CHAPTER 1

INTRODUCTION

1.1 Introduction

Zinnia is a genus of beautiful ornamental plants. Novel flowers colors of this plant will be of great commercial and aesthetic value. It is not famous like chrysanthemum or sunflower. However, Zinnia hybrids were induced too many cultivars because it can be a cut flower in the flower markets of many countries. Zinnia is a cosmopolitan ornamental plant with a long history. Many breeding companies have induced new cultivars with different attractive morphological characters. Zinnia cultivar classification is complicated by the fact that the typical characters are of qualitative nature and under strong environmental control such as height, flower size and leaf shape. To overcome these problems, in the present research *Zinnia* species and cultivars were analyzed on the genotypic level (DNA content, DNA sequence).

Furthermore, the hybrids of zinnias are propagated by seedling. So the seeds of zinnia plants are produced by cross-breeding of parents. Then it is difficult to conserve cultivars. In addition, *in vitro* culture is a type of propagation method that enables multiplication of plant and genetic variation to be observed in a relatively short time. Therefore the conditions required for *in vitro* culture of zinnia were determined because there is little information available on *in vitro* zinnia. The results from these studies can be used to generate basic data for manipulation of zinnia culture.

1.2 Literature review

1.2.1 Zinnia

1.2.1.1 Scientific classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: Zinnia

Species:

Zinnia acerosa

Zinnia angustifolia

Zinnia anomala

Zinnia elegans

Zinnia grandiflora

Zinnia haageana

Zinnia maritima

Zinnia peruviana

Etc.

1.2.1.2 Origin/history of zinnia (wikipedia, 2007)

Zinnia is a genus consisting of about 20 species of annual and perennial plants of family Asteraceae, originally from scrub and dry grassland in an area stretching from the Southwest to South America, but primarily Mexico.

Even after seeds of zinnias were sent back to Europe in the 18th century, the plants were not much to look at. Named for German botanist Dr. Johann Gottfried Zinn (1727-1759), who wrote the first description of the flower, the genus *Zinnia* had to wait for the late 19th century to become more successful as a garden annual. Breeding by selection occurred in Germany, Holland, and Italy: 'Pumila Mixed' (precursors of the 'cut-and-come-again' zinnias) and two selections from that strain, 'Mammoth' and 'Striata', were brought to Europe and enjoyed great success with gardeners. But the start of the zinnia's real popularity began around 1920 when Bodger Seeds Ltd. introduced the dahlia-flowered 'Giant Dahlia'. John Bodger discovered it as a natural mutation in a field of 'Mammoth' and within the next few years selected the large, flat-flowered 'California Giant' from the strain. It was available in separate colors and was considered to be a new trend in plant habit and flower form. It won a gold medal from the Royal Horticultural Society of England.

1.2.1.3 The advantages of *Zinnia*

1) Gardening

Zinnias are popular garden flowers and especially favored by butterflies, therefore zinnias are interested by many gardeners. They are usually grown from seeds, and preferably in fertile, humus-rich, and well-drained soil, in an area with full sun. They will reseed themselves each year. Zinnias also have an amazing number of colors. in fact, flowers come in almost every shade except blue. Most are solid, but some, in particular *Z. haageana*, are bicolor with a contrasting color at the tip of each petal. The colors include yellow, orange, cherry, pink, purple, scarlet, and white. Heights are an important consideration when planning a garden, and zinnias have growth habits to suit every need. The tall, 3- to 4-foot varieties are best for the middle or rear of a border or in a cutting garden. Dwarf plants grow 8 to 14 inches tall and do well in pots as well as at the front of a garden. *Z. angustifolia* plants reach only 8 to 15 inches in height with an equal spread. They are excellent in the ground, in pots or hanging containers and as summer-flowering ground covers.

In addition, dry flower of zinnia are used to decorate in houses. Alka *et al*. (2003) studied process of the drying zinnia flower such as *Z .angustifolia*. Zinnia flowers were embedded in different materials just as sand, borax and silica gel and dried at different temperatures to find the appropriate environment to exhibit well-maintained flower shape, smooth petals and attractive color of zinnia flower.

2) Ethno botanical use

Zinnia species are used as a medicinal herb in some rural regions in North America (Table 1.1).

Table 1.1 Use of zinnia species.

Scientific name	Use
Zinnia acerosa	Diarrhea, swelling, aches
Zinnia angustifolia	Poison
Zinnia elegans	Anodyne, Menstruation
Zinnia multiflora	Eye Altschul
Zinnia peruviana	Anodyne, Digestive, Menstruation
Zinnia grandiflora	Stomachache, Cathartic Heartburn,
	Nose, throat and kidney trouble
	Bath for excessive sweating

In addition, phytochem screening of *Z. elegans* indicats that it contains coumarin, tannins, alkaloids, cardiac glycosides, sitosterol and triterpenes. The alcohol extractions of different parts of zinnia (leaves, stems and flowers) exhibits estrogen like activity when it was administered orally to ovariectomized female rats. The extractions of leaves and stems of zinnia had a stimulatory effect on the isolated pregnant rat uterus, while the flower extraction has much less activity (Sharada *et al*, 1995).

The major components of *Z. elegans* flowers consist of the followings:

Germacrene D (12.4%) has been shown to be the major volatile component of several species of aster and is a sex stimulant of Periplaneta males that was isolated from various plant leaves (Kitamura *et al.*, 1976). The chemical structure is shown on Figure 1.1a.

Myrcene (7-methyl-3-methyleneocta-1, 6-diene) (11.8%) is a triply-unsaturated hydrocarbon used as an intermediate for the manufacture of flavor and fragrance chemicals. The chemical structure is shown on Figure 1.1b.

p-Cymene (9.1%) is one of the alkyl-substituted aromatic hydrocarbons which are found in volatile oils from over 100 plants. (Zoghbi *et al.*, 2000)

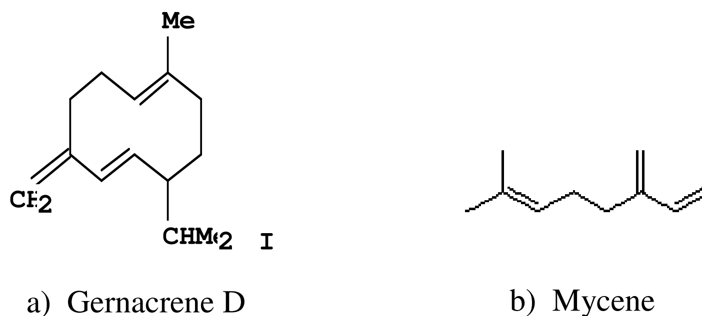

Figure 1.1 The formula structure of Gernacrene D (a) and Myene (b)

1.2.1.4 The cultivars of *Zinnia*

One of the reasons for the popularity of the zinnia is the cultivar diversity of its forms and colors. There are zinnias with white, cream, green, yellow, apricot, orange, red, bronze, crimson, purple, and lilac flowers; zinnias with striped, speckled and bicolor flowers; zinnias with double, semi-double and dahlia-like 'pompon' flowers; zinnias that range from dwarfs that do not exceed 6 in. (15 cm.) in height to cut flower that get 3 ft. (0.9 m.) tall.

Over 100 cultivars have been produced since selective breeding started in the 19th century. New varieties are resistant to powdery mildew and other diseases. 'Old Mexico' likes the wild plant with single, daisy like flower heads with wide purple rays. The first tetraploid zinnia, called 'State Fair', came from Ferry Morse Seed Company in the 1950's. (Tetraploids exhibit larger flowers on stronger stems, vigorous growth, and increased disease resistance.) Dwarf Zinnia (*Zinnia haageana*) was introduced for example, cv. 'Persian Carpet' and cv. 'Old Mexico'. John Mondry, who was working at the time for W. Atlee Burpee Company found a plant in the field with flowers that had no petals but was composed entirely of female reproductive parts. They could form seeds only after being cross-pollinated. That discovery led the way to the dwarf F1 Hybrid cv. 'Peter Pan' series introduced from 1971 to 1980. Yoshiro Arimitsu and Charles Weddle (National Garden Bureau, 2007) bred seven separate colors that were recognized as AAS Winners, now sold by Goldsmith Seeds, a wholesale seed company. Bodger Seed Ltd introduced the F1 Hybrid cv. 'Ruffles' series (cv. 'Scarlet', AAS 1974; cv. 'Cherry' and 'Yellow', AAS, 1978, http://www.all-americaselections.org), developed by Mondry (who had resigned from Burpee) as cutting flower plants.

In 1999 the 'Profusion' zinnia, 'Cherry' zinnia and 'Orange' zinnia, from Sakata Seed Corporation won Gold Medals from AAS--the first in 10 years. They represent a breakthrough in breeding for zinnias. They are interspecific crosses; that is, the result of crossing two species, *Z. angustifolia* and *Z. elegans*. They represent the best of both: heat and humidity tolerance, disease resistance, easy maintenance (no deadheading of spent blooms required), pretty 2- to 3-inches single flowers, and compact growth (12 to 18 inches tall) (National Garden Bureau, 2007).

1.2.2 Propagation of zinnia

1.2.2.1 Traditional propagation

Zinnia has been propagated by seed and stem cutting. Seeding is an easy method, with a high survival of plants compared to stem cutting. However, seed propagation, is difficult to maintain the characters because of out-crossing behavior of zinnia. The seed companies usually cross parents of zinnia and bring its seeds to culture for sale because the morphological characteristics of new cultivars are stable.

a) Pests and diseases

Zinnias are basically pest free. For years, though, they have been known to be affected by two fungal diseases: powdery mildew and alternaria blight. Alternaria blight causes reddish brown spots on both foliage and flowers. It is a problem in the tropical area more than any other areas.

The types of herbicide protecting zinnia from pets and disease are Daconil, Dithane, Mancozeb, Tilt, Sulfur, Benlate, Comag, etc. The best defense against the fungus is prevention. The leaves should not allow to wet and there is space

between these plants so they have good air circulation. *Z. angustifolia* and *Z. haageana* are more mildew-resistant than most *Z. elegans*. The interspecific crosses of *Z. elegans* and *Z. angustifolia*, such as cv. 'Profusion', are very resistant to powdery mildew.

Furthermore, some researchers tried to develop cultivars for resistance to disease. Gombert *et al*. (2001) studied fifty-seven cultivars of zinnia (*Zinnia elegans* Jacq.) for 17 weeks to determine their resistance to alternaria blight (*Alternaria zinniae* Pape), powdery mildew (*Erysiphe cichoracearum* DC ex Merat) and bacterial leaf and flower spot [*Xanthomonas campestris* pv. zinniae (syn. X. *nigromaculans* f. sp. zinniae Hopkins & Dowson)]. They found that only two cultivars showed tolerance to powdery mildew and alternaria blight at week 17 and induced new cultivars (such as cv. 'Profusion') resistance.

Boyle and Wick (1996) found that *Zinnia marylandica* (allotetraploids of *Z. angustifolia* x *Z. violacea*) was backcrossed with *Z. angustifolia* and *Z. violacea,* after that seeds were generated. Backcross families were screened for resistance to alternaria blight (*Alternaria zinniae* Pape), bacterial leaf and flower spot [*Xanthomonas campestris* pv. Zinniae] and powdery mildew (*Erysiphe cichoracearum*). It was found that all backcross families exhibited high levels of resistance to alternaria blight and powdery mildew. Backcross that derived from crossing *Z. marylandica* with *Z. angustifolia* was highly resistant to bacterial leaf and flower spot. Backcross that derived from crossing *Z. marylandica* with *Z. violacea* was susceptible to this disease. *Z. angustifolia* genome in backcross, allotetraploids is sufficient to confer resistance to *A. zinniae* and *E. cichoracearum*. At least two *Z.*

angustifolia genomes were required in backcross to provide resistance to *X. campestris* pv. zinniae.

b) The environmental effects of zinnia growing and flowering

There are a few literatures about the growth and flowering condition of field culture of zinnias.

Miyajima (1998) investigated the process and condition of seed production of *zinnia violacea*. He found that there was a low yield of seed from double-flowered zinnias *(Zinnia violacea)* compared to single-flowered plants so seed selection of double-flowered zinnias was investigated. Zinnia was found to be poor pollination. In the double-flowered capitula, newly opened ray petals overlapped the pistils that unfolded the previous day and the pollen flow to ray stigmata was limited due to the infrequent visitation by pollinators. Discussion on the composition of capitula and their making by florets of *Z. violacea* to improve seed production is given by Miyajima and Nakayama (1995).

In addition, the seed germination and growth rate depend on seed harvest time (Miyajima, 1997). Percentage of germination increased in seed harvested from 10 to 19 days after pollination for all cultivars. Furthermore, seed germination is also depending on pericarp (Miyajima, 1996). Time to germination and variance of time to germination were different between intact seeds produced by ray florets (R seeds) and tubular florets (T seeds). For the seeds without pericarps, percentage of germination after 120 hours from the beginning of imbibition was close to 100%. Time to germination was shortened and variance of time to germination was lessened by pericarp removal. Imbibition speed and final water absorption were accelerated by pericarp removal. The structure of the pericarp was different between R and T seeds

and this was considered to be the cause of the difference in water absorption and germination between R and T seeds.

Finally, the increase in the capitula of zinnia plants (*Zinnia violacea*) was investigated by analyzing the production of shoots (Miyajima, 2000). The effects of removing the buds from capitula and application of BA on the production of shoots were also evaluated. Some of the chemicals could induce seed germination such as H_2O_2 (Ogawa, 2001), Flurprimidol (Premachandra, 1994, 1996). The seed yield of zinnia was affected directly by macronutrients (N, P and K) (Dar, 2002).

1.2.2.2. *In vitro* culture

a) Propagation

From traditional propagation, it was found that the phenotypes of morphological characters of zinnia were depended on many criteria such as environment and genetic stability. Genetic stability is the important impact of maintaining hybrids or new breeding cultivars. Normally, zinnias are propagated by seeds, so genetic variation is high and backcross on breeding is an uncomfortable technique. Although zinnias may be propagated by vegetative cutting techniques, greenhouse maintenance of selected zinnia genotypes is difficult due to high susceptibility to both disease and insect infestations and a tendency for stock plants to decline in vigor over time. An alternative *in vitro* technique for zinnia offers the ability to uniformly mass-produce parent line and a means for safe long-term maintenance of valuable germplasm developed through breeding programs. Therefore some researchers had attempted to manipulate zinnia by *in vitro* culture.

Z. marylandica (Spooner *et al*., 1991) is a disease-resistant zinnia (*Z. angustifolia x Z. elegans*). Self-pollination of zinnia amphipolyploid produces seed with essentially clonal uniformity. Susan (1992) studied *in vitro* culture of *Z. marylandica* and found that adventitious shoots formed on *in vitro* culture of embryo. Optimum conditions for adventitious shoot formation were 16-day-old cotyledons oriented adaxial surface down on MS salts and organics supplement with 0.2 µM TDZ, based on number of adventitious shoots formed and percentage of embryos forming adventitious shoots. Embryos cultured on 22.2 µM TDZ produced more callus and formed adventitious shoots when they were culture a long time. Furthermore, Susan *et al.* (1992) evaluated the variation of adventitious-derived plants from medium containing two levels of TDZ and found that 8% of plants derived from 0.2 µM TDZ and 13% from 22.2 µM TDZ had variant characters.

The introduction of new, uniform hybrids requires controlled cross-pollination of selected parental lines. Zinnias produce both unisexual female ray flower and bisexual disk florets within a single flower head, which interferes with production of 100% hybrid seed. A male sterile genotype exists in zinnia and has been beneficial in hybrid seed production (Cowen and Ewart, 1990). Rogers (1992) demonstrated that the sterile male *Z. elegans* can be long-term cultured by tissue culture technique. Shoot culture initiated from axillary buds of sterile male zinnia proliferated best on MS medium with 1µM BA.

b) Trachea element

Zinnia is a good model system like *Arabidopsis* for *in vitro* study of the interaction between different plant cell types and the consequences for commitment to particular cell fate such as trachea elementary (TE). The use of a remarkable synchronized *in vitro* cell system, the *Zinnia elegans* mesophyll cell (Fukuda and Komamine, 1980) allows us to establish the chronology of molecular and biochemical events during the commitment and differentiation to a specific cell fate (McCann and Roberts, 2000; Milioni *et al*., 2001). Cells isolated from the leaves of zinnia cv. 'Envy' were cultured in liquid medium and supplied with two plant growth regulators, auxin and cytokinin. Cells that were already differentiated as photosynthetic mesophyll cells in the leaf now transdifferentiate —change cell fate— to become tracheary elements (Fukuda and Komamine, 1980) (Figure 1.2). Sheets of epidermal cells can be induced to differentiate to TE fate (Church and Galston, 1989). TEs are cells, dead at maturity, that in the plant form a series of connected tubes that transport water and dissolved minerals from the root to the shoot. During the formation of TEs, hoops of secondary cell wall material are deposited and subsequently stiffened and waterproofed by the deposition of lignin. Finally, the end wall of the cell is broken down and the cell contents are autolysed. The differentiation of mesophyll or epidermal cells *in vitro* to a TE fate is analogous to the ability of cortical cells in wounded stems to transdifferentiate into xylem elements and reestablishes vascular continuity. Thus, TE fate is terminal. The zinnia mesophyll cell system has several key advantages for studying xylogenesis *in vitro* rather than in field plants.

First, the entry into a new developmental pathway is induced by adding plant growth factors that act as a molecular switch to start the process of transdifferentiation. (Fukuda, 1980)

Second, the cells that form TEs do with remarkable synchrony, making it possible to stage more precisely the events involved in building a TE (Edouard, 2003)

Hence, there are many medium formulas for *in vitro* culture study of that differentiation from *Zinnia elegans* mesophyll cell to trachea elementary. Kohlenbach and Schmidt (1975) originally reported tracheary element (TE) differentiation in suspension cultures of mesophyll cell from *Z. elegans*. Modified medium composition was investigated by Fukuda and Komamine that has been used to TE study and many subsequent investigations

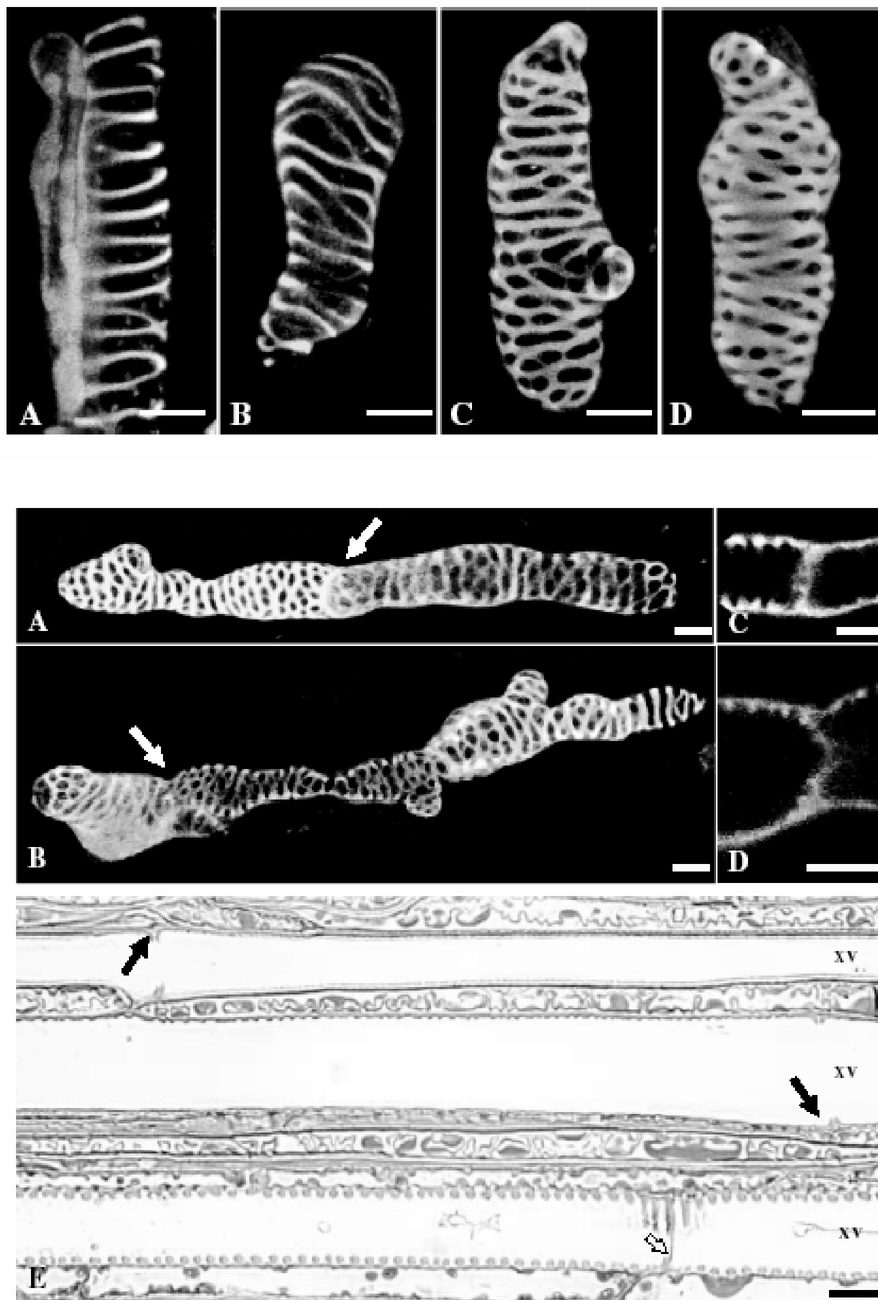

Figure 1.2 Tracheary element (TE) formation of *in vitro* isolated zinnia mesophyll cells (A - D) (Edouard *et al*., 2003)

1.2.2.3. Genetic variation

Normally, zinnias are propagated by seed. Then some breeders tried to select the attractive traits from genetic variation of seedling whereas some researchers studied the relationship between variation of morphological characters and genetic material. Some reports are shown below:

Susan *et al* (1992) reported that tissue culture induced plants had more variation than seed-derived parent plants. Aberrant characteristics in self-pollinated variants including plant height, fertility, flower color and morphology, were sexually transmitted, indicating genetic change had occurred. Aberrant characteristic were not observed in generated plants arose in progeny.

Roger (1992) found that *in vitro* male sterile zinnia were more compact, more heavily branched, and earlier flowering than seed-propagated zinnia.

Duffy and Ewart (1988) reported that some characteristics of zinnia are controlled by many genes such as leaf and flower shape. Zinnias have 2 dominant types of leaf shape: narrow and wide. The narrow-leaf form increases the amount of light penetration and air circulation, which may aid in prevention of disease problems during growth in bedding plant flats. From this paper, it was found that there are at least four genes involved in expression of leaf width and this character is greatly influenced by environment.

1.2.3. Cytogenetic and molecular biological scientific methods

1.2.3.1. Flow cytometry (Dolezel, 1991)

Flow cytometry (FCM) was originally developed as a method for rapid counting and analysis of blood cells. With the technical evolution and development of new fluorescent probes it becomes a useful analytical tool in many areas of biological research. Although the application of flow cytometry for the analysis of plant cells and subcellular organelles was delayed until the early eighties, the number of applications is continuously increasing. The opportunity to sort defined subpopulations of biological particles further increases the number of possible applications. The various applications of FCM including cell cycle analysis, sorting of protoplast and somatic hybrid, and analysis of gene expression.

a) Principle (Dolezel, 1991)

A flow cytometer is an instrument which analyzes optical parameters (light scatter, fluorescence) of particles in flow moving with respect to the point of measure. This contrasts with other techniques of quantitative analysis of isolated nuclei and chromosomes, such as microspectrophotometry or automatic image analysis, which require the particles to be fixed on a flat surface. The measurement in flow permits analysis at a very high speed, typically 10^2-10^3 per second. Furthermore, the particles to be analyzed are selected randomly from the whole population without any bias.

The sample to be analyzed is introduced into the centre of the flow chamber filled with a fast moving sheath fluid. The particles contained in the sample

are hydrodynamically constrained in the centre of the narrow liquid stream and pass, one after another, through a focus of exciting light. The particles scatter the exciting light and, if they contain a natural fluorochrome, or if they have previously been strained with fluorescent dye, they will fluoresce. Pulses of fluorescence pulses are collected using an optical detector system, separated by optical filters and converted to electric current pulses by optical sensors. The output analog signals are digitized and processed by a computer. Because FCM analyses the particles individually and at a high speed, large populations of cells and organelles can be measures in a relatively short time and the presence of subpopulations may be detected.

b) Application of flow cytometry for plant genome research (Dolezel, 1991,1997)

Analysis of the nuclear DNA content (Genome size)

Estimation of the nuclear DNA content was first reported by Heller in 1973 and remains by far the most frequent use of FCM in plant science. Without any doubt, FCM may be considered as the most suitable method for this assay. Biochemical methods are not appropriate because they generate mean values for large numbers of nuclei and cannot detect subpopulations. Absorption microspectro-photometry, cytofluorometry and the most recently introduced image analysis cannot compete with FCM in speed and convenience.

FCM analysis of the nuclear DNA content is based on the analysis of the relative fluorescence intensity of nuclei stained with a DNA fluorochrome. The specimen to be analyzed by FCM must be in the form of a single particle suspension. Suspensions of protoplasts may be prepared from plant tissue by enzymatic digestion of cell wall. However, autofluorescence and low penetration of DNA stains through

the cell membrane preclude their use for DNA content estimation. Fixation of plant protoplasts in ethanol: acetic acid fixatives is not used for DNA analysis due to the decreased resolution of the distribution of DNA content (Galbraith, 1984)

Galbraith *et al*. (1983) demonstrated that suspensions of intact nuclei may be prepared by chopping a small amount of plant tissue with razor blade, and that these suspensions are suitable for DNA content analysis. Alternatively, nuclei may be gently released by lysis of protoplasts in a hypotonic buffer. High resolution histograms may be obtained in most species with a more sophisticated nuclei isolation procedure.

Various buffers have been used to isolate nuclei from plant cells. Their composition is generally dictated by a requirement to preserve the integrity of nuclei, to protect DNA from degradation, and to provide optimal conditions for DNA staining. The most frequently used buffers include Mg^{2+} cation, or polyamines, as stabilizing agents. With some plant species, addition of a reducing agent such as mercaptoethanol, dithiothreitol or polyvinylpyrrolidone, is necessary to inhibit the occurrence of phenolic compounds (Dolezel *et al*., 1994)

After the isolation, the nuclei are stained by a fluorescent dye which binds specifically and stoichimetrically to DNA. Six fluorochromes most frequently used for DNA content estimation are listed in Table 1.2. Propidium iodide and ethidium bromide quantitatively intercalate into double stranded DNA. Hoechst 33258 and DAPI bind preferentially at AT-rich region of DNA, while mithramycin and chromomycin A3 bind preferentially to GC-rich regions. The selection of a suitable fluorochrome depends on the type of analysis (Dolezel, 1991)

Table 1.2 List of fluorochrome used for flow cytometry

Fluorochome	Primary binding mode	Wavelength (nm)	
		Excitation	Emission
Ethidium bromide	Intercalation	525	605
Propidium iodide	Intercalation	535	620
Hoechst 33258	AT-rich regions	345	460
DAPI	AT-rich regions	360	460
Chromomycin A3	GC-rich regions	445	570
Mithramycin	GC-rich regions	445	575

Ploidy analysis

Analysis of the relative fluorescence intensity of nuclei isolated from young leaf tissue yields a histogram showing a dominant peak corresponding to nuclei in the G_1 phase of the cell cycle and a minor peak corresponding to G_2 phase. To estimate ploidy level, the position of the G_1 peak on a histogram is compared to that of a reference plant with known ploidy. The comparison can be made between individual histograms obtained under identical conditions. This procedure is called external standardization. A more reliable approach involves a comparison within a single histogram, comparing G_1 peak positions of two samples (reference + specimen), processed in one tube. This approach, is called internal standardization, eliminates errors due to machine instability and variability in sample preparation. Example ploidy analysis in banana (*Musa acuminata*) was shown in Figure 1.3.

FCM assay has some important advantages over chromosome counting. It is convenient (sample preparation is easy), rapid (several hundreds of samples can be analyzed in one working day), it does not require dividing cells, it is non-destructive (one sample can be prepared, e.g., from a few milligrams of leaf tissue), and it can detect mixoploidy. Therefore, the method is used in different areas ranging from basic research to plant breeding and production.

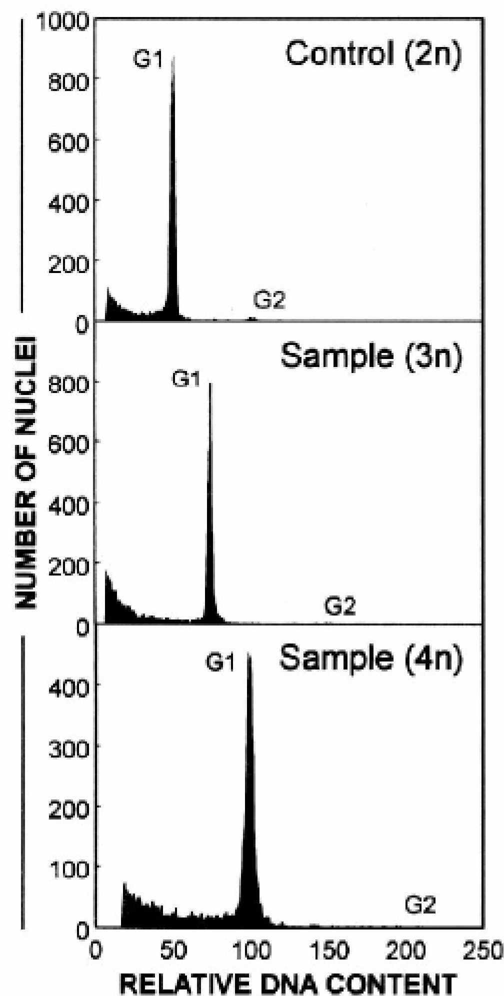

Figure 1.3 Example of ploidy analysis of banana (*Musa acuminata*) (Dolezel *et al.*, 1994)

In some crops, commercially used cultivars are triploid (e.g., sugar beet, hops, bananas), and their breeding is based on different crossing strategies to obtain the desired ploidy level. Therefore, FCM can be used to detect and screen populations of these plants quickly. *In vitro* cultures are usually characterized by a low frequency of mitotic cells. Many differentiated cells do not divide and their ploidy cannot be estimated by chromosome counting. FCM was found invaluable for estimating the ploidy stability of callus, cell or protoplast cultures, including the effects of aging and culture conditions. The applications also include assessment of ploidy stability after *in vitro* multiplication (Wang *et al*., 1992).

Interspecific crossing is used to transfer desired characters from one species into another. When parental species differ enough in their nuclear DNA content, FCM can detect interspecific hybrids according to their intermediate values (Keller *et al*. 1996). An important application of FCM ploidy screening involves plant taxonomy. Since chromosome counting is laborious, only a limited number of plants can be analyzed and phenotypically similar populations which differ in ploidy level may be overlooked (Lysak *et al*., 1997)

Even if FCM ploidy analysis is relatively simple, some precautions should be taken during the analysis and data interpretation. Ploidy is defined as the number of copies of the chromosome complement. Because FCM analyses DNA content, a term “DNA ploidy” should be used to distinguish the results obtained by chromosome counting from those obtained by FCM.

Genome size estimation

Knowledge of genome size is important in many areas of research. It is useful in studies aiming to resolve phylogenetic relationships, to analyse the correlation between the genome size and physiological or agronomical characters, and to estimate the effect of environmental factors on genome size. Bennett and Leitch (1995) reported that nuclear genome sizes of many angiosperm species (99%) are still not known. in general FCM is the preferred method to handle this task because of its speed and precision. FCM estimation of nuclear genome size in pg DNA or Mbp can be achieved by simultaneous measurement of specimen and reference standard nuclei. The genome size of the specimen is then calculated from the ratio of G_1 peak positions of specimen/standard (Figure 1.4). The precision of FCM analysis is very high and detection of statistically significant differences in genome size as low as 0.02 pg has been reported (Dolezel *et al.*, 1994). In addition, the selection of a proper standard is crucial for correct estimation of nuclear genome size. A range of standards is needed to cover the range of genome size variation observed in plants.

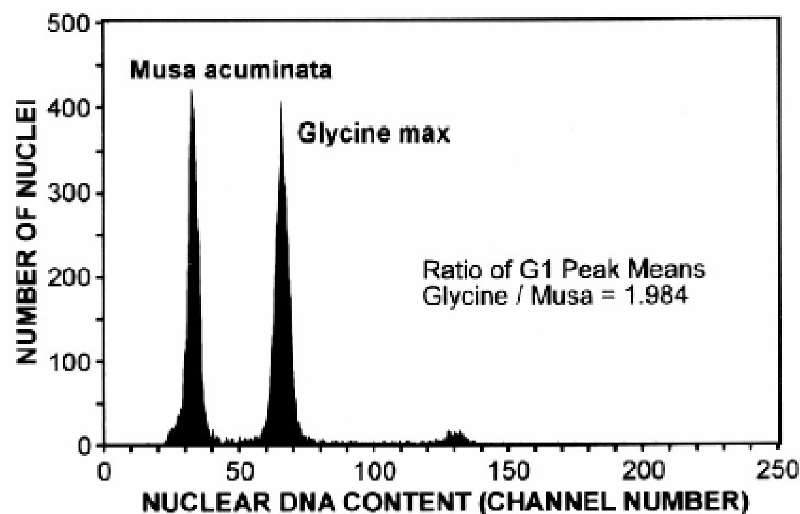

Figure 1.4 Example of genome size estimation of banana (*Musa acuminata*)

(Dolezel. *et al.*, 1994)

Chromosome analysis and sorting

Genome analysis and mapping in most cultivated crops is hampered by the large size of the genome. Its complexity may be reduced by purification of isolated chromosomes by flow cytometry and sorting (Dolezel *et al.*, 1994). In this approach, suspensions of intact chromosomes are prepared from synchronized cells, chromosomes are stained by a DNA specific fluorochrome and their fluorescence is measured and used to quantitatively classify each chromosome (Dolezel, 1995). Fluorescence distribution (flow karyotype) usually consists of several peaks representing individual chromosomes and chromosome groups (Figure 1.5). Chromosomes which can be unambiguously discriminated on a flow karyotype may be sorted in high numbers and used for subsequent molecular analysis.

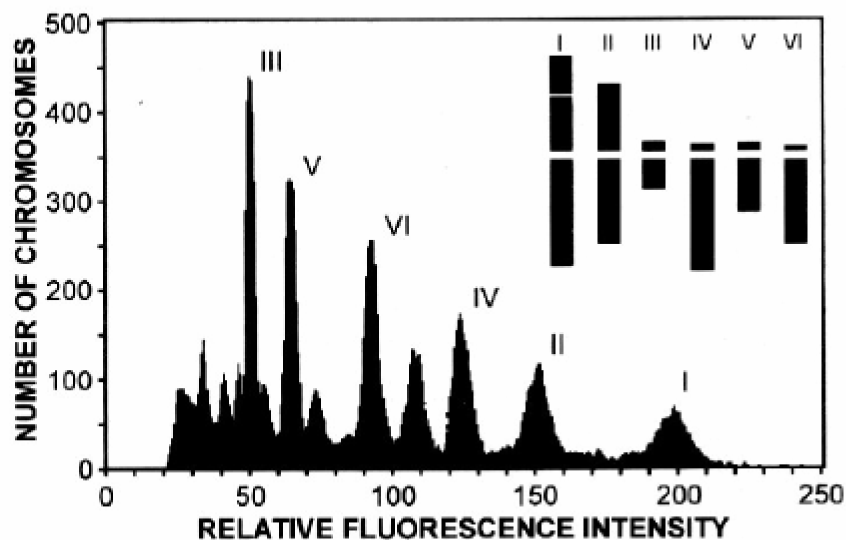

Figure 1.5 Example of chromosome analyzed by flow cytometry (each peak shows chromosome size in genome) (Dolezel and Lucretti, 1995)

1.2.3.2. RAPD-PCR

RAPD (random amplified polymorphic DNA) (Williams *et al*., 1990) is an easy and cost-effective profiling assay based on PCR (polymerase chain reaction) with arbitrary primers. Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence. The identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel. Therefore RAPD can detect genetic polymorphisms. The process concept of this technique was shown by Figure 1.6.

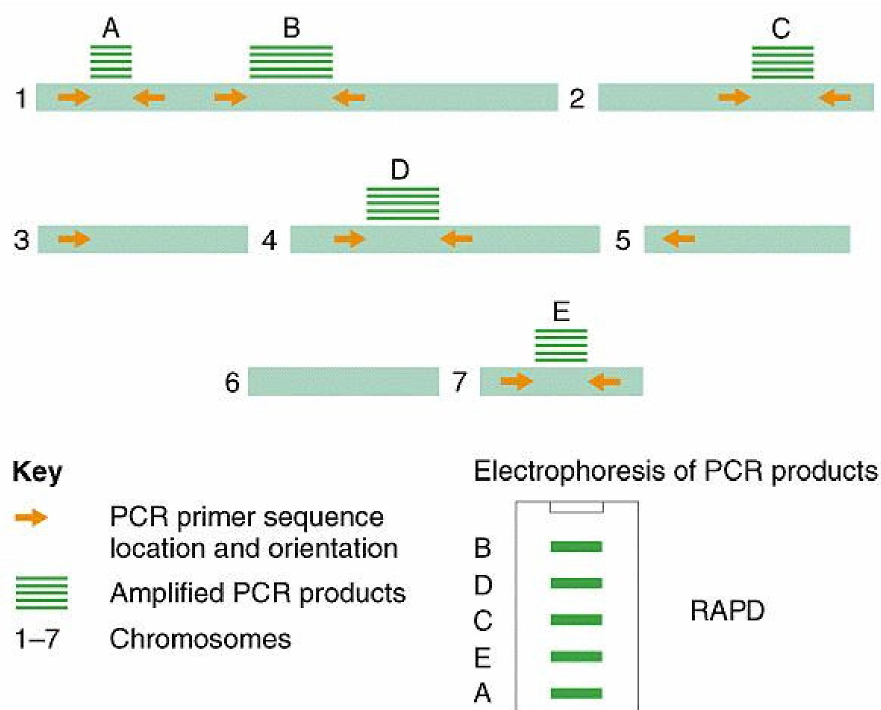

Figure 1.6 The process of random amplified polymorphic DNA (RAPD)

(Darcy, 2002)

Several factors have been shown to affect the number, size and intensity of bands. These include PCR buffers, dNTPs, Mg^{2+} concentration, cycling parameters, source of Taq polymerases, conditions and concentrations of DNA and primer concentrations. Results obtained by RAPDs have been criticized as highly prone to use because they can vary considerably between different analyses of the same sample. The limitation is, however, easy to overcome through practice and many articles have reported consistency in the profiles obtained from different runs of the same sample. Mailer *et al*. (1994) reported identical banding patterns obtained using different thermocyclers and depend mainly on a defined set of conditions, which should be maintained constant when using different machines. While RAPDs have been used successfully in many genetic diversity studies, the technique has been subjected to a range of criticisms. Some of these include the fact that RAPDs are dominant markers, and hence homozygotes are the only genotypes that can be definitively classified by the presence or absence of the band. Furthermore, it is assumed that the presence of a DNA band with identical molecular weight in RAPDs gels of different individuals indicates that the individuals share a common genetic feature. This has raised concerns over the nature of data generated by this technique and its interpretation. RAPD bands have also been reported to comprise several co-migrating amplification products and there can be uncertainty in assigning markers to specific loci if pedigree analysis is not done in advance.

RAPD have been use as tools for the following studies:

a) Genetic variation in cultivars

A number of scientists have used RAPD markers successfully to study polymorphism in various plant species. For examples, Arvind *et al.* (2004) employed RAPD to study the genetic diversity and interrelationships among twelve domesticated and three wild mulberry species. They found that RAPD is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different species of *Morus*, with polymorphism levels sufficient to establish informative fingerprints with 19 primers.

Betal *et al.* (2004) isolated DNA from 14 cultivars of Mungbean (*Vigna radiata)* and subjected to RAPD analysis using 14 random decamer primers. RAPD results were correlated with morphological characters like plant height, leaf and seed size, seed color.

In *Chrysanthemum*, genetic variation is very high between cultivars. These cultivars can be distinguished by using only two different primers based on RAPDs (Wolff, 1993, 1995, 1996). High levels of polymorphisms at the DNA level in *Chrysanthemum* have been determined (Wolff and Peters-Van Rijn, 1993), and identical DNA patterns from different accessions of the same *Chrysanthemum* cultivar can be detected by using RAPDs (Wolff *et al.*, 1995). Furthermore, spotting and chimerism of *Chrysanthemum* also revealed different DNA patterns among cultivars in two families and among the layers of one cultivar by RAPD analysis (Wolff, 1996).

Sheng (2000) used RAPDs in *Chrysanthemum* hybrid combinations including parents and offspring. Forty-five random primers were screened, of which twenty-two primers were selected to detect the molecular marker in three hybrid

combinations of *Chrysanthemum* by using RAPD. The patterns of molecular markers could be classified into seven types.

b) *In vitro* culture

RAPD can be used to prove that some *in vitro* plants have genetic stability or not. Some species show a high genetic stability even though they have been cultured for a long time. Lu´ýsa *et al.* (2004) explained that RAPD can be used as a tool to assess the clonal identity of four *in vitro* propagated chestnut rootstock hybrids (*Castanea sativa* × *C. crenata*). To confirm genetic stability after *in vitro* multiplication for more than 4 years, RAPD patterns of *in vitro* and donor plants were compared. No polymorphism was detected between the material propagated *in vitro* and the donor plants they originated from.

In contrast, some species easily develop genetic variability by somaclonal variation. Neeta (2001) showed that *in vitro* callus cultures of Turmeric (*Curcuma longa* Linn.) were initiated and transfer to the new medium for shoot induction. After they were transferred to medium devoid of phytohormones, complete rooted plants were obtained. RAPD analysis of regenerated plants using 14 primers showed 38 novel bands. About 51 bands that present in the control plant were absent in the regenerants. The result indicated that variation at DNA level has occurred during *in vitro* culture.

Ochatt *et al.* (1999) explained that RAPD with 70 different primers confirmed that the salt tolerant regenerants of potato also differed genotypically from the control.

1.3 objectives

1. To investigate cytogenetic variation of zinnia by flow cytometry, chromosome number counting and morphology of guard cells.

2. To investigate molecular genetic variation of *Zinnia elegans* cv. 'Dreamland' and *Z. haageana* cv. 'Persian carpet' by RAPD technique.

3. To propagate *Z. angustifolia, Z. haageana* and *Z. elegans* via *in vitro* culture.