (2:1-V/V) solution at about 60^0 C for 20 seconds and again the specimens were washed 3-4 times with distilled water within 3 hours. During that time filter paper was used for absorbing excess water from the specimens.

5. The whitish parts from inside the root tips were removed and used for the conventional squashing technique.

6. The whitish part of the specimen was put on a glass slide, covered with a cover slip, and pressed by using a thumb to make a single layer.

7. The glass slide was placed on dry ice. After few minutes the cover slip was removed properly without the specimen.

8. The glass slide was placed in the desiccators for 3 days.

9. The glass slide was removed from the desiccators and 1-2 drops of 50% silver nitrate staining solution was put on the specimen, covered with a cover slip, and the slide was placed in the moist glass chamber and was incubated 3-4 hours at 60^0 C.

10. After that the specimen slide was placed on a microscope and then nucleoli numbers were counted and recorded with a photographic image.

CHAPTER 4

RESULTS

Vegetative phase

Length of shoot

After removing the chemically soaked cotton wool from treatments, treated axillary buds' new growth was delayed from emerging in comparison with the control. Moreover, the shoot length revealed a different height (Figure 1, Tables 1 and 2). Additionally, the height of the treated shoot showed a negative relationship with chemical concentrations in sweet calamondin except 0.03% of colchicine and 0.01% of the trifluralin treatment while in sour calamondin, treated shoot heights showed a mixed relationship among treatments and they were less than the control (Figure 2). Moreover, colchicine and trifluralin treated shoots showed 25% and 75% mortality rate in sweet calamondin, respectively, while a 25% shoot mortality rate was found from the trifluralin treatment in sour calamondin. In sweet calamondin, the shoot length did not show statistically significant differences from colchicine and trifluralin treatments ($p > 0.05$), however, it was significantly less than the control from the colchicine treatment but in trifluralin treatment the control was less compared to treated shoots (Table 1). Furthermore, in sour calamondin, statistically significant difference was found from treated shoot length from the colchicine treatment ($p < 0.05$) and no significant difference was found from trifluralin treatment ($p > 0.05$) but the data pattern of shoot length was significantly less than control (Table 2). Shoot lengths were also statistically evaluated using paired t - test, but no significant difference was found between colchicine and trifluralin treated shoots in sweet calamondin ($p > 0.05$) (Table 3). However, a highly significant difference showed ($p < 0.01$) in sour calamondin between colchicine and trifluralin treated shoot lengths from the paired t - test and the shoot length was found to be 3.39 cm and 11.55 cm, respectively (Table 4). Furthermore, no significant difference was seen in the length of shoots between sweet and sour calamondin and the shoot lengths were nearly similar (7.42 cm and 7.02 cm) (Table 5).

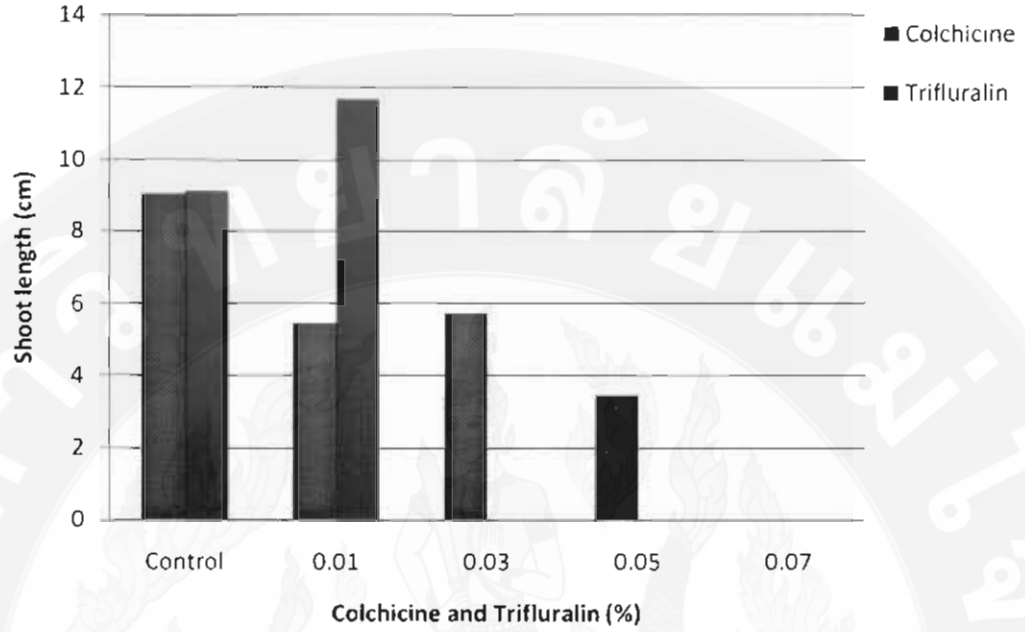

Figure 1 Shoot lengths of sweet calamondin after different colchicine and trifluralin concentration applications

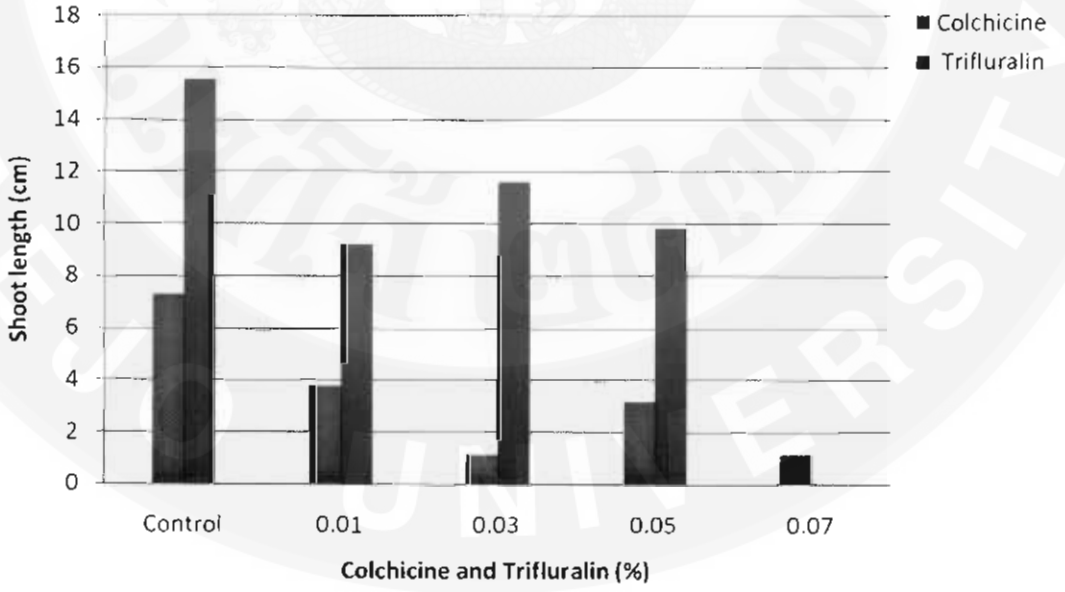

Figure 2 Shoot lengths of sour calamondin after different colchicine and trifluralin concentration applications

Number of leaf

Regarding the tabulated data pattern, statistically non significant difference was found from colchicine and trifluralin treatments in sweet calamondin, however, in the colchicine treatment, the leaf number was significantly less than the control and data patterns were 0.00 (15.63), 0.03% (12.22), 0.01% (10.67), and 0.05% (9.83), respectively, while 0.01% of the trifluralin treatment showed a maximum leaf number (14.37) compared to the control (14) (Table 1). On the other hand, in sour calamondin, the maximum leaf number was found from the control (11.6 and 28.13) in both colchicine and trifluralin treatments followed by 0.05% (8.08), 0.01% (6.76), 0.03% (4.13), and 0.07% (3.89) in the colchicine treatment while 0.03% (18.04), 0.05% (14.42) and 0.01% (11.96) in the trifluralin treatment, respectively, with no significant difference ($p > 0.05$) (Table 2). According to paired t - test, there was no statistically significant difference found from leaf numbers between colchicine and trifluralin treatment in sweet calamondin (Table 3) and between sweet and sour calamondin (Table 5). However, the leaf number pattern showed a significant difference between colchicine (6.89) and trifluralin (18.14) treatment in sour calamondin (Table 4).

Thickness of leaf

The leaf thickness result showed a statistically non significant difference from the colchicine and trifluralin treatments in sweet calamondin, but the data pattern differed. Moreover, the maximum leaf thickness (0.36 mm) was found from 0.01% of the colchicine and trifluralin treatments followed by 0.03% (0.31 mm), 0.00 (0.27 mm) and 0.05% (0.26 mm) from colchicine and 0.00 (0.26 mm) from trifluralin treatments in sweet calamondin (Table 1). A high statistically significant difference was found in the thickness of the leaf from colchicine treatment in sour calamondin ($p < 0.01$). The control leaf had maximum thickness (0.23 mm) followed by the treated leaf 0.05% (0.21 mm), 0.01% (0.19 mm), and 0.07% (0.15 mm) from colchicine treatment, respectively, while in sour calamondin, trifluralin treatment showed no significant difference and the leaf thickness pattern was followed by the control (0.21 mm), 0.01% (0.21 mm), 0.03% (0.18 mm), and 0.05% (0.18 mm), respectively (Table 2). Furthermore, there was no

significant difference found between the colchicine and the trifluralin treatment in sweet and sour calamondin in paired t - test (Tables 3 and 4). However, the thickness of the leaf had a highly significant difference between sweet (0.30 mm) and sour (0.19 mm) calamondin in paired t - test ($p < 0.01$) (Table 5).

Area of leaf

The result showed the maximum leaf area (13.12 cm^2) from 0.01% trifluralin treatment followed by the control and 0.00, 0.01%, 0.03%, and 0.05% from colchicine treatment in sweet calamondin, respectively, however, it did not differ significantly ($p > 0.05$) (Table 1). In sour calamondin, statistically non significant difference was found in leaf area from colchicine and trifluralin treatments ($p > 0.05$) (Table 2), but the data pattern showed the maximum leaf area from 0.00 (11.86 cm^2) from colchicine treatment followed by 0.05% (8.53 cm^2), 0.01 % (8.46 cm^2), 0.03% (6.99 cm^2), and 0.07% (2.29 cm^2) while the maximum leaf area from 0.03% (15.79 cm^2) from trifluralin treatment followed by 0.00 (14.44 cm^2), 0.01 % (13.62 cm^2) and 0.05(11.05 cm^2). In paired t - test analysis, the area of the leaf from colchicine (10.75 cm^2) and trifluralin (12.40 cm^2) did not differ significantly in sweet calamondin (Table 3) while in sour calamondin the area of the leaf showed a significant difference ($p < 0.05$) from colchicine (7.62 cm^2) and trifluralin (13.72 cm^2) (Table 4). However, no significant difference was found in the area of the leaf between sweet (11.29 cm^2) and sour (10.33 cm^2) calamondin by t - test analysis (Table 5).

Chlorophyll content

In sweet and sour calamondin, there was no statistically significant difference in the chlorophyll content (SPAD values) ($p > 0.05$); however, chlorophyll content (Chc) data pattern differed from each other. In sweet calamondin, the maximum Chc was found from the control (70.66 – 67.83) followed by 0.01% (69.99) from trifluralin, 0.05% (66.47), 0.01% (65.82) and 0.03% (64.89) from colchicine treatments, respectively (Table 1). Furthermore, the highest Chc data pattern was found to be 0.03% (62.92) > 0.01% (61.56) > 0.00 (59.81) > 0.05% (58.09) in trifluralin treatment, and a maximum Chc was found from the control (53.26) followed by

0.01% (52.58), 0.07% (51.80). 0.03% (51.22), and 0.05% (43.42) in colchicine treatment, respectively, in sour calamondin (Table 2). However, paired t - test analysis showed highly significant difference between the colchicine Chc (50.46) and trifluralin Chc (60.59) treatment in sour calamondin ($p < 0.01$) (Table 4) but there was no significant difference found in Chc between colchicine and trifluralin treatments in sweet calamondin (Table 3). Moreover, the Chc of sweet (67.61) and sour (54.96) calamondin showed a highly significant difference according to the paired t - test analysis ($p < 0.01$) (Table 5).

Stomatal number

In sweet calamondin, the data pattern of stomatal density revealed the maximum number of stomata (80.11) per film from 0.03% followed by 0.05% (75.37), 0.01% (69.78) of colchicine treatment, 0.01% (66.64) of trifluralin treatment, and in the control (64.72 and 65.86) (Figure 3), but the data were statistically non significant (Table 1). Moreover, in sour calamondin, the data pattern revealed the lowest number of stomata (87.63) per film from 0.07% of colchicine followed by 0.05% (90.94), 0.00 (94.19), 0.01% (102.36), and 0.03% (103.95) of colchicine treatment but was statistically no significant (Figure 4) while the maximum stomata number was seen to be 0.05% (93.92) followed by 0.01% (89.09), 0.00 (87.00) and 0.03% (85.25) from trifluralin treatment (Figure 5) and was statistically significant ($p < 0.05$) (Table 2). The paired t - test analysis showed a statistically non significant ($p > 0.05$) result in the stomatal number between colchicine and trifluralin treatment in sweet and sour calamondin (Tables 3 and 4) while there was a highly significant difference in the stomatal number between sweet and sour calamondin ($p < 0.01$) (Table 5).

Stomatal guard cell length

The maximum stomatal guard cell length was revealed to be from 0.01% of trifluralin and colchicine treatments and the length was 7.48 μ and 7.31 μ, respectively, followed by 0.05% (6.87 μ), 0.00 (6.69 and 6.15 μ), and 0.03% (5.54 μ) of colchicine treatment in sweet calamondin and treatments were statistically significant different ($p < 0.05$) (Table 1). Sour

calamondin showed a statistically no significant difference in the guard cell length ($p > 0.05$). Moreover, the highest guard cell length was found to be 0.07% (6.76 μ) followed by 0.05% (6.06 μ), 0.00 (5.73 μ), 0.01% (5.31 μ), and 0.03% (5.15 μ) from colchicine treatment, while 0.01% (5.73 μ) of trifluralin treatment had a maximum guard cell length followed by 0.05% (5.56 μ), 0.00 (5.56 μ) and 0.03% (5.48 μ) in sour calamondin, respectively (Table 2). The paired *t* - test analysis of colchicine and trifluralin treatments did not show a statistically significant result in stomatal guard cell length in sweet and sour calamondin ($p > 0.05$) (Tables 3 and 4) while between sweet and sour calamondin the stomatal guard cell length (6.67 - 5.70 μ) showed a highly significant difference ($p < 0.01$) (Table 5).

Stomatal guard cell width

In sweet calamondin, the data pattern of the stomatal guard cell width was found maximum from 0.05% (5.09 μ) followed by 0.01% (4.31 μ), 0.00 (4.27 μ) and 0.03% (3.54 μ) in colchicine treatment and data showed statistically significant difference ($P < 0.05$) while trifluralin treatment had no significant difference and data pattern was 0.00 (4.31 μ) and 0.01% (3.73 μ) (Table 1). Moreover, there was not a statistically significant result found in sour calamondin ($P > 0.05$), but the highest stomatal guard cell width pattern was 0.05% (4.56 μ) > 0.07% (3.98 μ) > 0.00 (3.90 μ) > 0.01% (3.56 μ) > 0.03% (3.48 μ) from colchicine treatment, and 0.03% (4.23 μ) > 0.01% (4.06 μ) > 0.00 (3.90 μ) > 0.05% (3.81 μ) from trifluralin treatment (Table 2). From the paired *t* - test analysis, a statistically non significant result was found between colchicine and trifluralin and also between the sweet and sour calamondin ($P > 0.05$) (Tables 3, 4 and 5).

Table 1 Shoot length, leaf numbers, thickness and area, chlorophyll content, and stomatal numbers, length and width after colchicine and trifluralin applications of sweet calamondin

Chemicals (%)		Shoots length (cm)	Leaf numbers	Leaf thickness (mm)	Leaf area (cm^2)	Chlorophyll content (SPAD values)	Stomatal numbers	Stomatal length (μ)	Stomatal width (μ)
Colchicine	0.00	9.06	15.63	0.27	11.38	70.66	64.72	6.69a	4.27ab
	0.01	5.46	10.67	0.36	11.01	65.82	69.78	7.31a	4.31ab
	0.03	5.73	12.22	0.31	10.63	64.89	80.11	5.54b	3.54b
	0.05	3.47	9.83	0.26	9.97	66.47	75.37	6.87a	5.09a
	0.07	-	-	-	-	-	-	-	-
	Total	23.72	48.35	1.20	42.99	267.84	289.98	26.41	17.21
	$\bar{X}$	5.93	12.09	0.30	10.75	66.96	72.50	6.60	4.30
	F-Test	ns	ns	ns	ns	ns	ns	*	*
	CV (%)	48.18	29.42	26.25	22.78	11.02	12.18	8.62	11.88
Trifluralin	0.00	9.13	14.00	0.26	11.68	67.83	65.86	6.15b	4.31
	0.01	11.65	14.37	0.36	13.12	69.99	66.64	7.48a	3.73
	0.03	-	-	-	-	-	-	-	-
	0.05	-	-	-	-	-	-	-	-
	0.07	-	-	-	-	-	-	-	-
	Total	20.78	28.37	0.62	24.80	137.82	132.50	13.63	8.04
	$\bar{X}$	10.39	14.18	0.31	12.40	68.91	66.25	6.81	4.02
	F-Test	ns	ns	ns	ns	ns	ns	*	ns
	CV (%)	89.22	70.24	38.34	48.70	14.37	6.91	9.36	20.51

Remark: Means with in columns followed by different letters differ significant by the DMRT

Table 2 Shoot length, leaf numbers, thickness and area, chlorophyll content, and stomatal numbers, length and width after colchicine and trifluralin applications of sour calamondin

Chemicals	(%)	Shoots length (cm)	Leaf numbers	Leaf thickness (mm)	Leaf area (cm^2)	Chlorophyll content (SPAD values)	Stomatal numbers	Stomatal length (μ)	Stomatal width (μ)
Colchicine	0.00	7.32a	11.60	0.23a	11.86	53.26	94.19	5.73	3.90
	0.01	3.80ab	6.76	0.19ab	8.46	52.58	102.36	5.31	3.56
	0.03	1.13b	4.13	0.15b	6.99	51.22	103.95	5.15	3.48
	0.05	3.18ab	8.08	0.21a	8.53	43.42	90.94	6.06	4.56
	0 07	1.55b	3.89	0.15b	2.29	51.80	87.63	6.76	3.98
	Total	16.98	34.46	0.93	38.13	252.28	479.07	29.01	19.48
	$\overline{X}$	3.39	6.89	0.18	7.62	50.45	95.81	5.80	3.89
	F-Test	*	ns	**	ns	ns	ns	ns	ns
	CV (%)	72.18	76.04	15.33	45.88	26.67	15.48	10.46	16.19
Trifluralin	0.00	15.53	28.13	0.21	14.44	59.81	87.00b	5.56	3.90
	0.01	9.25	11.96	0.21	13.62	61.56	89.09ab	5.73	4.06
	0.03	11.60	18.04	0.18	15.79	62.92	85.25b	5.48	4.23
	0.05	9.82	14.42	0.18	11.05	58.09	93.92a	5.56	3.81
	0.07	-	-	-	-	-	-	-	-
	Total	46.20	72.55	0.78	54.90	242.38	355.26	22.33	16.00
	$\overline{X}$	11.55	18.14	0.20	13.72	60.59	88.81	5.58	4.00
	F-Test	ns	ns	ns	ns	ns	*	ns	ns
	CV (%)	42.41	51.39	15.12	16.56	5.53	4.13	5.24	7.31

Remark: Means with in columns followed by different letters differ significant by the DMRT

Table 3 Comparisons of shoot length, leaf numbers, thickness and area, chlorophyll content, and stomatal numbers, length and width after colchicine and trifluralin applications of sweet calamondin

Chemicals (%)	Shoots length (cm)	Leaf numbers	Leaf thickness (mm)	Leaf area (cm^2)	Chlorophyll content (SPAD values)	Stomatal numbers	Stomatal length (μ)	Stomatal width (μ)
Colchicine	5.93	12.09	0.30	10.75	66.96	72.50	6.60	4.30
Trifluralin	10.39	14.18	0.31	12.40	68.91	66.25	6.81	4.02
T-Test	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	29.61	17.37	17.44	6.45	3.46	8.23	12.07	13.91

Table 4 Comparisons of shoot length, leaf numbers, thickness and area, chlorophyll content, and stomatal numbers, length and width after colchicine and trifluralin applications of sour calamondin

Chemicals (%)	Shoots length (cm)	Leaf numbers	Leaf thickness (mm)	Leaf area (cm^2)	Chlorophyll content (SPAD values)	Stomatal numbers	Stomatal length (μ)	Stomatal width (μ)
Colchicine	3.39	6.89	0.18	7.62	50.45	95.81	5.80	3.89
Trifluralin	11.55	18.14	0.20	13.72	60.59	88.81	5.58	4.00
T-Test	**	*	ns	*	**	ns	ns	ns
CV (%)	37.41	44.06	15.43	28.38	6.05	6.37	8.61	8.77

Table 5 Comparisons of shoot length, leaf numbers, thickness and area, chlorophyll content, and stomatal numbers, length and width after colchicine and trifluralin applications of calamondin

Chemicals (%)	Shoots length (cm)	Leaf numbers _	Leaf thickness (mm)	Leaf area (cm^2)	Chlorophyll content (SPAD values)	Stomatal numbers _	Stomatal length (μ)	Stomatal width (μ)
Sweet	7.42	12.78	0.30	11.29	67.61	70.41	6.67	4.20
Sour	7.02	11.89	0.19	10.33	54.96	92.70	5.70	3.94
T-Test	ns	ns	**	ns	**	**	**	ns
CV (%)	60.08	50.57	15.61	31.54	8.43	7.69	9.60	10.47

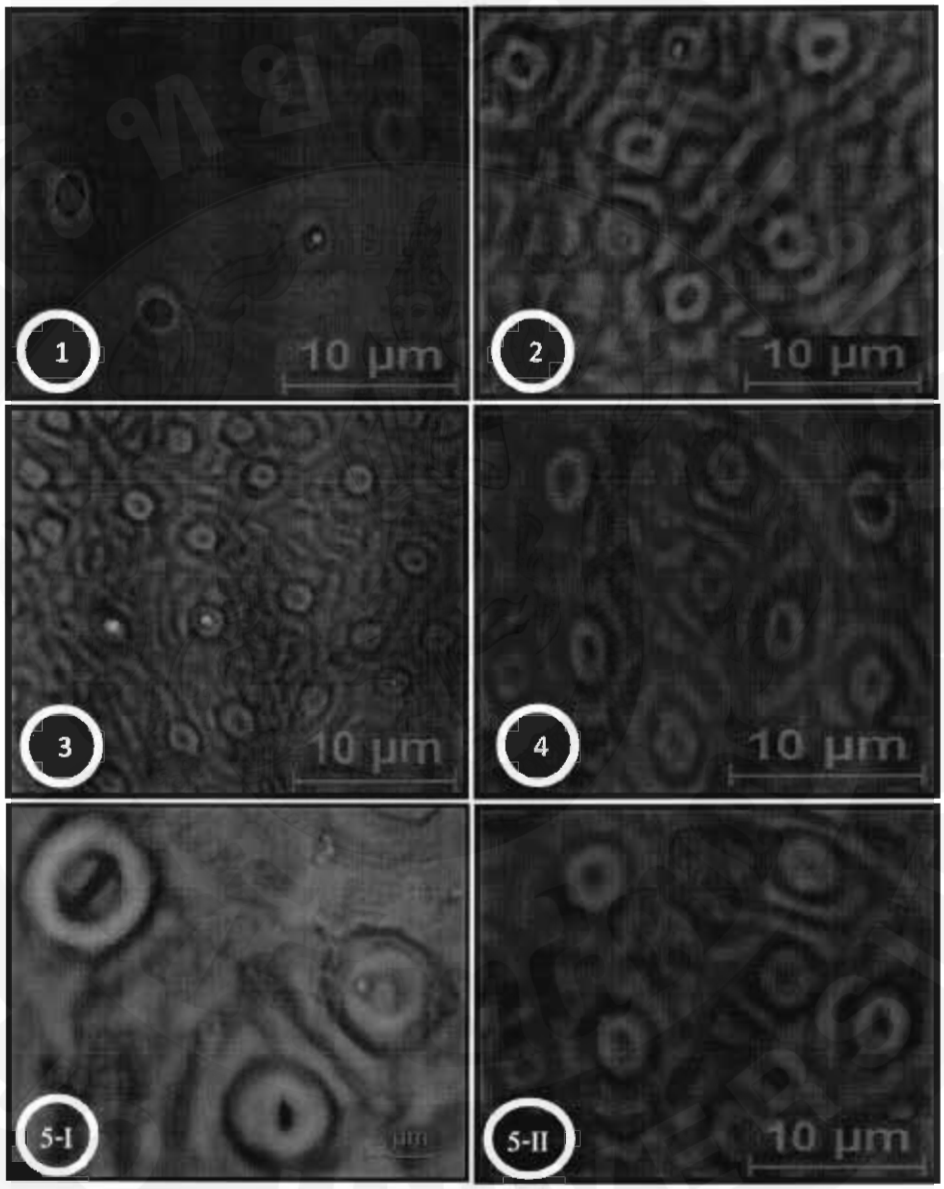

Figure 3 Stomatal observations after applications of different colchicine and trifluralin concentrations in sweet calamondin

1. control
2. colchicine 0.01%
3. colchicine 0.03%
4. colchicine 0.05%
5-I. trifluralin 0.01%
5-II. trifluralin 0.01%

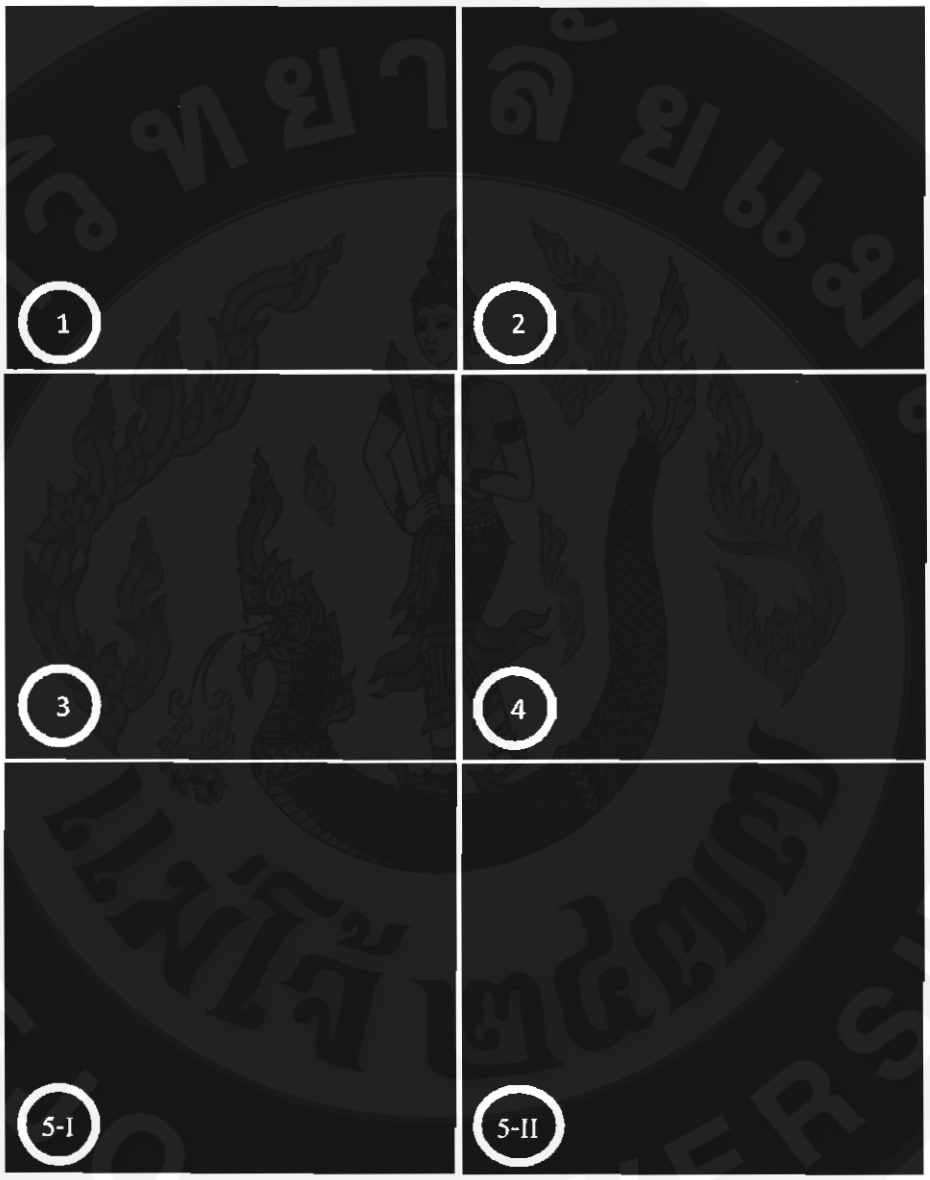

Figure 4 Stomatal observations after applications of different colchicine concentrations in sour calamondin

1. control
2. colchicine 0.01%
3. colchicine 0.03%
4. colchicine 0.05 %
5-I. colchicine 0.07%
5-II. colchicine 0.07%

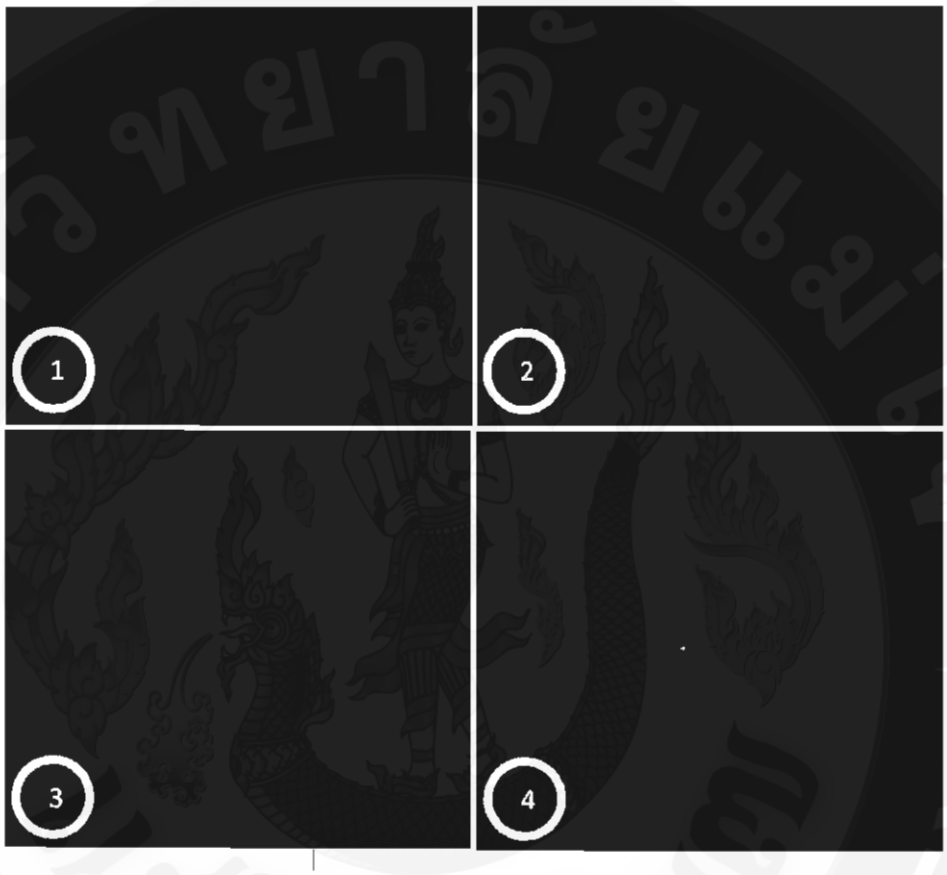

Figure 5 Stomatal observations after applications of different trifluralin concentrations in sour calamondin

1. control
2. trifluralin 0.01%
3. trifluralin 0.03%
4. trifluralin 0.05%

Reproductive phase

Number of pollinated flower and fruit sets

In the reproductive phase, chemicals showed the negative relationships between the numbers of flowers and the chemical concentrations compared to the control in sweet and sour calamondin except 0.03% of trifluralin in sour calamondin. Moreover, the percentage of fruit setting showed positive relationships among treatments in sweet calamondin but in sour calamondin the fruit set showed a negative relationship except in 0.07% from colchicine. The maximum number of pollinated flowers were found from the control (165 - 182) followed by 0.01% (129) from trifluralin treatment, and 0.01% (71), 0.03% (46) and 0.05% (42) from the colchicine treatment in sweet calamondin. However, the maximum fruit setting percentage was found to be 0.01% (40.85) followed by 0.05% (28.57) and 0.03% (26.08) from the colchicine treatment and 0.01% (23.25) from the trifluralin treatment while in the control (15.54 - 18.68) the fruit setting was lower in sweet calamondin (Table 6, Figure 6). Furthermore, in sour calamondin, the control (62) had the maximum number of pollinated flowers followed by 0.03% (62), 0.01% (54) and 0.05% (40) from the trifluralin treatment, 0.00 (38), 0.01% (25), 0.07% (15), 0.05% (14), and 0.03% (10) from the colchicine treatment. However, the maximum number of fruit setting was found to be 0.07% (9) followed by 0.00 (8), 0.01% (5) and 0.03% (2), 0.05% (2) from the colchicine treatment while the maximum number of fruit setting was found from control (29) followed by 0.03% (25), 0.01% (17) and 0.05% (11) from the trifluralin treatment in sour calamondin (Table 7, Figure 7). The fruit setting percentage was found to be higher in colchicine (27.51) compared to trifluralin (20.96) in sweet calamondin (Table 6), while in sour calamondin, it was higher from the trifluralin treatment (36.52) compared to the colchicine treatment (22.34) (Table 7).

Number of seeds and seeds per fruit

In sweet calamondin, the maximum number of seeds were found from the control (141) followed by 0.05% (73), 0.03% (48) and 0.01% (39) from the colchicine treatment while the trifluralin treatment 0.01% (132) had fewer numbers of seeds compared to the control (162).

However, the seeds per fruit revealed a maximum of 0.05% (6.08) followed by 0.00 (5.87), 0.03% (4.0) and 0.01% (1.34) from the colchicine treatment, and 0.00 (4.76), 0.01% (4.40) from the trifluralin treatment in sweet calamondin (Table 6). The 0.07% (14) of colchicine revealed a maximum number of seeds followed by 0.00 (6), 0.01% (5), 0.03% (5), and 0.05% (2) but the maximum number of seeds per fruit was found to be 0.03% (2.5) followed by 0.05% (2), 0.07% (1.55), 0.01% (1), and the control (0.75) from colchicine treatment in sour calamondin. Moreover, the maximum number of seeds from the control was 31 followed by 0.03% (29), 0.01% (18), and 0.05% (14) from the trifluralin treatment, however, the seeds per fruit were 0.05% (1.27), 0.03% (1.16), 0.00 (1.07), and 0.01% (1.06) from the trifluralin treatment in sour calamondin, respectively (Table 7). The seeds per fruit were nearly similar from colchicine (4.32) and trifluralin (4.58) in sweet calamondin (Table 6) while in sour calamondin seeds per fruit were 7.80 and 4.56 from colchicine and trifluralin treatment, respectively (Table 7).

Number of embryos and embryos per seed

In sweet calamondin, the control revealed the maximum number of embryos (524) followed by 0.03 % (217), 0.05% (214), 0.01% (154) and the maximum number of embryos per seed 0.03% (4.52) followed by 0.01% (3.94), 0.00 (3.71) and 0.05% (2.93) from the colchicine treatment as well as 0.01% in the trifluralin treatment showed the highest number of embryos per seed (3.72) and the total number of embryos (492) and in the control embryos per seed (3.31) and total number of embryos (537) was obtained (Table 6). In sour calamondin, the maximum numbers of embryos were found from 0.07% (55) followed by 0.00 (25), 0.03% (18), 0.01% (16), and 0.05% (5) of colchicine treatment while the maximum number of embryos were found from the control (118) followed by 0.03% (104), 0.01% (76) and 0.05% (68) from the trifluralin treatment. However, the maximum number of embryos per seed was found in the control (4.16) followed by 0.07% (3.92), 0.03% (3.60), 0.01% (3.20), and 0.05% (2.50) from the colchicine treatment and the highest number of embryos per seed was 0.05% (4.86) followed by 0.01% (4.22), 0.00 (3.80) and 0.05% (3.58) in the trifluralin treatment in sour calamondin (Table 7). The embryos per seed were nearly similar between the colchicine (3.78) and trifluralin (3.52) treatment in sweet calamondin while it was different between colchicine (3.48) and trifluralin (4.12) in sour calamondin (Tables 6 and 7).

Number of seedlings and seedlings per seed

The maximum seedling percentage was obtained from 0.05% (41.58) and followed by 0.01% (32.46), 0.00 (29.38) and 0.03% (24.88) in the colchicine treatment while the control seedling percentage (35.19) was higher compared to 0.01% (30.48) from the trifluralin treatment in sweet calamondin. However, the seedlings per seed pattern were found to be 0.01% (1.28) > 0.05% (1.22) > 0.03% (1.13) > 0.00 (1.09) from the colchicine treatment and 0.00 (1.17) > 0.01% (1.14) from trifluralin treatment in sweet calamondin (Table 6). Moreover, in sour calamondin, the maximum seedling percentages were found from the control (52 - 51.69) followed by 0.03% (50), 0.07% (45), 0.05% (40), and 0.01% (37.50) from the colchicine treatment, and 0.03% (42.30), 0.01% (40.78), and 0.05% (36.76) from the trifluralin treatment. Furthermore, the seedlings per seed pattern were found to be 0.00 (2.16) > 0.03% (1.80) > 0.07% (1.78) > 0.01% (1.20) from colchicine and 0.00 (1.97) > 0.05% (1.78) > 0.01% (1.72) > 0.03% (1.52) from trifluralin treatments in sour calamondin (Table 7). The seedling percentages were found to be 32.07 and 32.83 between the colchicine and trifluralin treatments in sweet calamondin, respectively, (Table 6) while 44.99 and 42.88 percentages of seedlings were found from colchicine and trifluralin treatments in sour calamondin, respectively (Table 7). Furthermore, the number of seedlings per seed was higher from the colchicine treatment compared to the trifluralin treatment in sweet calamondin as well as in sour calamondin (Tables 6 and 7).

Number of triploid seedling progenies

In this study, a total number of 2,138 embryos and 686 (32.09%) seedlings were found from sweet calamondin (Table 6) while 485 embryos and 216 (44.54%) seedlings were found from sour calamondin (Table 7). In addition, 902 (34.38%) number of seedlings were observed from 2,623 embryos. Three (0.33%) were triploid seedling progenies and the rest remained diploid seedlings (Tables 6 and 7, Figure 9-1). In the case of the triploid seedling progenies, three seedlings were recovered from the treated female flower where two from 0.03% and one from 0.05% of colchicine concentrations in sweet calamondin (Table 6, Figure 9-2).

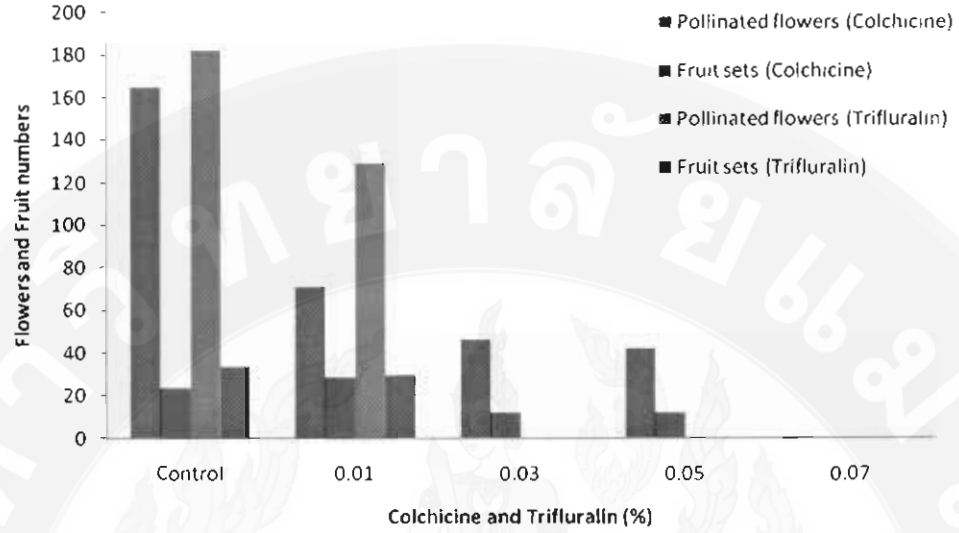

Figure 6 Effect of different colchicine and trifluralin concentrations on pollinated flowers and fruit sets in sweet calamondin

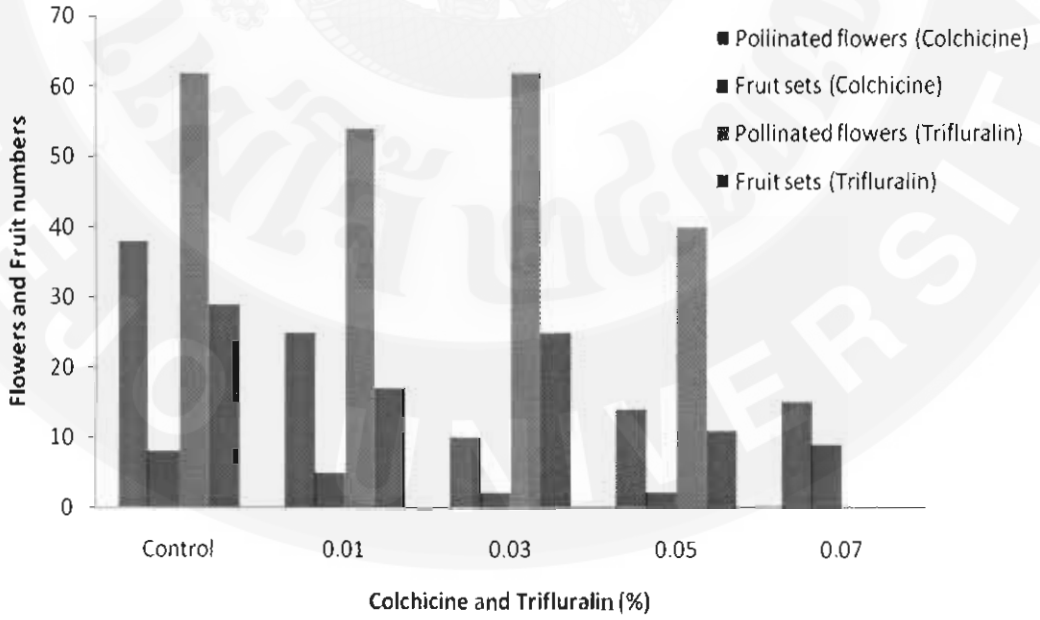

Figure 7 Effect of different colchicine and trifluralin concentrations on pollinated flowers and fruit sets in sour calamondin

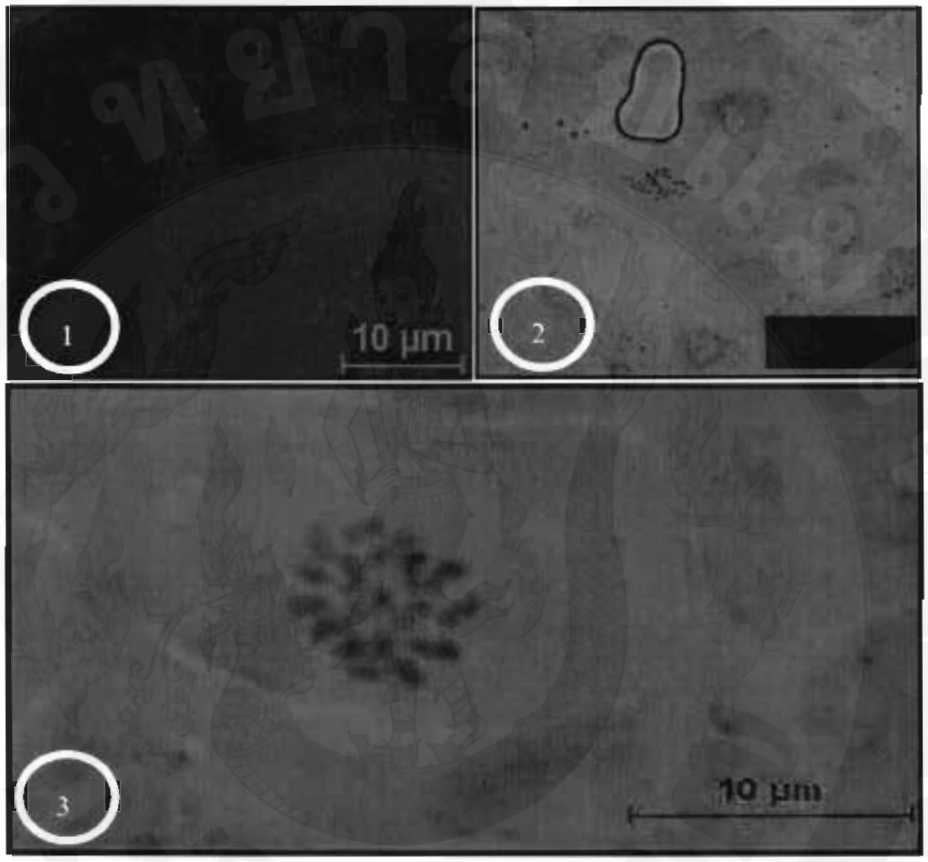

Figure 8 Chromosome numbers of sweet calamondin from root tip cell

1. – 2. triploid [2n=3x=27] 3. diploid [2n=2x=18]

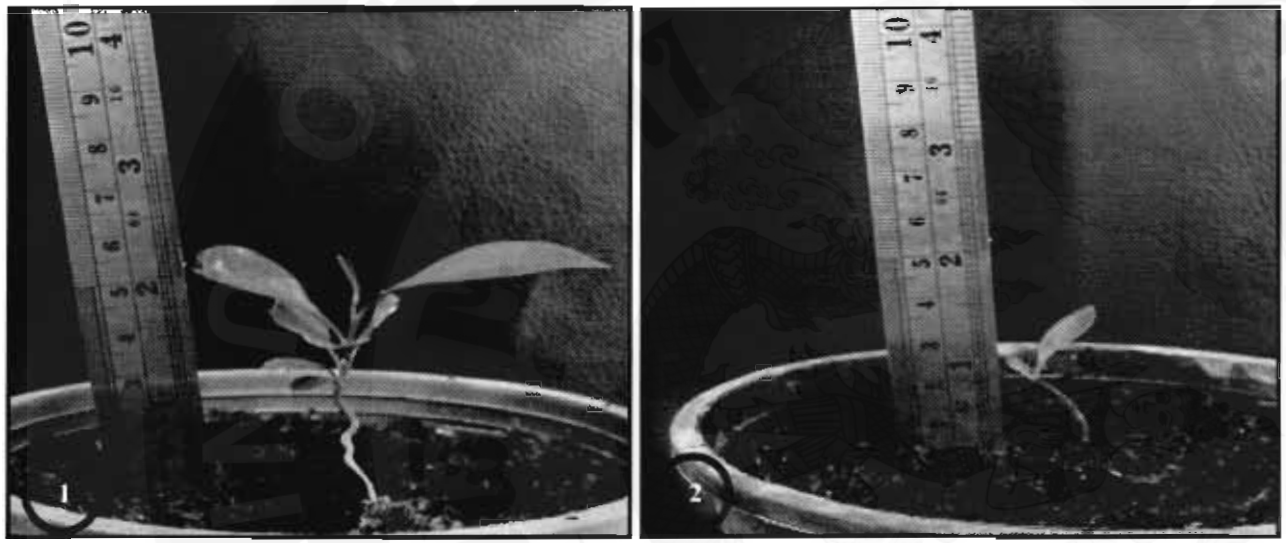

Figure 9 One hundred day old seedlings of sweet calamondin

1. diploid 2. triploid seedlings

Table 6 Flowers, fruits, seeds, embryos, and triploid seedlings' numbers after colchicine and trifluralin applications of sweet calamondin

Chemicals (%)	Number of									
	Poll. flowers	Fruit sets (%)	Seeds	Seeds/ Fruit	Embryos	Embryos/ Seed	Seedlings (%)	Seedlings/Seed	3X Seedlings (%)	
									TFF x UTMF	UTFF x TMF
Colehicine 0	165	24 (14.54)	141	5.87	524	3.71	154 (29.38)	1.09	-	-
0.01	71	29 (40.85)	39	1.34	154	3.94	50 (32.46)	1.28	-	-
0.03	46	12 (26.08)	48	4.00	217	4.52	54 (24.88)	1.13	2 (3.7)	-
0.05	42	12 (28.57)	73	6.08	214	2.93	89 (41.58)	1.22	1 (1.12)	-
0.07	-	-	-	-	-	-	-	-	-	-
Total	324	77.00 (110.04)	301.00	17.29	1109.00	15.10	347 (128.30)	4.72	3 (4.82)	
$\overline{X}$	81	19.25 (27.51)	75.25	4.32	277.25	3.78	86.75 (32.07)	1.18	1.5 (2.41)	
Trifluralin 0	182	34 (18.68)	162	4.76	537	3.31	189 (35.19)	1.17	-	
0.01	129	30 (23.25)	132	4.40	492	3.72	150 (30.48)	1.14	-	
0.03	-	-	-	-	-	-	-	-	-	
0.05	-	-	-	-	-	-	-	-	-	
0.07	-	-	-	-	-	-	-	-	-	
Total	311.00	64 (41.93)	294	9.16	1029.00	7.03	339 (65.67)	2.31	-	
$\overline{X}$	155.50	32 (20.96)	147	4.58	514.50	3.52	169.5 (32.83)	1.16	-	

Remarks: Poll. = Pollinated, TFF = Treated Female Flower, UTMF = Untreated Male Flower, UTFF = Untreated Female Flower, TMF = Treated Male Flower

Table 7 Flowers, fruits, seeds, embryos, and triploid seedlings' numbers after colchicine and trifluralin applications of sour calamondin

Chemicals (%)	Number of								
	Pollinated flowers	Fruit sets (%)	Seeds	Seeds/ Fruit	Embryos	Embryos/ Seed	Seedlings (%)	Seedlings/Seed	3X Seedlings (%)
Colchicine 0	38	8 (21.05)	6	0.75	25	4.16	13 (52.00)	2.16	-
0.01	25	5 (20.00)	5	1.00	16	3.20	6 (37.50)	1.20	-
0.03	10	2 (20.00)	5	2.50	18	3.60	9 (50.00)	1.80	-
0.05	14	2 (14.28)	2	2.00	5	2.50	2 (40.00)	1.00	-
0.07	15	9 (36.36)	14	1.55	55	3.92	25 (45.45)	1.78	-
Total	102.00	26 (111.69)	32.00	7.80	119.00	17.38	55 (224.95)	7.94	-
$\overline{X}$	20.40	5.2 (22.34)	6.40	1.56	23.80	3.48	11 (44.99)	1.58	-
Trifluralin 0	62	29 (46.77)	31	1.07	118	3.80	61 (51.69)	1.97	-
0.01	54	17 (31.48)	18	1.06	76	4.22	31 (40.78)	1.72	-
0.03	62	25 (40.32)	29	1.16	104	3.58	44 (42.30)	1.52	-
0.05	40	11 (27.50)	14	1.27	68	4.86	25 (36.76)	1.78	-
0.07	-	-	-	-	-	-	-	-	-
Total	218.00	82.00 (146.07)	92	4.56	366.00	16.46	161.00 (171.53)	6.99	-
$\overline{X}$	54.50	20.50 (36.52)	23	1.14	91.50	4.12	40.25 (42.88)	1.75	-

Chromosome observation

For the chromosome observation, a total numbers of 902 seedlings' newly emerging root tips were checked and it was found that three of them were triploid seedlings and the remaining were (899) diploid seedlings (Tables 6 and 7; Figures 8 and 9).

Nucleoli observation

Table number 8 shows the results of the nucleoli number related to the ploidy level, where thirteen diploid sweet calamondin seedling root tip cells were checked and found to be 87.48% haploid and 12.52% diploid nucleoli while no triploid nucleoli were found from them (Figure 12). Moreover, one root tip of a sweet calamondin triploid seedling was checked one nucleolus (87.01%), two (11.68%) and three (1.29%) nucleoli were found (Table 8, Figure 10).

Table 8 Observations of chromosomes and nucleoli from root tip cells of sweet calamondin

Numbers of					
Seedlings	Samples	Chromosomes	Nucleoli (%)		
			Nucleolus	2 Nucleoli	3 Nucleoli
13	13	18	2390 (87.48)	342 (12.52)	-
1	1	27	67(87.01)	9(11.68)	1(1.29)

Figure 10 Nucleoli numbers (1) diploid and (2) triploid from root tip of sweet calamondin seedlings

CHAPTER 5

DISCUSSION

Vegetative phase

Length of shoot

The effect of polyploidization on vegetative and reproductive parts of plant is difficult to predict. However, polyploidy provides genome buffering, increased allelic diversity and heterozygosity, so that novel phenotypic variation is generated (Udall and Wendel, 2006). While Kermani et al. (2003) found significantly shorter or longer internodes within induced polyploids. The first evidence for chemical activity in treated shoots was delayed in the emerging of new growth from the treated buds in comparison with the untreated ones; probably this is why the shoot lengths showed different heights. Moreover, the slow growth may be due to a physiological disturbance caused by colchicine, resulting in a reduced rate of cell division (Swanson, 1957). Furthermore, treated shoot lengths showed a negative relationship with chemical concentrations in sweet calamondin except 0.03% of colchicine and 0.01% of the trifluralin treatment (Figure 1) while sour calamondin showed a mixed relationship among the treatments but less than the control (Figure 2). This result is supported by the finding of Barrett (1974), who found a delay in shoot emergence when citrus is treated with colchicine to develop polyploids, and also the nature of anti-mitotic agents to disturb the cell division. Moreover, colchicine and trifluralin showed a mortality rate of 25%, 75% in sweet calamondin, respectively, while a 25% mortality rate was found in trifluralin treated shoots in sour calamondin. The mortality rate of treated shoots was high due to the chemical nature, which might be related to the concentration level or long time of exposure of colchicine and trifluralin. Moreover, because of the herbicidal properties of trifluralin (Alder et al., 1960) the morality rates were quite high compared to colchicine treated shoots. It is similar to the finding of Zlesak et al. (2005), who found a 65% mortality rate in trifluralin-mediated polyploidization of *Rosa chinensis* minima Sims Voss seedlings. However, colchicine and trifluralin treatments from sweet calamondin and trifluralin treatment from sour calamondin did not show statistically significant differences in the

shoot length ($p > 0.05$) (Table 1). This finding was supported by Fatima (2004), where the colchicine treated shoot length did not show statistically significant differences in Kinnow, Musmabi and Succari (Pakistani citrus) but they were significantly less than the control, while a significant difference found in treated shoot lengths from colchicine treatment in sour calamondin ($p < 0.01$) (Table 2). Furthermore, the relationships between the physiological processes of plants are not well understood, although some cultural techniques (irrigation, fertilization, *etc.*) can improve them (Marini and Trout, 1984; Sansavini et al., 1985).

Number of leaf

The number of leaves was significantly larger in the diploid ancestral plants compared to chemically induced *Spathiphyllum wallisii* (Peace lily) tetraploid plant (Vanstechelman et al., 2009). The maximum leaf numbers were found from the control followed by colchicine and trifluralin treatments in sour calamondin but there was no significant difference between them ($p > 0.05$) (Table 2). This finding was nearly similar to Chaicharoen et al. (1995), where they found a higher number of leaves in diploid (12) compared of tetraploid (10) in mulberry (*Morus alba* Var. S54) plants treated with colchicine. However, there was no statistically significant difference found from the leaf number in sweet calamondin, but the data pattern of leaf numbers revealed a maximum in the control followed by the colchicine treatment while 0.01% of trifluralin showed a high leaf number (14.37) compared to the control (14.00) (Table 1). Furthermore, physiological processe of plants can be improved by irrigation, fertilization, *etc.* (Marini and Trout, 1984; Sansavini et al., 1985).

Thickness of leaf

The leaf thickness is a good diagnostic feature of induced polyploids. From the result, the maximum leaf thickness was found from 0.01% (0.36mm) of colchicine and trifluralin treatment followed by 0.03% (0.31mm) colchicine treatment in sweet calamondin (Table 1). The result was statistically non significant from colchicine and trifluralin treatments in sweet and trifluralin treatment in sour calamondin but among the treatments data pattern revealed some

inductive features in sweet and sour calamondin which might be related with ploidy levels, as the increased leaf thickness is an indicative characteristic of polyploidy in citrus (Barrett, 1974). Moreover, Vanstechelman et al. (2009) and Chaicharoen et al. (1995) found the thickness of leaves was significantly larger in the tetraploid plants compared to diploid plants. However, in sour calamondin, the significant differences were seen in the thickness of the leaf from colchicine treatment but not in a positive way as a ploidy level (Tables 1 and 2). This result might be influenced by external and internal physiological process of plants (Marini and Trout, 1984; Sansavini et al., 1985).

Area of leaf

In most instances, the leaf shape and size is a useful indicative feature to easily find out differences between diploids and polyploids because generally ploypoid leaf size and width is increased compared to diploids (Ray, 2002). However, polyploidy increases leaf size mainly by increasing the cell elongation rate (Sugiyama, 2005). The data pattern indicated that 0.01% of trifluralin treated twigs' leaf area (13.12 cm^2) was the maximum followed by the control and the colchicine treatment in sweet calamondin, however, it did not differ significantly ($p > 0.05$) (Table 1). In sour calamondin, the area of the leaf showed no statistically significant differences ($p > 0.05$) (Table 2) but the maximum leaf area was found from 0.03% (15.79 cm^2) of the trifluralin treatment followed by other treatments. Barrett (1974); Khan et al. (1992) and Jaskani et al. (1996) reported that the longer and broader leaves are related to tetraploids in citrus. Nevertheless, in acid citrus cultivars, Wakana et al. (2005) found a relatively large variation in leaf length between and among the original and colchicine induced tetraploid plants, probably due to the extent of the tree's vigor.

Chlorophyll content

In citrus, *Citrus sinensis* (L.) Osbeck, cellular nitrogen and chlorophyll contents were found to increase by only 25 percent from diploid to tetraploid citrus (Romero-Aranda et al., 1997), and the tendency of chlorophyll content to increase with an increase in ploidy level is not

always clear. However, the chlorophyll contents as a diagnostic measure of ploidy level and can be successfully used to differentiate between diploids and tetraploids. The highest chlorophyll content was found 0.03% (62.92) from trifluralin treatments and followed by others treatments (Table 2) in sour calamondin. This finding is nearly supported by Mathura et al. (2006), where the tetraploid black wattle (*Acacia mearnsii*) had more chlorophyll content than diploid black wattle and also the polyploid induced plants had an increased leaf chlorophyll content in *Coccinia palmata* and *Lagenaria sphaerica* (Ntuli and Zobolo, 2008). Furthermore, the maximum chlorophyll content was found from control in swcet calamondin (Table 1) but the chlorophyll contents from colchicine and trifluralin treatments in sweet and sour calamondin was not significantly different. However, the chlorophyll concentration of a leaf is strongly affected by numerous external factors, for example; light, pollutants, low salinity *etc.* (Karacan, 2006; Jaleel et al., 2008). Also the mesophyll cells did not respond to changes in ploidy like the bundle sheath cells. Furthermore, Warner and Edwards (1989) showed that chlorophyll content remained constant in various levels of ploidy in *Atriplex confertifolia* Torr. & Frem. (a dicot, Shadscale) diploid, tetraploid, hexaploid, octaploid and decaploid.

Stomatal number, Stomatal guard cell length and width

Stomatal size and number is related to ploidy level. But, the number of stomata on leaf surface varies widely among different species of plants and ploidy levels (Case, 1994). Moreover, in sour calamondin, the data pattern revealed the lowest stomatal number (87.63) per film by 0.07% of colchicine and 0.03% (85.25) of trifluralin treatment (Table 2). Furthermore, the stomatal number decreased with increase in chromosomes number (Achyuta and Vishveshwara, 1960) and stomatal frequency can be predicted with 83 and 87% accuracy by measuring stomatal guard cell length in coffee (Mishra, 1997). However, a study of the stomatal density revealed a lower stomatal number (64.72 - 65.86) from the control in colchicine and trifluralin treatments in sweet calamondin (Table 1) which is nearly similar to the finding of van Duren et al. (1996) and Azhar et al. (2000), where they found that the stomatal density was significantly higher in mixoploid than in diploid plants in banana.

According to the data pattern, the highest stomatal guard cell length was found from 0.01% of trifluralin and colchicine treatment and was followed by colchicine 0.05%, and other treatments in sweet calamondin. Similarly, the highest guard cell length was found from 0.07% of colchicine and 0.01% from trifluralin treatments in sour calamondin (Table 2). The maximum stomatal guard cell width was found from 0.05% of the colchicine treatment in sweet calamondin, and 0.05% of colchicine and 0.03% of trifluralin treatments in sour calamondin (Table 2). In *Scutellaria baicalensis*, the stomata of autotetraploids were larger but fewer compared to the controls (Gao et al., 2002) and for ornamental Alocasia, the stomatal length increased with the ploidy level (Thao et al., 2003). In contrast to this, Fatima (2004) found shorter stomatal guard cell length in tetraploid Musambi (4x) compared to diploid (2x), and also Klaewtanong (2009) found that 0.05% of colchicine gave a minimum stomatal length in calamondin, which are supportive of the above result.

Furthermore, in sweet calamondin, the stomatal guard cell length was statistically significant from colchicine and trifluralin treatments, stomatal guard cell width from colchicine treatment and stomatal numbers from trifluralin treatment in sour calamondin. This is similar to the finding of Fatima (2004) from stomatal studies in citrus. But there was no significant difference found in stomatal numbers from colchicine and trifluralin treatments and stomatal width from trifluralin treatments in sweet calamondin, stomatal numbers from colchicine treatment, stomatal guard cell length and width from the colchicine and trifluralin treatments in sour calamondin, (Tables 1 and 2). The result is similar to Madon et al. (2005), where they found no significant difference in the stomatal density between polyploids and controls in oil palm (2n). However, the effect of ploidy in stomatal frequencies, stomatal guard cell length, and genotypic difference in stomatal frequency were observed among cultivars of the same ploidy level (Mishra, 1997).

Nevertheless, using only the stomatal count is not an effective method to find out the exact ploidy level in calamondin plant. Moreover, it is known that various physiological factors such as, light intensity, leaf development and water content of the plant can influence the stomatal density (Marini and Trout, 1984; Sansavini et al., 1985; van Duren et al., 1996).

Reproductive phase

Number of pollinated flower and fruit sets

The fruit set process related to the flower bud induction process, climatic stress and internal limitations from a lack of pollination, adverse hormonal levels or competition for carbohydrate and/or nutrients may lead to unsuccessful fruit (Albrigo and Sauco, 2004). However, in this study, the chemicals showed negative relationships between the number of flowers and chemical concentrations compared to the control in sweet and sour calamondin. But the percentage of fruit setting showed a positive relationships among treatments in sweet calamondin and a negative relationship except in 0.07% of colchicine compared to the control in sour calamondin (Tables 6 and 7). This result probably showed the effectiveness of chemicals. Moreover, Lai and Chen (2008) found that the flower bud formation of calamondin was not influenced whether plants were held in high or low temperature conditions. Generally the colchicine doubling experiment does not result in larger whole plants or greater yields (Sanford, 1983), and also the fertility was a problem with colchicine-induced polyploids, but crosses among American cranberries have improved fertility (exceeding 80% fruit set in greenhouse tests) (Zeldin and McCown, 2004). However, the relationships between the physiological processes of plants and their productivity are not well understood, although some cultural techniques (irrigation, fertilization, *etc.*) can improve it (Marini and Trout, 1984; Sansavini et al., 1985)

Number of seeds and seeds per fruit

The maximum numbers of seeds were found in the control followed by other colchicine treatments while the trifluralin treatment had fewer seed number compared to the control in sweet calamondin, however, in sour calamondin 0.07% of colchicine revealed a maximum seed number among the colchicine treatment and the control had a maximum number of seeds in the trifluralin treatment. The number of seeds per fruit was the highest at 0.05% (6.08) from the colchicine treatment and the control (4.76) from the trifluralin treatment in sweet calamondin (Table 6), as well as a maximum number of seeds per fruit were found at 0.03%

(2.50) from the colchicine treatment and at 0.05% (1.27) from the trifluralin treatment in sour calamondin (Table 7). The mentioned results are similar to the finding of Promtong (2007), as number of seeds was not dependent on the concentration of the substance (colchicine and trifluralin) in Calamondin, Dankwean lime and Sainumpheng. Moreover, the highest number of seeds were found in Bitter sweet orange (27.9) followed by Sour orange (14.8) (Jaskani et al., 2006).

Number of embryos and embryos per seed

Most species of the genus *Citrus* are characterized by polyembryony and they have more than 90% nucellar seedlings (Roose and Kupper, 1992). They can produce 1–40 adventive embryos by the nucellus (Fusurato, 1957) so that two or more embryos are developed in a single seed, but the ratio deviates from crosses depending on the species involved (Cameron and Soost, 1979). The maximum number of embryos was in the control followed by other treatments in sweet calamondin (Table 6). Moreover in sour calamondin, the maximum numbers of embryos were found at 0.07% of colchicine from the colchicine treatment while the highest numbers of embryos were found from control in the trifluralin treatment (Table 7). Furthermore, the maximum embryo per seed was found in 0.03% (4.52) of colchicine and 0.01% (3.72) of trifluralin treatments in sweet calamondin, while in sour calamondin the control showed more (4.16) embryos per seed in the colchicine treatment, and 0.05% (4.86) of trifluralin showed the highest number of embryos per seed in trifluralin treatment (Table 7). This result is nearly similar to the finding of Morton and Miami (1987), they showed that calamondin seeds are polyembryonic and have 3-5 embryos. However, the number of embryos per seed varies greatly even on one tree and the average number differs greatly according to the variety with no general consistency within many of the species in which polyembryony is present (Frost and Soost, 1968).

Number of seedlings and seedlings per seed

The polyembryonic cultivar seeds generally contain two or more embryos, and very small embryos could not germinate (Toolapong, et al., 1995) that is why the total number of seedlings decreased compared to the total number of embryos. Moreover, the average number of seedlings produced per seed is commonly much smaller than the total number of embryos (Frost and Soost, 1968). The highest percentage of seedlings found from embryos in 0.05% of colchicine treatment while the maximum percentage of seedlings was found from the control in the trifluralin treatment in sweet calamondin. In sour calamondin the maximum percentage of seedlings was from the control followed by colchicine and trifluralin treatments. The highest number of seedlings per seed was found from 0.01% (1.28) of colchicine treatment from sweet calamondin (Table 6) and the maximum was in the control (2.16) among the colchicine and trifluralin treatments in sour calamondin (Table 7). This finding is supported by Promtong (2007), as the number of seedlings per seed was not dependent on the concentration of the substance (colchicine and trifluralin) in Calamondin, Dankwean lime and Sainumpheng.

Number of triploid seedlings

The high proportions of 3x hybrids were obtained from crosses of 4x sexual seed parents by 2x pollen parents in citrus (Cameron and Burnett, 1978). In this study, 902 (34.38%) seedlings were observed from 2,623 embryos where three (0.33%) triploid seedlings were found and the rest remained diploid seedlings. In the case of the triploid seedling progenies, three seedlings were recovered from treated female flower where two from 0.03% and one from 0.05% colchicine concentrations in sweet calamondin. Three triploids seedlings were found from colchicine treated shoots, suggesting chromosome polyploidization, and probably that seedling being inherently triploid through the union of a $2n$ and n gamete because the nucellar embryony does not prevent normal sexual reproduction in citrus (Esan and Soost, 1977; Wilms et al., 1983). This result is nearly similar to Kuntad (2005), who found that the six triploid seedlings from treated female flowers, two from 'Lime' (0.08% colchicine) and four from 'Meiwa Kumquat' where one each from 0.02 and 0.06% of colchicine while two from 0.08% of colchicine

concentrations. Moreover, Promtong (2007) recovered two triploid seedlings by using 0.08% of colchicine from treated female flowers in Sainumpheng variety (Mandarin). Additionally, the lateral buds in citrus cultivars were treated with colchicine and grafted onto monoembryonic citrus cultivars, and 7.68 triploids per fruit were found when the tetraploid 'Kiyomi' was used as the seed parent (Kaneyoshi *et al.*, 2008). Moreover, colchicine treatment at concentrations of 0.05–0.2% produced 40–76% of the tetraploid seedlings and was superior to 0.02% colchicine in Japanese Barberry (Lehrer et al., 2008).

Chromosome observation

It is extremely difficult to distinguish between zygotic and nucellar seedlings because of identical morphological characteristics in close crosses (Frost and Soost, 1968). Therefore, chromosome counts have great significance in *citrus* species because of the taxonomic complexity of varieties and species. However, the chromosomes were counted from root tips of 902 seedlings. Three triploid seedlings found among them and the remaining were diploid seedlings. Geraci (1978) counted chromosomes of immature embryos at the cotyledonary stage and on seedlings coming from the same crosses of 2x X 4x lemons and observed 2% to 4% of triploids in the embryos and 0 to 0.56% of triploids in the seedlings. Additionally, an interspecific allotetraploid somatic hybrid plant and two autotetraploid lemon plants were regenerated from a chemical protoplast fusion of Valencia sweet orange and Femminello lemon. The plants were characterized according to their chromosome number (Tusa et al., 1990). All the seedlings from underdeveloped seeds were of a tetraploid nature (Oiyama et al., 1991) while from fully developed seeds were tetraploid as determined by a chromosome count from root tip cells (Oiyama, 1981). Therefore, the only sure way to determine whether chromosome doubling has occurred is to make chromosome counts (Ray, 2002).

Nucleoli observation

The nucleolar number has been used as an index of ploidy level in plants. The genome size and the number of nucleoli may be used to determine the ploidy level as an alternative to chromosome counting (Vilhar et al., 2002). However, in early stage of cell division, the nucleolus formation is not clear so the one nucleolus or two nucleoli does not matter that plant is not a triploid but at matured stage of cell division triploid plant cells have three nucleoli number Toolapong (1999). In this study, only haploid and diploid nucleoli numbers were found from root tip of cells in diploid seedlings of sweet calamondin while haploid, diploid and triploid nucleoli numbers were found from the root tip of triploid sweet calamondin seedling (Table 8, Figure 10). This finding is nearly similar to Toolapong (1999), where he observed the haploid and diploid numbers of nucleoli in diploid seedlings while the haploid, diploid and triploid number of nucleoli were found in triploid seedlings in 'Banpeiyu Pummelo' crossed with 'Ruby Red' Grapefruit progenies. A similar result was found by Klaewtanong (2009), where the triploid nucleoli number from the root tips of 'Ocean' citrus seedling progenies was related to the triploid ploidy level.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Conclusion

The induced ploidy manipulations have played a significant role in the domestication and improvement of crops. Anti-mitotic agents have great potential to bring about sudden changes in genotypes of plants. This is why breeders have been allowing sexual recombination between mutated plants (anti-mitotic treated plant) and normal plants to find triploid seedlings. However, various methods have been used to produce triploid seedlings such as crossing diploids with tetraploids (2n x 4n), crossing tetraploids with diploids (4n x 2n), using the embryo rescue technique, endosperm culture, irradiation *etc.* But, the somatic polyploidization method is easily produces triploid cultivars.

In this study, five doses, 0, 0.01, 0.03, 0.05, 0.07% of colchicine and trifluralin were applied to meristematic buds of sweet and sour calamondin. After treating the meristematic buds, new growth started to emerge slowly in comparison with the control. Moreover, treated shoots started to initiate flower buds, and those matured flower buds were emasculated in the evening and covered with a small paraffin paper bag. The next morning the treated emasculated flower was pollinated by untreated emasculated pollen grains and vice versa. After pollination, the fertilized flower started fruit setting, then removed the paraffin paper bag completely and fruits became mature.

According to the results, the shoot lengths revealed different heights and showed 12.5% and 50% mortality rates from colchicine and trifluralin treatments respectively. Statistically significant results were found in stomatal guard cell length and width from the colchicine and stomatal guard cell length from the trifluralin treatments in sweet calamondin while in sour calamondin statistically significant different revealed in shoots length and leaf thickness from the colchicine and in stomatal numbers from the trifluralin treatments. Moreover, there were no significant differences found in the shoots length, leaf numbers, thickness and area, chlorophyll content, and stomatal numbers from the colchicine and trifluralin treatments and stomatal guard cell width from the trifluralin treatment in sweet calamondin. The leaf numbers and area, chlorophyll content, stomatal

guard cell length and width from the colchicine and the trifluralin treatments, stomatal numbers from the colchicine treatment and shoots length from the trifluralin treatment in sour calamondin also revealed non siginificant difference. Furthermore, the testing equality of the mean between sweet and sour calamondin after chemical application, thickness of leaf, chlorophyll content, stomatal number and length were revealed to have a highly significant difference while shoots length, leaf number, area and stomatal width showed no significant difference.

The number of flowers and chemical concentrations from sweet and sour calamondin showed negative relationships while the percentage of fruit setting showed positive relationships among treatments in sweet calamondin and negative relationship in sour calamondin except in 0.07% of them. However, the seed number, embryos and seedlings revealed variations among the treatment but was not related to the chemical concentrations. From mature fruits seeds were extracted, and the outer and inner coats of seeds were removed. After removing the outer and inner seed coats, seeds were germinated on moistened double thickness filter papers in petri dishes at room temperature. In addition, chromosomes were observed from 902 seedlings in 2,623 embryos and three triploid (3x) seedlings were found while the others remained diploid (2x). Moreover, two triploid seedlings were found from 0.03% of colchicine concentrations and one triploid seedling from 0.05% of colchicine concentrations in sweet calamondin.

Recommendations

The following recommendations are suggested on the basis of results and experiences of conducted experiments:

1. The colchicine concentration 0.03% and 0.05% are effective to produce triploid seedling progenies in sweet calamondin.

2. The mentioned results proposed that somatic polyploidization by artificially induced diploid shoots (colchiploid shoots) could be a powerful method to create novel variations in the breeding of calamondin.

3. If possible seeds should be germinated in tissue culture media to encourage for all embryos germination, so the exact number of ploidy levels can be found from the treatments in calamondin.

4. The plant mortality occurred, might be due to the solution being too strong or exposure for too long, or a combination of both. Therefore, it should be considered and avoided in future studies.

5. During rainy season research should be conducted in greenhouse to maintain the pollination of calamondin plant.

6. If possible, flowcytometry equipment should be used to check DNA contents of mutated shoots to confirm their ploidy level in a short amount of time.

7. Such kind of studies should be continued because it helps to improve citrus cultivar germplasms and farmers incomes.

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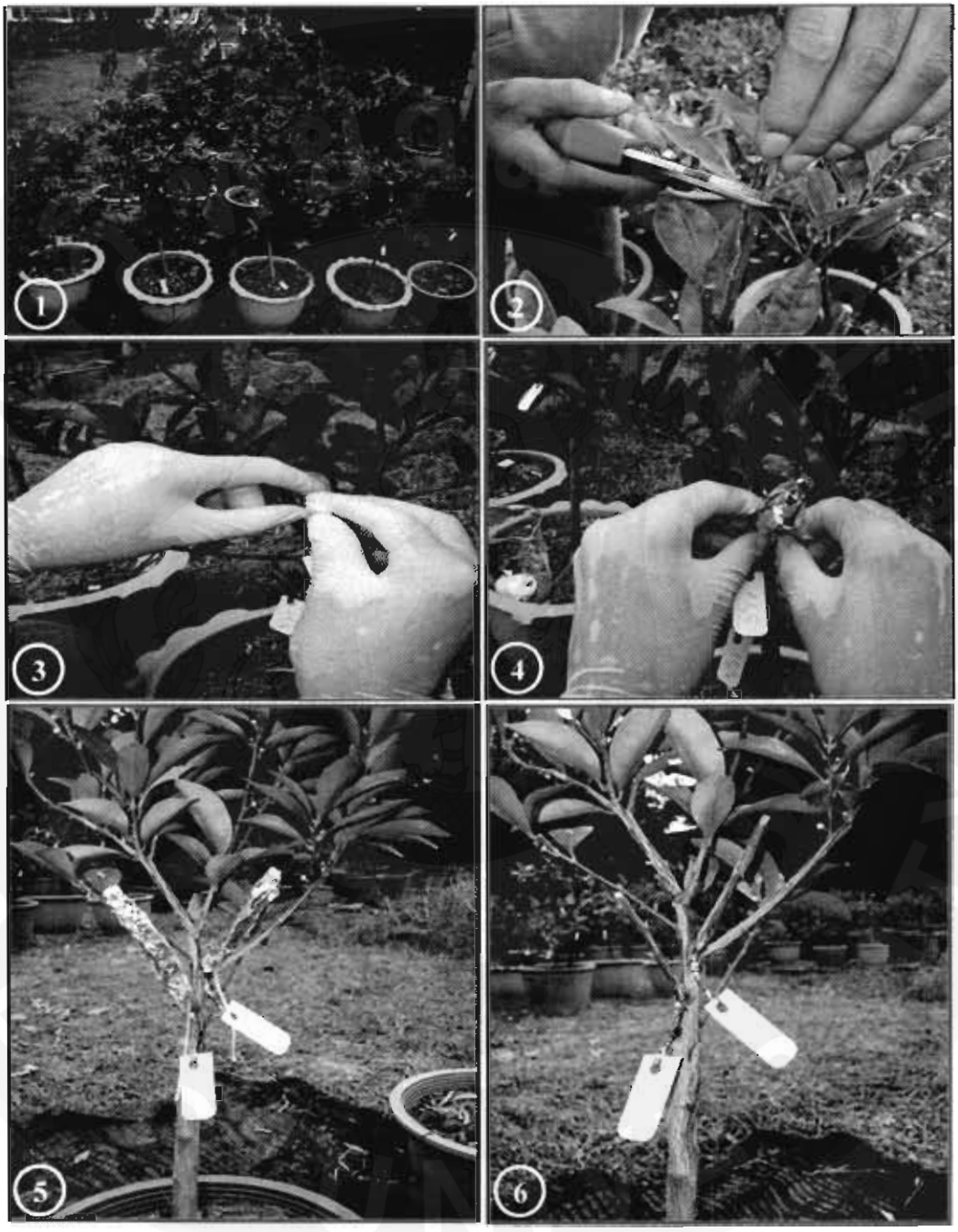
APPENDIX

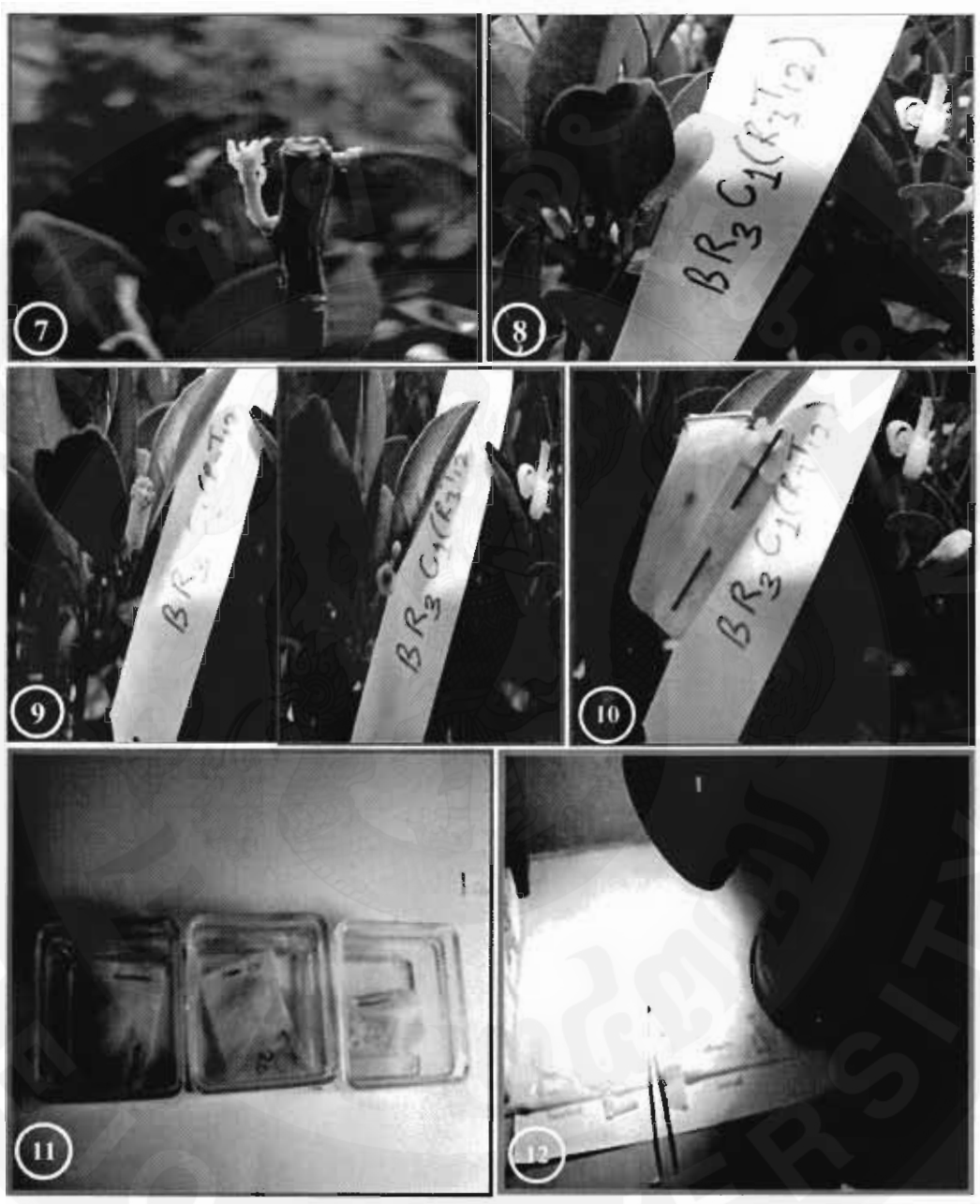
Appendix A

Research Figures



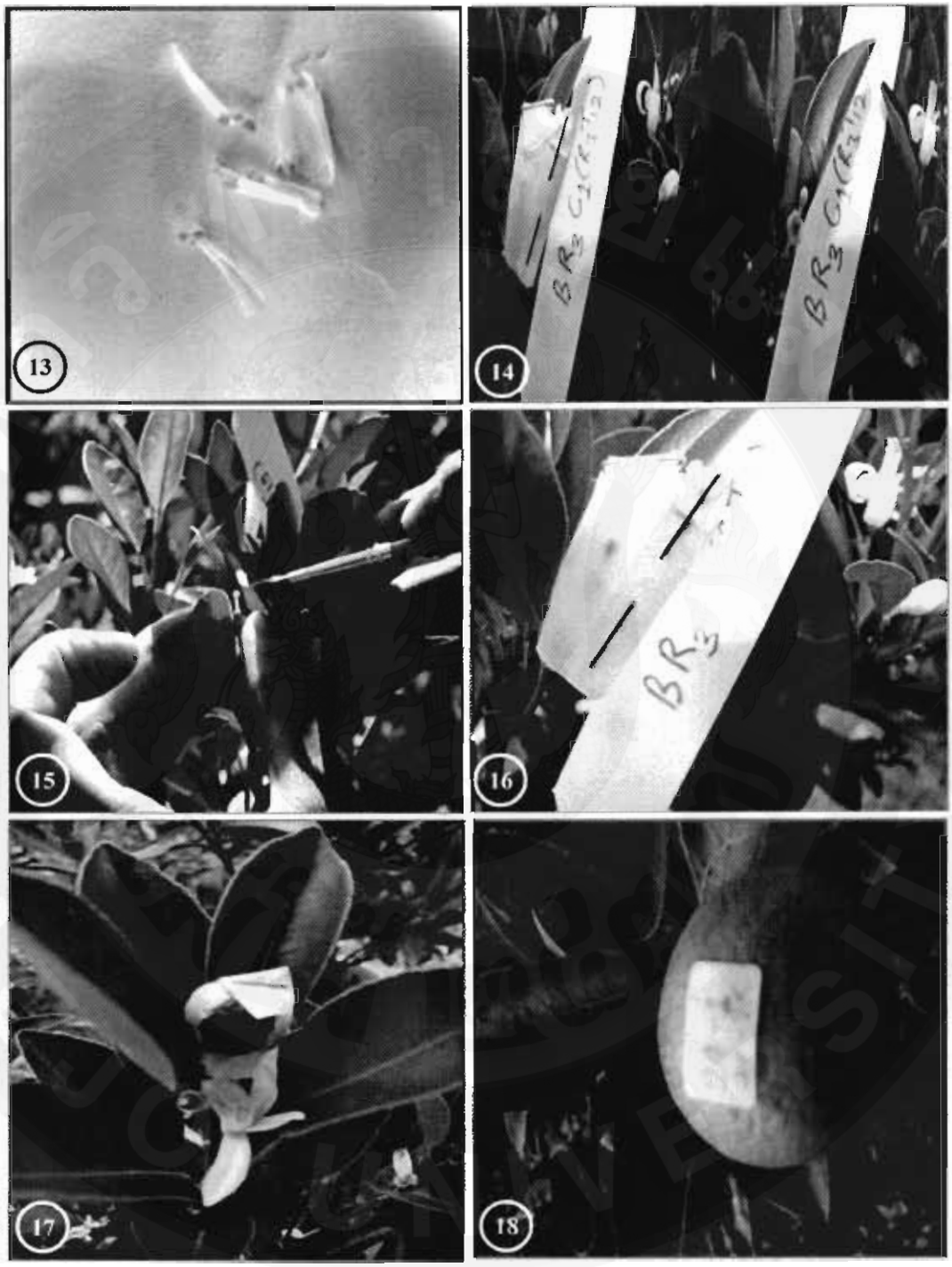
Appendix Figure 1 Experiment methodology related pictures I

1. selection of plant
2. cutting of branch
3. treating branch with chemical soaked in cotton wool
4. wrapping treated shoot with a plastic sheet and aluminum foil,
5. treated shoots
6. removed wrapping materials after 6 days



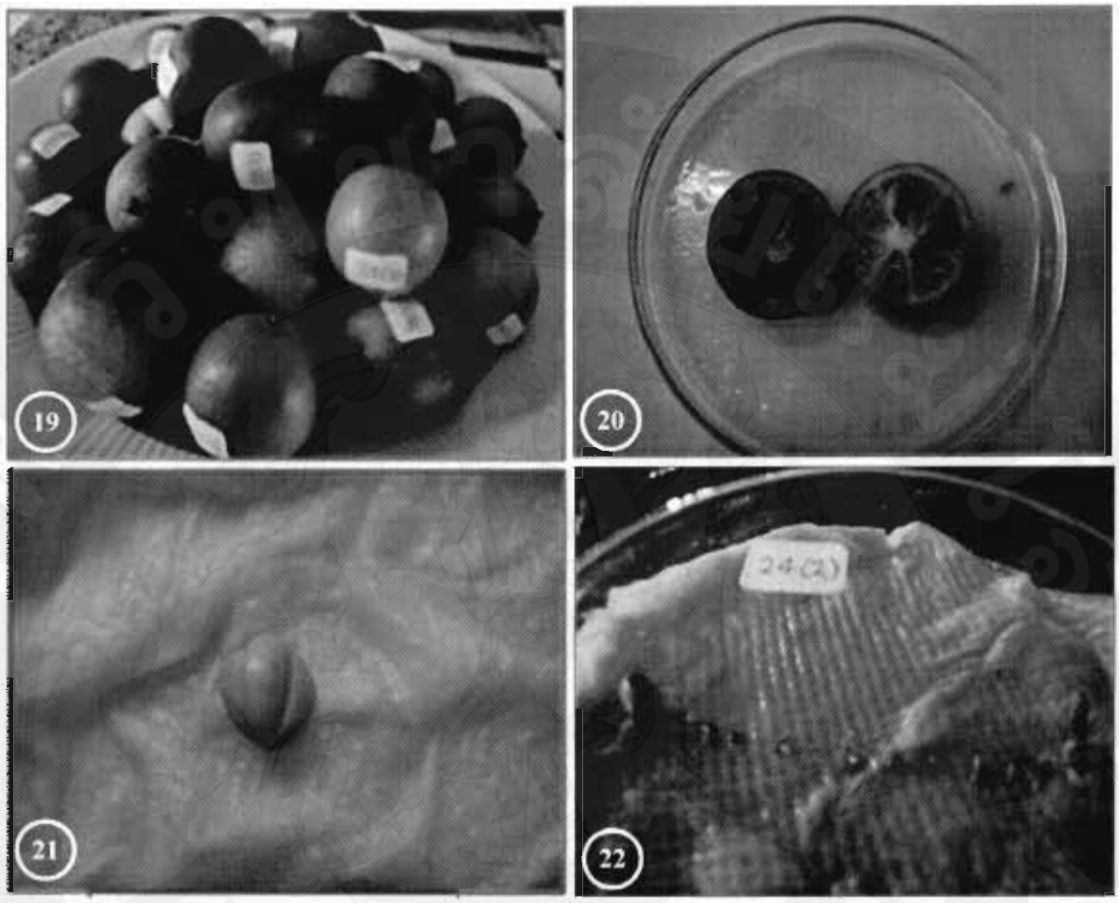
Appendix Figure 2 Experiment methodology related pictures II

7. new emerging treated shoots
8. start flowering
9. emasculation of flower
10. emasculated flower cover with paraffin paper
11. emasculated pollen grains inside paraffin paper
12. pollen grains under artificial light



Appendix Figure 3 Experiment methodology related pictures III

13. full bloom pollen grains
14. uncover the emasculated flower
15. pollination
16. pollinated flower cover with paraffin paper
17. fruit setting
18. matured fruit



Appendix Figure 4 Experiment methodology related pictures IV

19. harvested fruits
20. remove seeds
21. seed
22. seedlings

Appendix B

Curriculum Vitae

Curriculum Vitae

Name	Mr. Arbind Mani Tripathi	
Date of Birth	20th January, 1972	
Educational Background	1990-1994	B.Sc. (Ag.) Gorakhpur University of Gorakhpur, UP, India
	2008- 2010	M.S. Horticulture Maejo University, Chiang Mai, Thailand
Scholarship		Thailand International Development Cooperation Agency (TICA)
Work Experience	1995-2002	High School Teacher Shri Siddhartha Higher Secondary School, Phedikhola, Syangja. Under Ministry of Education and Social Welfare, Nepal
	2002-2009	Junior Technician (J.T.): District Agriculture Development Office (DADO), Gorkha, Under Department of Agriculture, Nepal
	2009 to Present	Crop Development Officer Department of Agriculture, Ministry of Agriculture and Cooperatives, Nepal

Publication

I. Paper presented in 16th National Graduate Research Conference held on 11-12 March, 2010 at Maejo University; Chiang Mai (Thailand)

II. Maejo University, Annual Conference 2010, on May 26-27, 2010