CHAPTER 4

DISCUSSION

4.1 Cultivar classification by flow cytometry

The flow cytometry analyses have been conducted at two different laboratories, and by using two different instruments as well as different preparation protocols. The first, preliminary studies have been done by using a FACSCalibur. Due to problems inherent to the method, and the lack of experience the results had to be confirmed later on. However, even after the preliminary measurements the following statements could be made:

1) Compared to other species of the family *Asteraceae* that have been analyzed so far, *Z. angustiflolia* (2C: 1 pg), *Z. haagena* (2C: 1.7 pg) and *Z. elegans* (2C: 2.6 pg) have a rather low DNA content (see PlantDNA C-values Database, Royal Botanical Gardens, Kew). The range of genome size of *Asteraceae* is 0.05 pg. (*Arabidopsis thaliana*) – 148.9 pg. (*Allium validum* Wats.)

2) The three analysed species of zinnia can be distinguished by their DNA content. *Z. angustifolia* has the smallest genome size followed *Z. haageana* and *Z. elegans.* Those differences are significant, and therefore, the estimation of the DNA content by flow cytometry can be used as a tool for species recognition in zinnia.

3) The preliminary studies have shown that there might be an intraspecific variation in the DNA amount in *Z. elegans*. However, those differences were not significant, and therefore they had to be confirmed by follow-up measurements (see below). In contrast to most of the analyzed cultivars in *Z. elegans*,

the cv. 'Jungle' has an extraordinary high DNA amount that differed from the other below). In contrast to most of the analyzed cultivars in *Z. elegans*, the cv. 'Jungle' has an extraordinary high DNA amount that differed from the other cultivars significantly. The DNA content of Jungle is nearly twice as high as in the other analyzed diploid *Z. elegans* cultivars indicating its tetraploid status.

As mentioned above, we did not consider the preliminary results as completely reliable. However, there are general problems inherent to flow cytometry that should be taken into account.

1) The proper calibration of the instrument should make sure that successive measurements are comparable. However, due to the warming up of the instrument it might take up to 2 hours to get stable results.

2) The plant cell does not only contain the nuclear DNA but quite a lot of secondary metabolites that are able to interfere with the staining. Those secondary metabolites (e.g. phenolic compounds) compete with the fluorescent dye for binding sites on the DNA (Dolezel, 1997). The actual amount of secondary metabolites depends on the actual physiological status of the plant, and therefore, it depends on several factors (time of day, nutritional condition, cultivar etc.). Furthermore, in some species DNA is degraded by nucleases released after chopping the plant material. In both cases buffer additives might reduce the problems mentioned above. In order to bind the phenolic compounds we added 1 to 2 % PVP which resulted in an improved staining efficiency (DAPI staining). We also observed that in zinnia better staining could be obtained by using actively growing parts (e.g. young leaves) of the plant. We assume that in those materials the concentration of secondary metabolites is lower in general.

The secondary metabolites as well as the nuclease activity might be affected (positive as well as negative) by the composition of the nuclei isolation buffer (lysis buffer). Noirot *et al*. (2000) could show that the dilution of the lysis buffer by distilled water decreased dye accessibility.

3) The way how nuclei are isolated from the plant tissue is also of a great importance for the accuracy of flow cytometry. A) Chopping (by using a razor blade) is the cheapest and fastest way to get the nuclei out of their cells. However, some of the nuclei might get damaged in this process, and thus, giving false signals with a lower fluorescent intensity. This can be avoided by using B) protoplasts. Unfortunately, there is no protocol available for the preparation of protoplasts in zinnia.

4) The concentration of the fluorescent dye is another critical factor, in general (Noirot *et al*, 2000). Especially when chopping is the used isolation technique the exact concentration of isolated nuclei in the staining solution can never be calculated. However, due to the usual rather low concentration of nuclei after chopping the risk of under-staining can be neglected. Quenching of fluorescent signals due to high levels of unbounded molecules of the fluorescent dye might be considered as another problem. Problems of under-staining as well as of over-staining might be solved by measuring the concentration of the isolated nuclei after every preparation. However, this is too time-consuming to be considered a practical approach of those problems.

Fortunately, most of the difficulties mentioned above can be avoided by implementing a so-called internal reference standard (IS). The IS should have nearly but not exactly the same DNA amount as the analyzed sample. The histogram

resulting from a measurement of the sample mixed with the IS should show two clearly separated peaks. Plant material (usually seeds) with a proven DNA amount is provided by several international laboratories on request (for review see Dolezel, 1998). In order to get the best possible results the IS plant material (e.g. leaves) should be chopped together with the plant material of the samples. In this case both types of nuclei are facing the same interferences by secondary metabolites, nucleases etc.

In some laboratories so-called pseudo-internal standards (PSI) are used in order to reduce the labor input by mixing the already isolated nuclei of the IS and the sample if required (e.g. if subsequent measurements of samples give unexpected results). The main disadvantage of the PSI is that in the case of different concentrations of secondary metabolites it takes some time to equalize those differences after mixing the two nuclei isolation solutions. If the nuclei solutions also contain high amounts of nucleases the mixed sample might deteriorate during that waiting time.

In our experiments we used *Raphanus sativus* cv. Saxa which was kindly provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic (DNA content = 1.1 pg., Dolezel, 1992), and later on *Z. angustifolia* cv. 'Starbright' (standardized by using *Raphanus sativus*) as IS.

The intensity of the fluorescence signals of a sample might be influenced by the factors described above. However, even if all of those disturbances are eliminated there is still the problem that there are two different types of

fluorochromes that might give different results when analyzing the same sample (for review see Galbraith *et al.* 1983).

Table 4.1 Binding and spectral properties of selected DNA fluorochromes

Dye	Primary binding mode	Wavelength (nm) [a]	
		Excitation	Emission
Ethidium bromide	Intercalation	530	605
Propidium iodide	Intercalation	540	615
Hoechst 33258	AT binding	365	465
Hoechst 33258	AT binding	360	460
DAPI	AT binding	365	450
DIPI	AT binding	365	450
Chromomycin A3	GC binding	445	570
Mithramycin	GC binding	445	575
Olivomycin	GC binding	440	560

Note: Actual binding mode as well as spectral properties depends on many factors. Including DNA/dye ratio. Ionic strength. and pH of the staining solution.

[a] Dye-DNA complex.

There are many studies demonstrating that data obtained by DNA intercalators (ethidium bromide and propidium iodide, Table 4.1) are strongly correlated with those obtained by Feulgen densitometry. Dolezel *et al*. (1992) clearly

showed that the use of dyes showing base preference may lead to large errors in DNA content estimation. Subsequently, Godelle *et al*. (1993) described a non-linear relationship between changes in fluorescence intensity of dyes showing base preference. Some authors prefer to use DAPI as the fluorochrome for genome size estimation and find propidium iodide (PI) less reliable (e.g. Rayburn *et al.*, 1992). Therefore, both DAPI and PI are used to investigate all cultivars of zinnia (Table 3.3).

Using the Partec (PAS) flow cytometry at the Department of Botany (State Research Center Geisenheim, Germany) we tried to confirm the preliminary measurements.

1) Absolute DNA amounts analyzed by using PI (laser excitation)

The PAS measurements differed slightly from those made with the FACSCalibur, but were more uniform. Since the main goal of this study was not to determine the absolute amount of DNA in zinnia species (cultivars) but to find out whether flow cytometry can be used to distinguish between species and/or cultivars, we could confirm the preliminary results in general. The three analyzed species have a clearly different DNA amount. Further on, we could show that the hybrid cv. 'Profusion' has a DNA amount that is different from its parents. This has been reported in other species, too (Brandham 1998). Since the DNA amount of cv. 'Profusion' comes up to the sum of the DNA amount of its parents we conclude that cv. 'Profusion' is an allopolyploid hybrid of *Z. angustifolia* and *Z. elegans* (National Garden Bureau, 2007). Narayan (1998) investigated the genome size of a *Brassica* hybrid that was a little bit less than the expected sum of its parent's DNA. The cv. 'Jungle' is the only out of the analyzed cultivars in *Z. elegans* that has a clearly

different DNA amount. Due to the fact that it has twice as much DNA than the other cultivars we assume that it is a tetraploid cultivar.

2) Analysis of the intraspecific differences in the DNA amount by using DAPI (UV excitation)

The use of DAPI as a fluorochrome is more convenient because the preparation protocol is simpler, cheaper and therefore, less time consuming. It is still under discussion whether DAPI can be used for the estimation of the absolute DNA amount. In our studies we did not want to know the absolute amount of DNA in zinnia species, but we wanted to find out whether there is an intraspecific variation in *Z. elegans*. In this case, the use of a base-specific fluorescent dye might be justified because the cultivars of a species should not differ significantly regarding their AT/GC-ratio.

When intraspecific variation of *Z. elegans* was investigated, it was found that no evidence of two separate 2C peaks was found in any of the five combinations of two co-chopped cultivars examined (see Fig. 3.2, 3.3). As noted above, comparisons of the shapes of peaks for populations of 2C nuclei for pairs of cultivars run singly or together should provide a telling test for stoichiometric error within *Z. elegans* cultivars. Comparing 2C curves for known pairs of cultivars run either singly or together showed no evidence of increased variance in the latter. We could clearly show that there is no intraspecific variation of the DNA content in *Z. elegans.*

3) Analysis of the AT/GC ratio in zinnia by comparing PI and DAPI stained samples

The ratio of AT/GC of zinnia is a kind of specific characters (because there are 57-59% of AT in the genome of *Zinnia* genus), as does the percentage of AT of *Chrysanthemum multicolor, Haplopappus gracilis* and *Lactuca sativa,* which is 63.5, 61.7 and 60.3, respectively (Martin, 2002). Because the AT/GC ratio is not constant in plant species, some researchers suggest that the use of dye showing base preference is not advisable - at least on a theoretical basis. However, the results of zinnia show that the ratio of DAPI-stained and PI-stained DNA content is nearly 1.00. Therefore, the absolute DNA content of zinnia can be investigated by DAPI staining.

In general, the fluorescent dye should have access to all possible binding sites. But in reality this cannot be achieved. Several physical and chemical treatments consist of heating, addition of bivalent cations, pH modification and NaCl content variation. These are known to modify chromatin condensation and thus cause fluorescence variation (Darzynkiewicz *et al.*, 1984). There are reports that estimates of genome size decrease because chromatin condensation increases (Manicardi *et al*., 1995). O' Brien *et al.* (1996) also reported variation in staining intensity of *Pinus* nuclei. They attributed this to differences in the amount of chromatin condensation affecting the binding of propidium iodide. The difference in DNA content between *Z. haageana* and *Z. elegans* may be the result of a vast number of genomic changes because their chromosome number is similar. Repetitive DNA elements, including retrotransposons, are major components of eukaryotic genomes and such elements have a tendency towards amplification (Bennetzen and Kellogg, 1997).

It is well known that there is always some degrees of chromosomal variation within genus involving duplication and deletions, spontaneous aneuploidy and polyploidy, heterochromatin segments, B-chromosomes. Chromosomal variation will naturally cause some variation of the interspecific DNA content.

Genome plasticity (quantitative changes in genomic DNA of an organism in response to environmental or developmental stimuli) might be another problem. It seems to be particularly important and influential. When several species designated as having a 'plastic genome' are subjected to a careful reanalysis, non-significant or negligible intraspecific variation is investigated. For example, significant intraspecific variations of 15 and 12% are revealed in soybean (*Glycine max* Merrill) by Graham *et al*. (1994) and Rayburn *et al.* (1997), respectively. The reinvestigation of the same soybean accessions showed non-significant variation in the species (Greilhuber and Obermayer, 1997; Obermayer and Greil-huber, 1999). The variation investigated by the former authors is probably due to the use of external standardization instead of internal standardization, which is generally recommended for reliable flow cytometric assays of DNA content (Dolezel el *et al*., 1998).

In *Aloe* (Brandham, 1998), there is a trend towards an increase in nuclear DNA content with evolutionary advancement. The advanced species of *Aloe* have nuclear DNA values that are higher than those of primitive species. On this basis, it can be interpreted that *Z. angustifolia* is a more primitive species and *Z. elegans* is an advanced species according to DNA volume.

4.2 Cultivar classification by RAPD

The five primers were chosen for RAPD screening because they produced many DNA fragments during PCR. RAPD yielded more DNA fragments in *Z. haageana* than in *Z. elegans.* However, the diversity of DNA segments within each population didn't differ as the diversity ratios in *Z. haageana* and *Z. elegans* were 0.49 and 0.46, respectively. The small fragments of RAPD products (350 bp DNA fragments of UBC89 product) can be used use to classified intraspecific variation. It was not observed in *Z. haageana.* Then this fragment can be used to distinguish between *Z. elegans* and *Z. haageana* .. The separation of *Z. elegans* and *Z. haageana* can be divided into two main braches based on RAPD patterns indicated that *Zinnia* species possibly was classified by RAPD makers.

Phenotypic variation within populations of *Z. haageana* and *Z. elegans* is seen in the color of ray floret. *Z. elegans* has a bright color and the florets have varieties of color. *Z. haageana* had only two colors (brown and yellow). The ray florets were bicolor and the strip color ratio differed within populations. Hence, the value of intraspecific variation of *Z. haageana* was more than value of intraspecific variation of *Z. elegan*s. However, variation between *Z. elegans* and *Z. haageana* was low.

Sources of polymorphism in the RAPD assay may include base changes within priming site sequences, deletions of priming sites, insertion that render priming sites too distant to support amplification, and deletions or insertions that change the size of DNA fragments without preventing it amplification (Williams, 1990).

The identification of cultivars or breeding lines is very important in all horticultural and agricultural species in order to protect the rights of plant breeders (Woff, 1995). In chrysanthemum, traditional identification has been based on morphological characters such as flowering trials, and breeders' rights are presented by cultivar characteristics including flower, leaf and growth morphology (Woff, 1995). There are many cases that the origins of varieties are unknown. The development of scientific techniques has allowed basing these analyses on DNA information (RAPD). It had high efficiency to investigate (Martýn, 2002) therefore RAPD possibility could be used to identify cultivars of zinnia as with chrysanthemum.

4.3 Cultivar classification by morphology of guard cells

The positive correlations between DNA content, flower diameter and height in this study were similar to other species such as soybean (Chung *el al.,*1998), *Allium* (Bennett *et al.*, 1972) and *Crepis* (Jones and Brown, 1976). However, some species had negative correlation between DNA content and morphology characters such as seed weight and seed number per plant in maize (Biradar *et al.*, 1994) or *Senecio* species (Lawrence, 1985)

The characteristic data compared with DNA content from the first part are shown on Fig. 3.7. It was found that the size of guard cell, the numbers of chloroplasts in guard cells and flower diameter had a positive relation with DNA content. Similarly, Masterson (1994) demonstrated that there were positive correlation between stomatal guard cell size and nuclear DNA amount for species in the

paleodicot family such as Magnoliaceae (Magnoliales) and this relationship was used to estimate DNA content of the fossil of the Magnolia species.

In addition, numbers of chloroplasts and guard cell size were used to classify the species of zinnia. It's similar with many genuses such as orchid (Stern, 2002). In the case of cv. 'Jungle' and cv. 'Profusion', the guard cell sizes and numbers of chloroplast per guard cell were higher than others significantly. Therefore cv. 'Profusion' and cv. 'Jungle' should be hybrid. Hence chloroplast counts and guard cells measurement can be used to distinguish between diploid and hybrid zinnia.

The degree of relationship between DNA content and some phenotypic characters may vary depending on plant species and the environmental conditions (Chung *el al.*, 1998). In hybrid (cv. 'Profusion'), many characters were different from other cultivars. There are small plants with branching and flowers like *Z. angustifolia* but leaf morphology like *Z. elegans* and flower sizes were intermediate between *Z. angustifolia* and *Z. elegans*.

4.4 Chromosome number

The chromosome number of *Z. angustifolia* reported here (2n = 22) was the same as those obtained by Keil *et al*. (1988) and Razaq *et al.* (1988).. The chromosome number of *Z .haageana* confirmed with those reported by Lane and Li (1993), Jose and Mathew (1995). In addition, the chromosome numbers of *Z. elegans* (2n=24) agreed with those reported by Husaini and Iwo (1990); Zhao *et al.,* (1990); Nirmala and Rao, (1990); Razaq *et al.*, (1994); Jose and Mathew, (1995); Huang and Zhao (1995) except cv. 'Jungle'. However, the cv. 'Jungle' has a chromosome number of 48 and thus it is a tetraploid cultivar.

We could show that the DNA content of *Z. haageana* and *Z. elegans* is significantly different while the number of chromosomes is identical. It might be caused by the evolutionary accumulation of repetitive DNA sequences and/or retrotransposons. Those sequences are generally considered to have an inherent tendency towards amplification, and thereby, increasing the DNA content and the size of chromosomes, respectively (Kubis *et al.* 1998).

4.5 *In vitro* culture

Research on *in vitro* culture showed regeneration ability of zinnia species and cultivars. KS medium served as the basal medium for *in vitro* culture of *Z. angustifolia* cv. 'Starbright', *Z. haageana* cv. 'Percient carpet', and *Z. elegans* cv. 'Dreamland'. It was found that KS medium was very effective in this study since number of shoots and shoot length were the best in the three zinnia cultivars especially for *Z. angustifolia* cv. 'Starbright'. KS medium differed from FK and MS medium mainly with respect to ammonium nitrate, adenine sulphate and glutamine. The influence of the absolute and relative amounts of nitrate and ammonium on the induction and differentiation of plant cell cultures has been reported for a number of *in vitro* systems (Grimes and Hodges, 1990; Cousson and Tran Tan Van, 1993). Considering the variable, KS medium has less concentration of nitrate and ammonium ion than the rest of the media. Therefore it is worth noting that the three zinnia cultivars are sensitive to nitrate and ammonium ion in the culture medium. Furthermore, the effect of adenine sulphate and glutamine in KS medium resulted in better axillary shoot production. This finding was in concurrence with Laliberte *et al.* (1985) who reported that the organic addenda showed positive effect in *Gerbera*

jamesonii when the medium was supplemented with adenine sulphate and tyrosine. All tested media were very effective for callus induction since an average of 30-40 % of the calluses from the three zinnia cultivars regenerated.

Beside media composition, the effect of several phytohormones on the regeneration from shoot tip explants was investigated as well. The dosage of plant growth regulators is known to be critical for shoot organogenesis. In the present study, kinetin was found to be more potent compared to BA. This is probably due to the ability of plant tissue to utilize kinetin more readily that BA. The promotive effect of kinetin in inducing multiple shoots has been previously reported in gerbera (Murashige *et al*., 1974), gladiolus (Boonvanno and Kanchanopoom, 2000). Incorporation of IBA in the medium containing kinetin did not have a deleterious effect on shoot bud formation but encouraged callus formation. The variable response of different zinnia cultivars to auxin supplemented media may be due to different endogenous levels of auxins. Genotypic effects are well established in tissue culture responses. This study demonstrated explant genotype in *in vitro* shoot regeneration and callus formation. Different genotypes had different physiological requirement of plant growth regulators for *in vitro* shoot organogenesis (Palmer, 1992). Therefore, genotype must be considered while developing an *in vitro* regeneration system for zinnia.

TDZ, a synthetic cytokinin, strongly enhanced callus formation in the three zinnia cultivars as it does in other species such as *Z. marylandica* (Susan *et al.,* 1992). Research on *Z. marylandica* showed that adventitious shoots could be derived from either 0.2 or 22.2 µM TDZ. In this study, the reproduction of zinnia cultivars through callus was characterized by the presence of TDZ. TDZ has been used to

induce shoot organogenesis in callus culture of carnation (Nakano *et al*., 1994), roses (Hsia and Korban, 1996) and orchid (Chen *et al*., 1999). Furthermore high activities of TDZ in plant regeneration were also reported in watermelon (Compton and Gray, 1993); and woody species (Huetteman and Preece, 1993).

The addition of $AgNO_3$ to the medium improved the regeneration frequency and reduced callus formation in all tested cultivars. Many reports have shown the positive effect of $AgNO_3$ on plant tissue culture e.g. cucumber (Mohiuddin *et al.,* 1997); apple (Ma *et al*., 1998); Chinese cabbage (Zhang *et. al*, 1998); cassava (Zhang *et al.,* 2001); pearl millet and sorghum (Oldach *et al.,* 2001); date palm (Al-Khayri and Al-Bahrany, 2001) and rapeseed (Akasaka-Kennedy, 2005). $AgNO_3$ at the concentration of 2 mg/l was very beneficial to shoot regeneration in *Z. angustifolia* cv. 'Starbright' while this concentration had no effect on *Z. haageana* cv. 'Percient carpet' and *Z. elegans* cv. 'Dreamland' suggested that both the response to and the optimum concentration of $AgNO_3$ were cultivar or genotype dependent. Zhang *et al.* (2001) reported the genotype and the developmental stage of explants affected the response to $AgNO_3$ in cassava. The capacity of shoot organogenesis in a number of cassava cultivars can be improved by supplementing the medium with $AgNO_3$. The positive effect of $AgNO_3$ at 2 mg/l in *Z. angustifolia* cv. 'Starbright' is consistent with those reported in Chinese cabbage (Zhang *et. al*, 1998) and cassava (Zhang *et al.*, 2001). $AgNO_3$ is a potent inhibitor of ethylene action, and ethylene is considered to suppress shoot organogenesis *in vitro*. Zhang *et al.* (1998) considered that the increased of shoot regeneration frequency by $AgNO_3$ is caused by the interruption of an ethylene signal transduction pathway. Adding $AgNO_3$ to the medium resulted in the mode of shoot regeneration without the callus phase indicating that these two physiological

processes are inversely correlated. In barley (Castillo *et al.,* 1998) and pea (Madsen *et al*., 1998), the use of $AgNO_3$ has reduced rooting of regenerated shoot but in the three zinnia cultivars no adverse effect on root formation were observed.