CHAPTER 2

MATERIALS AND METHODS

Plant Materials

Sixteen cultivars of Zinnia *spp.* including *Z. angustifolia* cv. 'Starbright', *Z. haageana* cv. 'Pebrsian carpet', cv. 'Chippendale daisy', *Z. elegans* cv. 'Short stuff', cv. 'Dreamland', cv. 'Jupiter', cv. 'Border beauty', cv. 'Peter pan', cv. 'Dahlia', cv. 'Candy cane', cv. 'Jungle', cv. 'Giant', cv. 'Gold medal', cv. 'Piccolo', cv. 'Sinnita' and a hybrid (*Z.elegans x Z. angustifolia*) cv. 'Profusion' were used as plant materials in this studied. Seed of Zinnia *spp.* were taken from the AFM Flower Seed & Agriculture Co. Ltd. Seed size are shown in Figure 2.1 and picture of all cultivars have shown in Table 2.1 and Figure 2.2 - 2.6

Figure 2.1 The seed size of *Z. angustifolia* cv. ' Starbright' (A), *Z. haageana* cv. 'Persian carpet' (B), *Z. elegans* cv. ' Dreamland' (C) and Z. *elegans* cv. 'Profusion'(D).

Table 2.1 Comparison of morphology of cultivars

Type / Cultivars	Flower Diameter (cm.)	Color	Height (cm.)	Usage
'Chippendale daisy'	3 - 4	Bicolor	30	Pot plants and field plants with big flowers
'Persian carpet'	5	Bicolor	30	
'Sinnita'	4 - 5	Yellow	25 - 30	
'Short stuff'	7 - 8	Scarlet, Red, Orange, Gold, Pink, White	20 - 30	
'Dreamland'	9 - 10	Scarlet, Red, Pink, Yellow, Ivory, Rose	25 - 30	
'Jupiter'	5 - 6	Cherry, Pink	30 - 35	
'Piccolo'	4	Red, Orange, Gold, Pink, White	45 - 50	
'Peter pan'	7 - 8	Red, Orange, Gold, Pink, White	30 - 40	
'Border beauty'	5 - 7	Red, Orange, Gold, Pink, White	40 - 45	
'Starbright'	2 - 3	Gold, Orange,White	25 - 30	Pot and field plants with small flowers
'Profusion'	3 - 4	Cherry, Orange, White	30 - 40	
'Candy cane'	9 - 10	Bi-color (Mix)	90	Cut-flower and field plants
'Jungle'	5	Red, Orange, Gold, Pink, White	60 - 70	
'Dahlia'	9 - 10	Red, Orange, Gold, Pink, White	100	
'Giant'	9 - 10	Red, Orange, Gold, Pink, White	70 - 80	
'Gold medal'	7 - 8	Red, Orange, Gold, Pink, White	70 - 80	

Figure 2.2 *Z. angustifolia* cv. 'Starbright'

Figure 2.3 *Z. haageana* cv. 'Persian carpet' and cv. 'Chippendale daisy'

Figure 2.4 *Z. elegans* cv. 'Profusion'

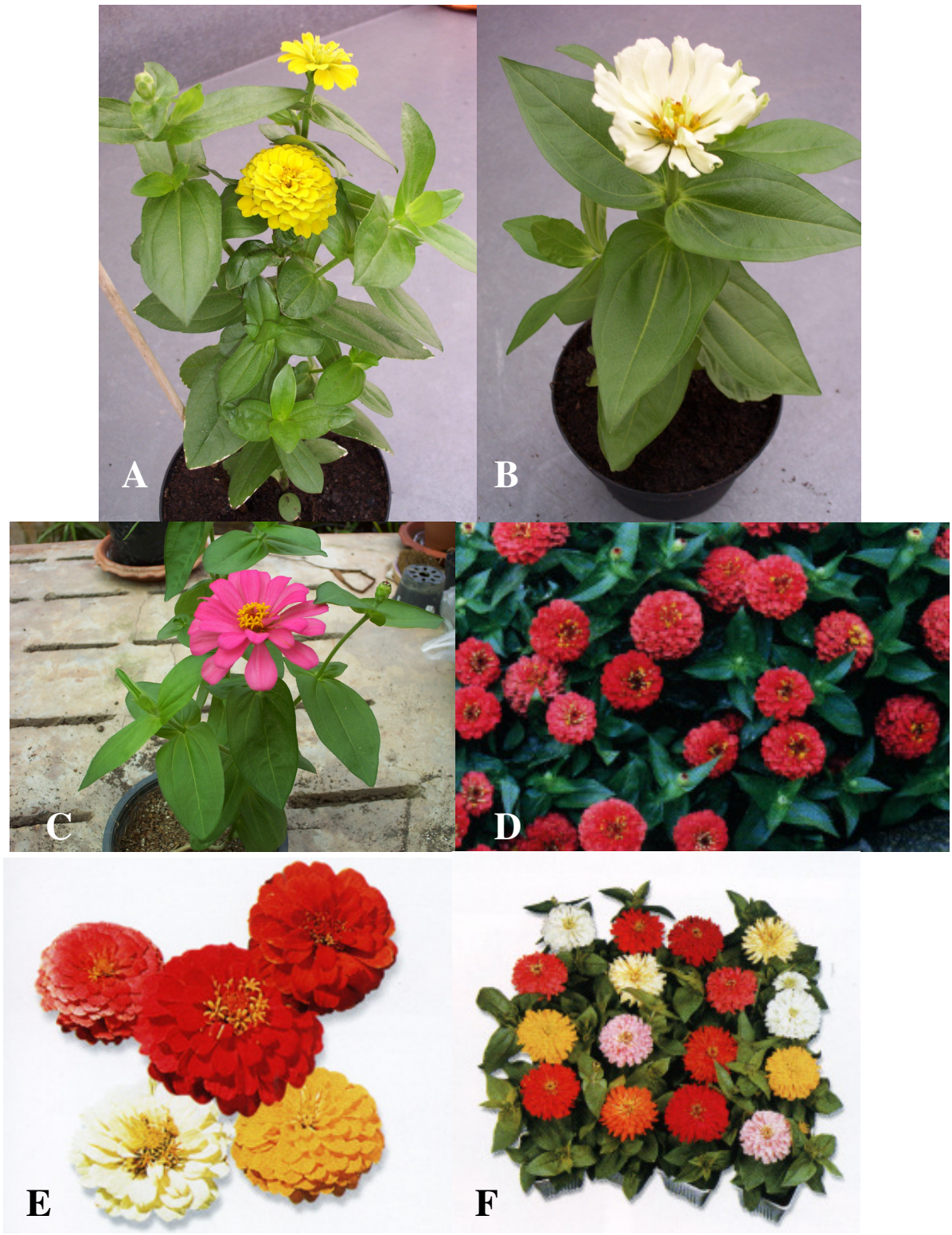

Figure 2.5 *Z. elegans* cv. 'Sinnita' (A), cv. 'Short stuff' (B), cv. 'Dreamland' (C), cv. 'Jupiter' (D), cv. 'Piccolo' (E) and cv. 'Peter pan' (F).

Figure 2.6 *Z. elegans* cv. 'Border beauty' (A), cv. 'Candy cane' (B), cv. 'Jungle' (C), cv. 'Dahlia' (D), cv. 'Giant' (E) and cv. 'Gold medal' (F)

Methods

2.1 Cultivar classification by flow cytometry

Sixteen cultivars were analysed using flow cytometry. Our routine internal standard for flow cytometry was diploid *Raphanus sativus* cv. 'Saxa' obtain from Institute of Experimental Botany Olomouc, Czech Republic, and The DNA content of *Raphanus sativus* was checked by Dolezel (1992) and its DNA content is 1.11 pg (Dolezel 1992). *Z. angustifolia* cv. 'Starbright' was used as standard DNA content for Partec PAS flow cytometry.

Seeds of Zinnia *spp*. were germinated in beds and one-week-old seedlings were transferred into pots containing fertile soil : sand = 1: 1 in a glasshouse at temperature 30°C. Meristem tissue of young leaves of 4- to 8-week-old zinnias was used for isolation of intact nuclei. Plant height (cm) and flower diameter (cm) were recorded when zinnias bloomed for the first time.

Two flow cytometry instruments

Two flow cytometry instruments from different institutes were used in this study then the difference results and precision of the instruments would be investigated.

FACSCalibur

FACSCalibur is equipment from Becton Dickinson, California, USA. It is the only four-color, automated benchtop flow cytometry system that can perform both analysis and cell sorting (Figure 2.7) (see Appendix). FACSCalibur instrument

was operated by the Scientific Equipment Center of Prince of Songkla University, Thailand.

Figure 2.7 FACSCalibur in Prince of Songkla University

Partec PAS (Particle Analysing System)

Partec PAS is an equipment from PARTEC, Münster, Germany. It has an argon ion laser and HBO lamp / 25mW 635nm laser diode to study both DAPI and PI fluorescence (see Appendix). For this reseach, it was provided by the Botany Department of The State Research Center Geisenheim, Germany (Figure 2.8)

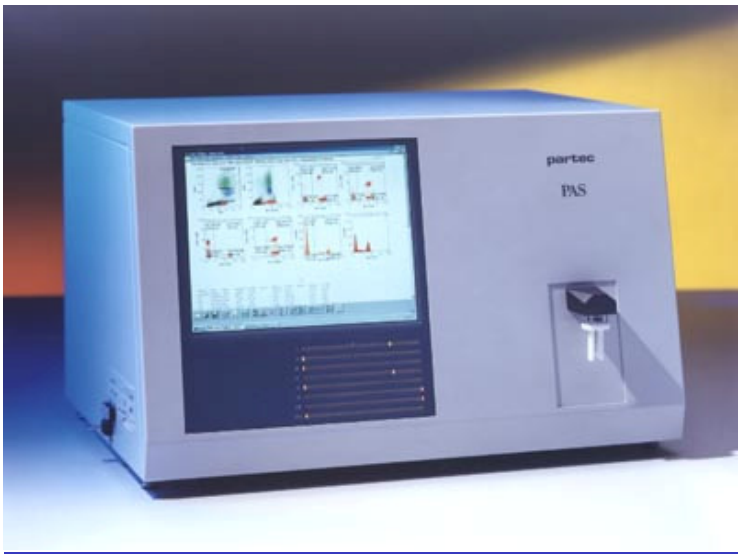

Figure 2.8 Partec PAS in The State Research Center Geisenheim, Germany

Methods

A) PI-stain for FACSCalibur

Nuclei were prepared from young leaf tissue and stained for flow cytometry according to Pfosser *et al.* (1995). Twenty milligram of zinnia leaves were chopped with a razor blade in 500 µl ice cold Tris $MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2$. $6H_2O$ and 0.5% Triton X-100). After filtration through a 42-µm nylon filter, 50 µg/ml propidium iodide (Sigma) and 4 units/ml ribonuclease II A (Sigma) were added. It was then incubated for 20 min. at room temperature. Young leaf DNA of *Raphanus sativus* was prepared by above methods and used as a standard DNA. Each cultivar analysis was repeated 2 times for at least 5 different glasshouse-grown plants.

B) PI-stain for Partec PAS

Nuclei were prepared from the leaf tissue and stained for flow cytometry with CyStain® PI Absolute P reagent kit originating form Partec Co.Ld. Young leaves were chopped with a razor blade in 400 µl extraction buffer then filtrated through a 42-µm nylon filter. Next, the 1.6 ml of staining buffer of the kit with 100 µg PI and 3 µl of RNase were added. DNA of *Z. angustifolia* cv. 'Starbright' leaves was prepared by the above methods and used as a standard DNA. Each cultivar analyzed was repeated 2 times for at least 5 different glasshouse-grown plants.

C) DAPI-stain for Partac PAS

Nuclei were extracted and stained using the high resolution DNA Precise P kit, (Partec, Münster, Germany). Young leaves were chopped with a razor blade, while in 0.5 ml. of solution A of the kit. The effect of PVP was tested. The 0.5 ml. of 1% PVP was added in some samples to eliminate secondary products of its cell. Samples were then filtered through a 42-µm nylon filter and stained in 1.5 ml. of

solution B (4′, 6-diamidino-2-phenylindole, DAPI staining) of the DNA kit. The DNA content of the nuclei was evaluated using a PARTEC equipped with a HBO lamp for UV excitation. Measurements were assigned according to relative fluorescence intensity. All analyses were performed using peak height detection. Each cultivar analyzed was repeated 2 times for at least 5 difference glasshouse-grown plants.

Data collection and statistical analysis

All analyses were performed using peak height detection with both linear and logarithmic amplification. The data were plotted on linear scale depending on the type of amplification, so that histogram peaks were evenly distributed along the abscissa. Histograms were stored on a floppy disk and processed off-line using the WINMDI software (version 2.7, copyright © 93–98 Joseph Trotter) for data analysis and correction of background noise. The fluorescence signals are presented as frequency distribution histograms of about 1024 channels, starting from channel 10. The amount of DNA in the nucleus is proportional to the fluorescence signal between sample peak and known-genome sizes species peak (standard). The gain settings were adjusted for the different times, so that signals of standard intact nuclei were registered within the same channel range. During the analysis of nuclei from different cultivars readjustments were not made.

DNA content estimation was analyzed by one-way ANOVA and Sheffe's test. Data were analyzed using SPSS version 10.0.1. In addition, percent of AT in each cultivar was analyzed. The data from DAPI and PI strain were used for calculation following the method as described by Meister (2001).

There were two parts of these experiments. It consisted of

a) Preliminary of the investigation DNA content in Zinnia

The DNA content of 11 cultivars of zinnia was investigated by FACSCalibur instrument. And 16 cultivar of zinnia was investigated by Partec PAS. *Raphanus sativus* cv. 'Saxa' was used as internal standard with FASCalibur instrument. The effect of PVP was tested when DNA content was observed by Partec PAS.

b) Comparative analysis of *Z. elegans* cultivars

Within *Z. elegans* cultivars, we could not observe significant differences in the DNA contents. (See result, Table 3.2 and 3.3). However, in order to rule out small intraspecific differences of the DNA content, we analyzed mixed samples of two different cultivars. Two experiments were designed.

Experiment 1: Two cultivars of *Z. elegans* were pooled in serial series: 'Border'/'Dreamland', 'Dreamland'/'Giant', 'Giant'/'Sinnita', 'Sinnita'/'Jupiter', 'Jupiter'/' Short stuff'.

Experiment 2: *Z. elegans* cv. 'Dreamland' was compared with 5 cultivars of *Z. elegans* (cv. 'Border beauty', cv. 'Giant', cv. 'Sinnita', cv. 'Jupiter' and cv. 'Short stuff'). Additionally, our internal standard (*Z. angustifolia*, cv. 'Starbright') has been added to these mixed samples.

2.2 Cultivar classification by RAPD

Plant material

Dry seeds of 16 different cultivars of zinnia [*Zinnia angustifila, Z. haageana* and *Z. elegans*] were studied. Seeds were germinated in beds and one-week-old seedlings were transferred into pots containing fertile soil: sand = 1: 1 in a glasshouse at temperature 30 °C. The first young leaves from shoot tip of seven- to ten-day-old etiolated seedlings were used for DNA extraction.

Methods

DNA extraction

Total genomic DNA from young seedlings of the plants was isolated following Reutter's method (2003). Two volumes of DNA extraction buffer containing 50 mM Tris-HCl pH 8.0, 312.5 mM NaCl, 20 mM EDTA pH 8, 1% Sarkosine and 7 M urea was added to the 100-500 mg. leaf tissue and ground gently in a mortar with a pestle. Then, it was vortexed strongly. The homogenate was centrifuged for 10 min. at 12,000-15,000 rpm at room temperature to remove tissue fragments. The supernatant was extracted twice with phenol-chloroform at 12,000 - 15,000 rpm for 5 min. After that ½ volume 5 M NaCl was added to the supernatant and mixed. The clear upper aqueous phase was collected carefully and 2 volumes of 95% ethanol was added and mixed gently. Finally, the homogenate was centrifuged for 10 min at 12,000-15,000 rpm at 4 °C. DNA spooled with sterile capillary was then washed with 70 % ethanol and air-dried. Finally the DNA was dissolved in 0.05 cm^3 of TE. The DNA isolated from each cultivar was run in 1 % (w/v) agarose gel to check the quality and was also scanned in Spectrophotometer and absorbances (A) at 260 and 280 were noted.

DNA amplification

Amplification was performed in a MJR PTC 100 thermo cycler in volumes of 25 µl consisting of 1× reaction buffer, 1.5 mM MgCl2, 200 µM dNTPs, 0.04 Units of Taq DNA polymerase, 0.2µM primer, 2.5 µg BSA and 25 ng. of genomic DNA. Following an initial denaturation at 95 °C for 5 min., the amplification program was set to 35 cycles of denaturation at 95 °C for 1 min., annealing at 35 °C for 15 sec. and extension at 72 °C for 2 min. with a final extension at 72 °C for 5min. Amplified fragments were resolved in 1.8 % agarose gels followed by staining with ethidium bromide (0.5µg/ml) and gels were viewed and photographed under UV transillumination. All primers used were 10-mer random DNA from the University of British Columbia (UBC), Vancouver, Canada. And 20 DNA samples amplified with 5 primers producing reproducible bands were considered for analysis. The 5 primers were chosen from the set of 10 random primers. Amplification was repeated twice.

RAPD data analysis

Distance matrix analysis of the RAPD data was calculated using Bionumberic ver. 3.0 software. The distance matrix based on RAPD data sets is graphically represented as dendrograms using the UPGMA method.

2.3 Cultivar classification by morphology of guard cells and plant height

Plant material

The seeds and cultures of 16 different cultivars of zinnia [*Zinnia angustifila, Z. haageana* and *Z. elegans*] were investigated as described in the RAPD section.

Methods

A certain amount of epidermal cells was obtained from the underside of 4–weeks leaves by tearing with nail and were then rubbed onto a microscope slide using a razor blade. In order to measure stomata diameter and length, the under surface of a leaf was placed onto a microscope slide after the addition of one drop of tap water, and covered with the cover glass (Dońe, 1986). Under a light microscope, the diameter and length of 10 stomata per leaf were recorded. The chloroplast number in each of the two guard cells of stomata was scored under a fluorescence microscope. Measurement and scoring were performed for 4 leaves of 2 greenhouse plants per cultivar

2.4 Chromosome numbers

Plant material

Chromosome numbers were counted in main root tips from greenhouse plants. Seeds of 4 cultivars of zinnia seedlings (*Z. angustifolia* cv. 'Starbright', *Z. haageana* cv. 'Persian carpet', *Z. elegans* cv. 'Peter pan' and cv. 'Jungle') were germinated in wet soft paper for 4 days. The root samples were collected and chromosome counting was performed.

Methods

The tips were pre-treated for 4 hours in saturated PDB (para-dichlorobenzene) then fixed for 24 hours in 1:3 glacial acetic acids: 95% ethanol. Next, they were hydrolysed by passing through 75% ethanol and then 1 N HCl for 15 min. at 60 °C. Root tips were stained with Feulgen solution for 4 hours and squashed in acetocarmin. The chromosome number in 5 cells was examined under light microscope.

2.5 *In vitro* culture

Plant material

The seeds of *Z. angustifolia* cv. 'Starbright', *Z. haageana* cv. 'Persian carpet' and *Z. elegans* cv. 'Dreamland' were used as plant materials for *in vitro* culture.

Methods

Seeds were first washed with distilled water and surfaced sterilized with 70% ethanol for 30 sec followed by sterilization with 20% Clorox for 20 min and 10% Clorox for 20 min, respectively. And they were then washed three times with sterile water. Finally, seed coats were cut off and seeds incubated on media. The basal medium used for incubation was MS (Murashige and Skoog, 1962) solid medium supplemented with 3% sucrose, 0.7% agar, and a pH of 5.7 and was autoclaved at 121 °C 1.06 kg/cm^2 for 20 min. After 30 days, the 1-2 cm. shoot segments of seedlings were cut and placed on MS solid medium and incubated at 25±2 °C under a 16-h photoperiod of white fluorescent tube.

The follow factors were investigated.

A. Effect of medium formula

Four different media namely MS, 1/2 MS, FK and KS (Table 2.2), supplemented with 1 mg/l $AgNO_3$, 4.4 μM BA and 2.5 μM IBA were used for *in vitro* culture.

Table 2.2 Different media composition for *in vitro* culture.

(Concentrations are given in μM)

	KS- Medium Kohlenbach & Schmidt (1975)	FK- Medium Fukuda & Komamine (1980)	MS Medium Murashige and Skoog (1962)	½ MS Medium
KNO_3	9,400	20,000	18,790	9,395
NH_4NO_3	9,000	-	20,612	10,306
NH_4Cl	-	1,000	-	-
$MgSO_4$	750	1,000	2,189	1,095
$CaCl_2$	1,500	1,000	2,992	1,446
KH_2PO_4	500	500	1,222	611
$MnSO_4$	100	100	131	60.5
H_3BO_3	160	160	100	50
$ZnSO_4$	35	35	30	15
$NaMoO_4$	1	1	1	0.5
$CuSO_4$	0.1	0.1	0.1	0.05
Na_2EDTA	100	100	100	50
$FeSO_4$	100	100	100	50
Glycine	27	27	27	13.5
Myo-inositol	550	550	925	462.5
Nicotinic acid	41	41	4.06	2.03
Pyridoxine	2.4	2.4	0.50	0.25
Thiamine	1.5	1.5	0.3	0.15
Folic acid	0.11	0.11	-	-
Biotin	0.2	0.2	-	-
Adenine	150	-	-	-
Glutamine	100	-	-	-
Sucrose	30,000	30,000	90,000	45,000

B. Effect of growth regulators

The appropriate medium from part A was supplemented with different auxins (2,4-D, IBA and NAA) at the concentration of 0.1 µM alone or in combination with different cytokinins (BA and Kinetin) at the concentration of 1 µM for 4 weeks.

The chosen types of growth regulators were supplemented with different concentration of auxin in combination with different concentration of cytokinin about 4 weeks, as shown in Table 2.3:

Table 2.3 The concentration combination of two growth regulators

Auxin / Cytokinin	0.05 µM	0.5 µM	5.0 µM
1 µM	+	+	+
5 µM	+	+	+
10 µM	+	+	+

C. Effect of $AgNO_3$

$AgNO_3$ is an inhibitor of ethylene activity. It has been successful induced shoot regeneration in many plant species such as buffalo grass (Shuizhang Fei, 2000), cassava (Peng Zhang, 2001), coffee (Sandra 2000), and cucumber (A.K.M. Mohiuddin, 1997). Therefore, the effect of $AgNO_3$ was investigated by culturing zinnia explants on MS medium containing 5 µM Kinetin, 0.05 µM IBA and different concentrations of $AgNO_3$ (0–16 mg/l).

D. Callus induction and organogenesis

Z. elegans cv. 'Dreamland' shoots (1-cm long) were cultured on MS with various growth regulators for about one month as follows:

- MS + 0.1 μM TDZ
- MS + 1.0 μM TDZ
- MS + 17.6 μM BA
- MS + 5.4 μM NAA
- MS + 9.0 μM 2,4-D
- MS + 9.9 μM IBA

There were 25 explants for each treatment. The number of shoots per explant and height were recorded after 4 weeks of culture. For root induction, three different ranks were established according to the number and length of root

Rank 1: small main root less than 2 cm and no hairy root.

Rank 2: a few hairy roots and main root length between 2-5 cm.

Rank 3: many hairy roots and main root length more than 5 cm.

The morphology of callus was observed and fresh weight was recorded.

Statistical analysis

The length and number of shoots of three zinnia species were compared and differences between means were tested for significance using ANOVA and Sheffe test. Data was analyzed by SPSS ver. 10.0.1