CHAPTER 3

RESULTS

3.1 Cultivar classification by flow cytometry

a) Preliminary of the DNA content investigation in Zinnia

The DNA content of *Zinnia* was investigated by FACSCalibur instrument. The 11 cultivas of zinnia were investigated. It was found that all three species had a relatively small genome (Table 3.1). The DNA content of species and hybrid were significantly different according to ANOVA ($p< 0.05$). *Z. angustifolia* had the lowest DNA content (1.00 pg). In comparison, the DNA content of *Z. haageana*, *Z. elegans* 2C DNA content was 1.87-2.15 and 2.32-2.85 pg, respectively. 'Profusion' hybrid (*Z. angustifolia x Z. elegans*) had the highest DNA content (3.88 pg). It was about four-time DNA content of *Z. angustifolia* or 2-time DNA content of *Z. elegans.* As shown in Table 3.1 SE values were more than 0.10 in many cases. Therefore, calculation of DNA content was not precised. However, DNA content was significant differences between species of the genus *Zinnia* but small variation was observed within species.

Table 3.1 DNA contents and intraspecific genome size variation in *Zinnia* by PI staining using FACSCalibur

Species	Cultivars	2C nuclear DNA content (pg) (mean ± SE)
Z.. angustifolia	'Starbright'	1.00 ± 0.03 [a]
Z. haageana	'Persian carpet'	1.87 ± 0.11 [b]
	'Chippendale daisy'	2.15 ± 0.11 [b]
Z. elegans	'Short stuff'	2.32 ± 0.13 [b]
	'Dreamland'	2.54 ± 0.10 [bc]
	'Jupiter'	3.10 ± 0.21 [cd]
	'Border beauty'	3.26 ± 0.08 [de]
	'Peter pan'	3.40 ± 0.35 [de]
	'Dahlia'	3.50 ± 0.18 [de]
	'Candy cane'	3.85 ± 0.13 [de]
Z.elegans x Z angustifolia	'Profusion'	3.88 ± 0.15 [e]

The few standard samples (*Raphanus sativus*) were limited. In order to perform the number of required analyses on our own plant material, we used *Raphanus sativus* to create our own internal standard. *Z. angustifolia* cv. 'Starbright' was chosen for that because this species had the lowest DNA content among the three analysed *Zinnia* species. From this species histograms of a very good resolution could be obtained (Figure 3.1).

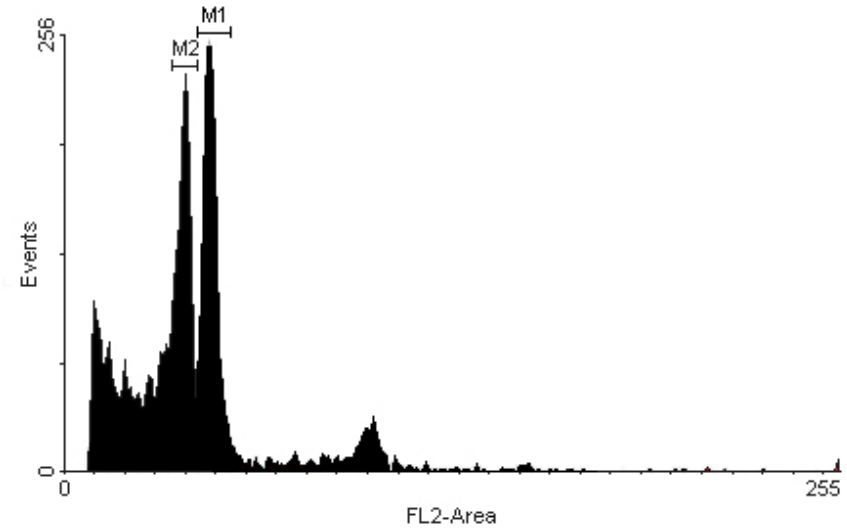

Figure 3.1 Histogram of *Raphanus sativus* (M1) and *Z. angustifolia* (M2)

The 16 cultivars were analyzed by Partec PAS. This instrument is equipped with a 488 nm laser as well as an UV arc lamp excitation light sources therefore the isolated nuclei can be analyzed by either PI or DAPI staining. PI is an intercalating fluorescent dye and it is used for the estimation of the absolute DNA content. DAPI is a base-specific dye (AT-specific) that allows comparative measurements of samples only. Two fluorescent stains have been used in order to estimate the DNA content of species and/or cultivars in the genus *Zinnia.* It is used for the estimation of the intraspecific variation of the DNA content in *Z. elegans* cultivars and results of two fluorescent stains (DAPI and PI) were used to calculate AT-GC ratio in genome.

PI

Table 3.3 showed that the DNA content of *Z. haageana* varied from 1.64 to 1.73 pg. and that of *Z. elegans* varies from 2.49 to 2.74 pg. Species could be

distinguished by DNA content because there was a significant difference. Among the analyzed cultivars of *Z. elegans,* there were 2 hybrids (cv. 'Profusion' and cv. 'Jungle') that had a significant higher DNA content. DNA content of cv. 'Profusion' and cv. 'Jungle' were 3.94 and 4.94 pg., respectively.

Results from two different instruments were compared; it was found that each species had a different DNA content. The accuracy of DNA content value depends on Standard error (SE). In the case of PAS, SE values of all samples were less than 10% of the DNA content value. SE values of FACSCalibur were high therefore results of PAS had more accuracy than FACSCalibure.

DAPI

The absolute DNA content of *Z. haageana*, *Z.elegans* (2C-value) stained by DAPI was 1.60-1.63 and 2.43-2.54 pg., respectively (Table 3.4). Within *Z. elegans,* two cultivars (cv. 'Profusion' and cv. 'Jungle') had significant different DNA content from other cultivars. DNA content of cv. 'Profusion' and cv. 'Jungle' were 3.49 and 4.95 pg, respectively. DNA staining was improved by adding PVP in order to minimize the interference of the staining by secondary metabolites (e.g. phenolic compound) present in the plant material. Consequently, the SE values in all analyzed samples were lower than 0.10.

Table 3.2 DNA contents of *Zinnia* by PI staining using Partec PAS.

Species	Cultivars	DNA content (pg) (mean±S.E)
Z. angustifolia	'Starbright'	1.00 [a*]
Z. haageana	'Persian carpet'	1.73 ± 0.02 [b]
	'Chippendale daisy'	1.69 ± 0.04 [b]
Z. elegans	'Short stuff'	2.53 ± 0.05 [c]
	'Dreamland'	2.51 ± 0.06 [c]
	'Jupiter'	2.56 ± 0.05 [c]
	'Border beauty'	2.51 ± 0.03 [c]
	'Peter pan'	2.61 ± 0.04 [c]
	'Dahlia'	2.66 ± 0.04 [c]
	'Candy cane'	2.65 ± 0.04 [c]
	'Jungle'	4.94 ± 0.04 [e]
	'Piccolo'	2.66 ± 0.02 [c]
	'Gold medal'	2.74 ± 0.04 [c]
	'Giant'	2.57 ± 0.03 [c]
	'Sinnita'	2.58 ± 0.02 [c]
Z.elegans x Z angustifolia	'Profusion'	3.63 ± 0.06 [d]

* Estimated by using *Raphanus sativus* cv. 'Saxa' as internal standard.

Table 3.3 DNA contents of *Zinnia* by DAPI staining with and without 1% PVP and using Partec PAS.

Species	Cultivars	2C nuclear DNA content (pg) (mean±SE)	
		Not adding PVP	Adding PVP
Z. angustifolia	'Starbright'	1.00 [a*]	1.00 [a*]
Z. haageana	'Persian carpet'	1.63 ± 0.06 [b]	1.60 ± 0.02 [b]
	'Chippendale daisy'	1.64 ± 0.09 [b]	1.67 ± 0.02 [b]
Z.elegans	'Short stuff'	2.45 ± 0.11 [bc]	2.50 ± 0.03 [c]
	'Dreamland'	2.40 ± 0.02 [bc]	2.45 ± 0.08 [c]
	'Jupiter'	2.43 ± 0.13 [bc]	2.45 ± 0.08 [c]
	'Border beauty'	2.41 ± 0.09 [bc]	2.50 ± 0.04 [c]
	'Peter pan'	2.53 ± 0.08 [bc]	2.48 ± 0.03 [c]
	'Dahlia'	2.43 ± 0.22 [bc]	2.51 ± 0.01 [c]
	'Candy cane'	2.54 ± 0.12 [bc]	2.47 ± 0.05 [c]
	'Jungle'	5.15 ± 0.07 [e]	4.95 ± 0.07 [e]
	'Piccolo'	2.47 ± 0.04 [bc]	2.54 ± 0.05 [c]
	'Gold medal'	2.59 ± 0.11 [c]	2.46 ± 0.04 [c]
	'Giant'	2.56 ± 0.06 [bc]	2.50 ± 0.04 [c]
	'Sinnita'	2.51 ± 0.14 [bc]	2.45 ± 0.04 [c]
Z.elegans x Z. angustifolia	'Profusion'	3.33 ± 0.14 [d]	3.49 ± 0.04 [d]

* Estimated by using *Raphanus sativus* cv. 'Saxa' as internal standard.

b) Comparative analysis of *Z. elegans* cultivars

Among the *Z. elegans* cultivars, we could not observe significant differences in the DNA contents. (see 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, the results were shown on Figure 3.2. It was found that histograms of those mixed samples always had only one peak. This clearly demonstrated that the DNA contents of analyzes cultivars were identical.

Experiment 2, results were shown on Figure 3.3 the DNA content of cv. 'Dreamland' was identical to all other cultivars of *Z. elegans.* All histograms consisted of two peaks only. The first peak represented *Z. angustifolia* and the second peak represented a combination of 'Dreamland' DNA with another *Z. elegans* cultivar. Therefore all *Z. elegans* cultivars contained the same DNA content. However, the *Z. elegans /Z. angustifolia* fluorescence ratio range from 2.28 ('Dreamland'/'Giant', Figure 3.2B) to 2.64 ('Dreamland'/'Sinnita', Figure 3.2C) representing a 15% difference in estimated DNA content. These differences were not expected; for possible explanation see discussion chapter.

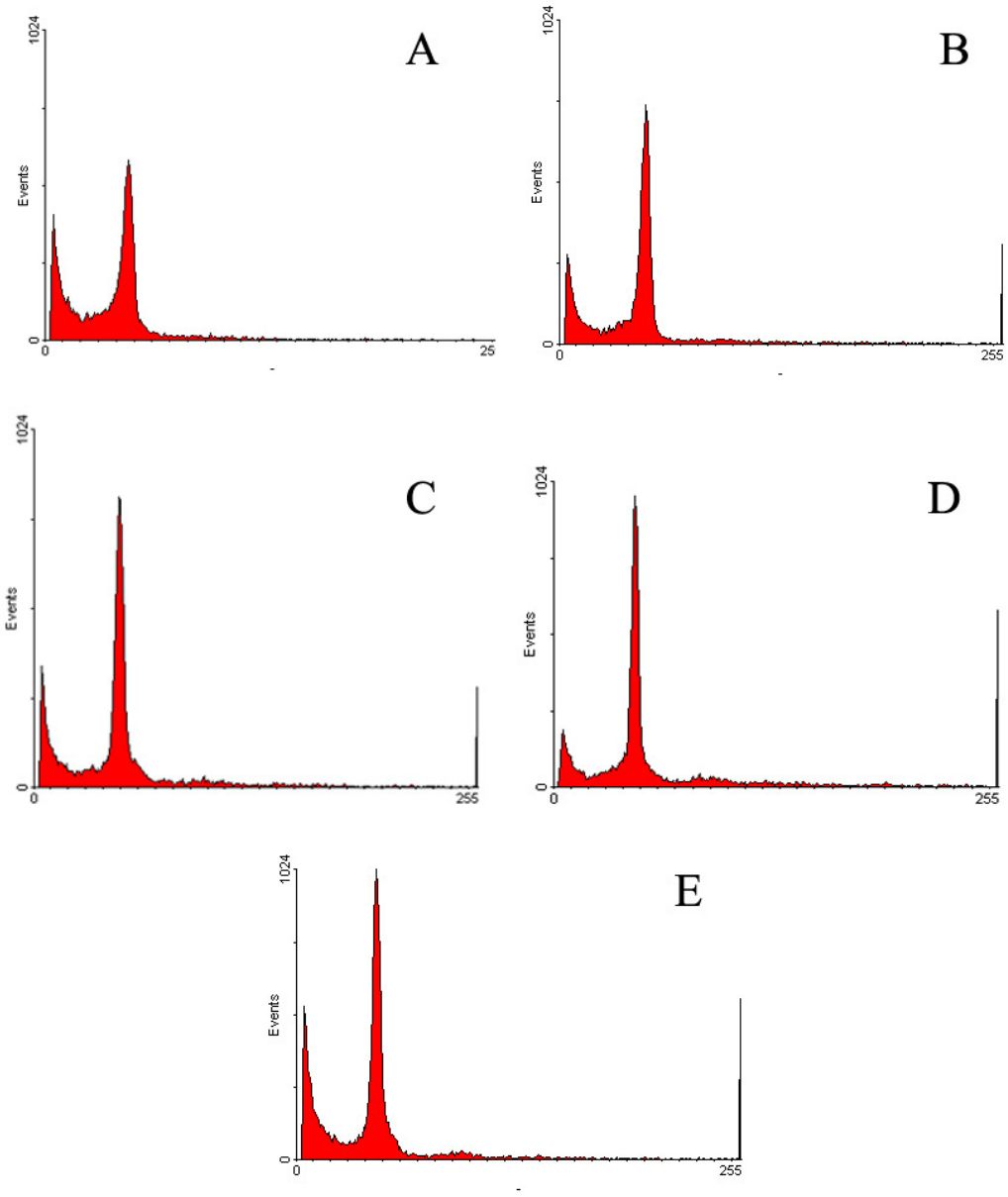

Figure 3.2 FCM histogram showing combination peak of

A) 'Border'/ 'Dreamland', B) 'Dreamland'/ 'Giant', C) 'Giant'/' Sinnita',

D) 'Sinnita'/ 'Jupiter' and E) 'Jupiter'/ 'Short stuff'.

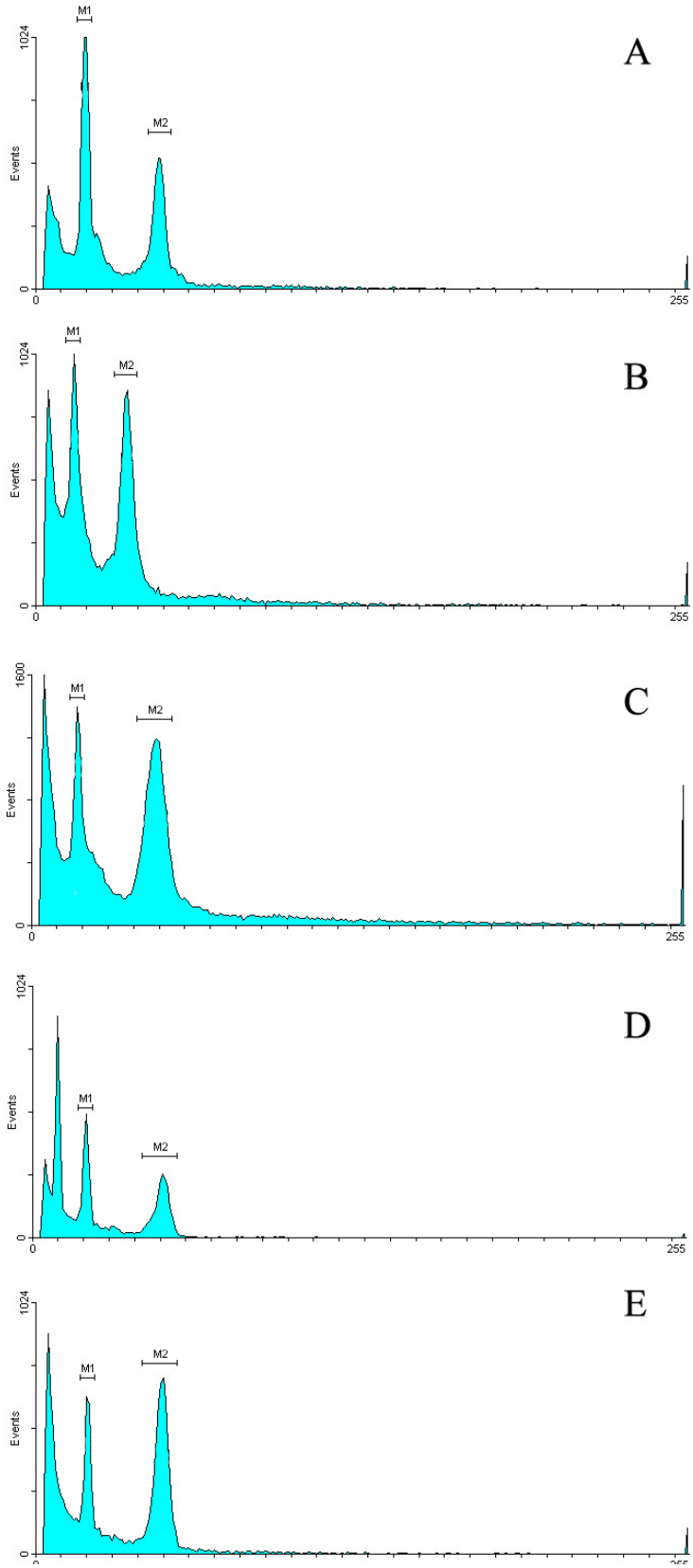

Figure 3.3 FCM histogram showing peaks of *Z. angustifolia* (M1) with *Z. elegans* cv. 'Dreamland' plus A) cv. 'Border beauty' B) cv. 'Giant' C) cv. 'Sinnita' D) cv. 'Jupiter' E) cv. 'Short stuff'

Tables 3.2 and 3.3 showed that a sample stained by DAPI had lower fluorescence intensity than the same sample stained by PI. This is due to the different binding mode of both fluorescent dyes (DAPI is an AT-base specific fluorochrome; PI is an intercalating fluorochrome) Thus these differences could be used in order to calculate the AT ratio (Dolezel *et al.*, 1992; see Table 3.4). The average AT-content in *Zinnia* is about 57 to 59%. The AT-content in *Z. angustifolia* was slightly higher than *Z. haageana* and *Z. elegans*. Whereas cv. 'Jungle' and cv. 'Profusion' had a significantly higher DNA content, the AT-content is not significantly different from other cultivars.

Table 3.4 Relative AT-Specific Fluorescence

Species	Cultivars	Ratio of DAPI / PI	AT (%)
Z. angustifolia	'Starbright'	1.00	59.00
Z. haageana	'Persian carpet'	0.92	57.45
	'Chippendale daisy'	1.00	59.00
Z. elegans	'Short stuff'	0.99	58.81
	'Dreamland'	0.98	58.62
	'Jupiter'	0.97	58.43
	'Border beauty'	1.00	56.96
	'Peter pan'	0.95	58.04
	'Dahlia'	0.94	57.84
	'Candy cane'	0.93	57.56
	'Jungle'	1.00	59.00
	'Piccolo'	0.95	58.04
	'Gold medal'	0.90	57.05
	'Giant'	0.98	58.62
	'Sinnita'	0.98	58.62
Z. elegans x Z. angustifolia	'Profusion'	0.98	58.62

3.2 Cultivar classification by RAPD

A total of 10 arbitrary primers were utilized for initial screening for their amplifying ability. Of these, 5 primers, comprising UBC 65, 87, 88, 89 and 95 (Figure 3.4), were selected for cluster analysis (Table 3.5). These primers produced a total of 76 bands, of which 40 were polymorphism in 30 samples of 2 cultivars. Diversity within the population of *Z. haageana* cv. 'Persian carpet' was higher than within population of *Z. elegans* cv. 'Dreamland' (Figure 3.5-3.6). In *Z. haageana* cv. 'Persian carpet' 28 out of 60 proved to be polymorphic bands, vs. 19 out of 45 bands in *Z. elegans* cv. 'Dreamland'.

When the similarity matrix was constructed, similarity level among accessions was very high (0.95). Most of the samples within each species shared the same bands, with a few exceptions. The cluster analysis separated *Z. elegans* cv. 'Dreamland' and *Z. haageana* cv.'Persian carpet' into two main branches (Figure 3.6). Within *Z. elegans* cv. 'Dreamland', three main secondary branches could be resolved. The top one in Figure 3.6 was comprised of samples coming from the same package of seeds which presumably originated from the same parent plant. The accessions clustered in second and third branches came from other sources.

Table 3.5 The list of used primers

Code	Sequence of primer (5’ to 3’ direction)
UBC 65	AGG GGC GGG A
UBC 87	GGG GGG AAG C
UBC 88	CGG GGG ATG G
UBC 89	GGG GGC TTG G
UBC 95	GGG GGG TTG G

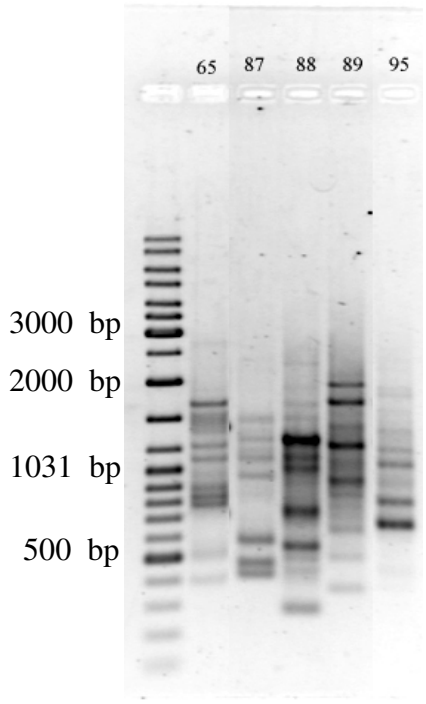

Figure 3.4 Primer screening in *Z. elegans* cv. 'Dreamland'

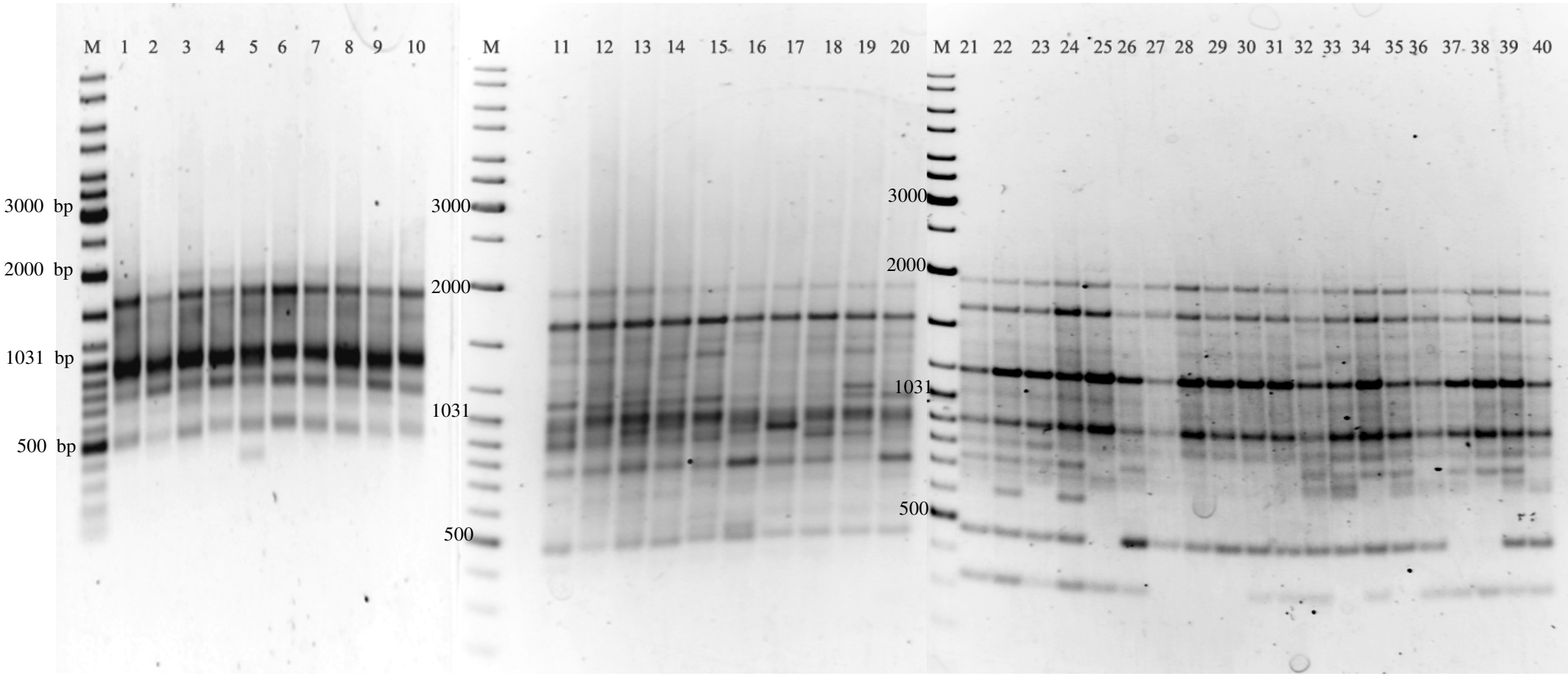

Figure 3.5 Electrophoretic analysis of amplification products obtained with the primer UBC 89. Lanes are as follows: M = marker ladder, 1-20 were samples of *Z. haageana* cv. 'Persian carpet' and 21-30 were samples of *Z. elegans* cv. 'Dreamland'

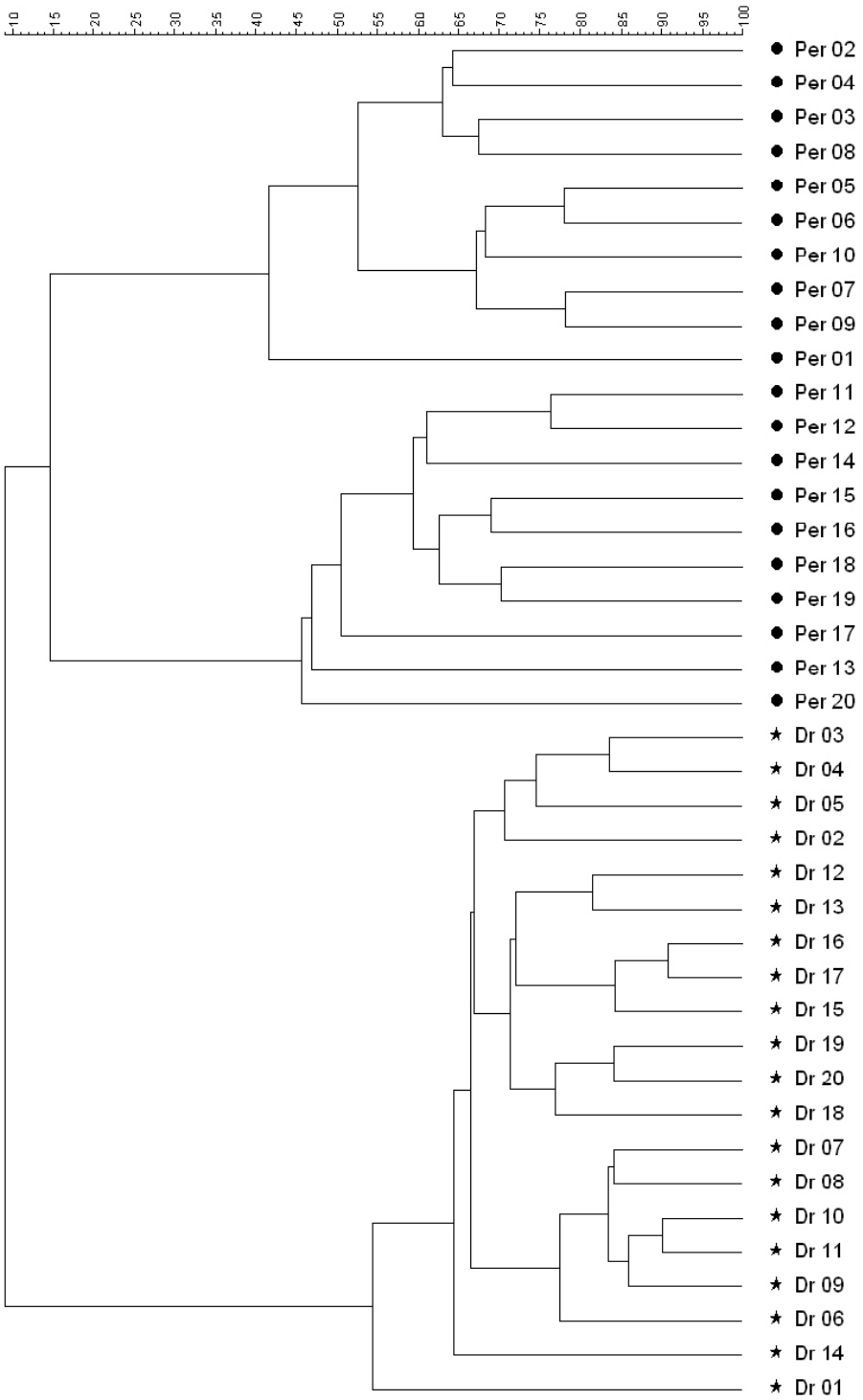

Figure 3.6 Dendrogram of *Z. elegans* cv. 'Dreamland' (Dr) and *Z. haageana* cv. 'Persian carpet' (Per) accessions (Bionumberic ver 3.0) based on cluster analysis of RAPDs with 5 primers.

3.3 Cultivar classification by morphology of guard cells and plant height

Some morphological characters of zinnia cultivars that were observed were shown in Table 3.6. The range of guard cell size and chloroplast number was different depending on the species. The length of guard cells was 28.7, 27.8 and 33.3-37.2 µm. in *Z. angustifolia, Z. haageana* and *Z. elegans*, respectively. *Z. elegans* had greater variation of guard cell size than other species, whereas the numbers of chloroplast were less in both *Z. angustifolia* and *Z. haageana,* about 4-6 units per cells, than in *Z. elegans,* which has 6-8 units per cell. Among *Z. elegans* cultivars, there were 2 cultivars (cv. 'Profusion' and cv. 'Jungle') which were different from other cultivars. The length of guard cells of cv. 'Profusion' and cv. 'Jungle' was 4.31 and 4.66 µm and the numbers of chloroplast was 9 and 12 unit/guard cell, respectively (Figure 3.7). In case of height, it was noted that there was no significant difference. *Z. angustifolia* and *Z. haageana* were small plants (16 - 23 cm.). *Z. elegans* had many sizes, distinguishing the plant into 3 groups. The short plants consisted of cv. 'Short stuff', cv. 'Dreamland', cv. 'Jupiter', cv. 'Border beauty', cv. 'Sinnita' and cv. 'Profusion'. The mediate-height plants consisted of cv. 'Peter pan', cv. 'Jungle' and cv. 'Piccolo' and the tall plants were cv. 'Gold medal', cv. 'Dahlia', cv. 'Candy cane' and cv. 'Giant'. Flower diameters were recorded and it was shown that even though some plants were short but the flowers were still large. Cultivar of 'Dreamland' and cv. 'Border beauty' were small plants (29-35 cm.) with flower sizes of 6.38 and 6.19 cm, respectively. Hence it was appropriate as a pot flower plant and these cultivars have been induced recently for pot flower plants. However, some cultivars have small

flowers but with many flowers per plant, such as cv. 'Sinnita' and cv. 'Profusion'. In *Z. elegans*, the flower size is significantly difference.

Table 3.6 The characteristics of guard cell, plant height and flower sizes of zinnia cultivars

Species	Cultivars	Guard cells		Height (cm.) (mean±SD)	Flower sizes (cm.) (mean±SD)
		Length (μm) (mean±SD)	Chloroplasts / guard cell		
Z. angustifolia	'Starbright'	28.7 ± 2.6 [a]	4.6 ± 0.7 [a]	16.64 ± 4.79 [a]	1.70 ± 0.27 [a]
Z. haageana	'Chippendale daisy'	27.5 ± 2.2 [a]	4.8 ± 0.7 [a]	23.38 ± 3.50 [ab]	2.75 ± 0.21 [a]
	'Persian carpet'	27.8 ± 2.1 [a]	5.6 ± 0.7 [b]	23.75 ± 2.75 [ab]	2.48 ± 0.17 [ab]
Z. elegans	'Short stuff'	36.2 ± 3.4 [cde]	7.3 ± 1.0 [ef]	25.70 ± 4.41 [abc]	5.03 ± 0.74 [cd]
	'Dreamland'	36.6 ± 3.0 [de]	7.5 ± 1.1 [fgh]	35.33 ± 4.13 [bc]	6.38 ± 0.84 [de]
	'Jupiter'	35.4 ± 2.8 [cde]	7.8 ± 1.0 [gh]	33.60 ± 6.43 [bc]	4.11 ± 0.49 [bc]
	'Border'	35.0 ± 3.8 [bcd]	7.9 ± 1.0 [h]	29.33 ± 2.08 [abc]	6.19 ± 0.85 [de]
	'Peter pan'	33.3 ± 2.5 [b]	7.3 ± 0.9 [ef]	40.44 ± 6.75 [c]	6.54 ± 0.63 [e]
	'Dahlia'	36.6 ± 3.3 [de]	6.6 ± 0.8 [cd]	73.80 ± 10.62 [e]	6.09 ± 0.67 [de]
	'Candy cane'	36.3 ± 3.1 [de]	7.3 ± 0.8 [ef]	71.75 ± 3.59 [e]	6.20 ± 0.57 [de]
	'Jungle'	44.6 ± 3.5 [f]	11.9 ± 1.3 [j]	58.75 ± 7.31 [d]	5.77 ± 0.25 [d]
	'Piccolo'	36.1 ± 3.4 [cde]	6.3 ± 0.8 [c]	58.40 ± 8.13 [d]	3.67 ± 0.76 [c]
	'Gold medal'	37.2 ± 3.2 [e]	7.3 ± 0.8 [efg]	62.13 ± 5.07 [de]	5.60 ± 0.53 [cd]
	'Giant'	34.6 ± 3.2 [bcd]	7.2 ± 0.9 [def]	66.00 ± 4.36 [e]	5.70 ± 0.66 [cd]
	'Sinnita'	34.2 ± 3.0 [bc]	6.8 ± 0.7 [cde]	21.60 ± 0.65 [ab]	3.77 ± 0.46 [c]
Z. angustifolia xZ. elegans	'Profusion'	43.1 ± 3.9 [f]	8.7 ± 0.9 [i]	27.57 ± 4.66 [abc]	3.06 ± 0.33 [ab]

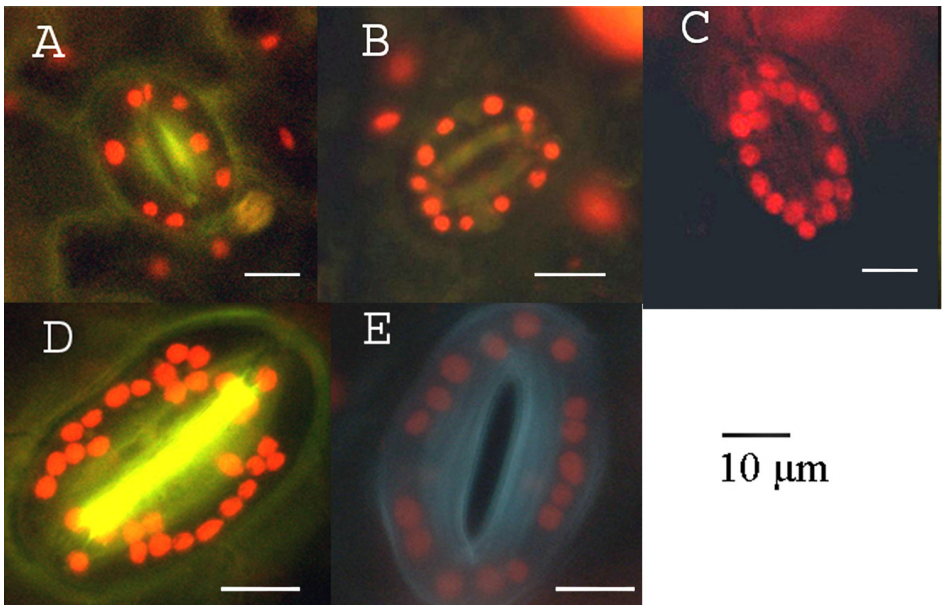

Figure 3.7 Micrographs of guard cells in zinnia: *Z. angustifolia* cv. 'Starbright' (A), *Z. haageana* cv. 'Persian carpet' (B), *Z. elegans* cv. 'Dreamland' (C), cv. 'Jungle' (D) and cv. 'Profusion' (E) in fluorescence optics.

The characteristic data compared with DNA content from flow cytometry studies were shown on Figure 3.8. It was found that the size of guard cell, the numbers of chloroplasts in guard cells had a positive relation with DNA content of zinnia. However, there was no correlation between height of plant and DNA content in *Z. elegans*.

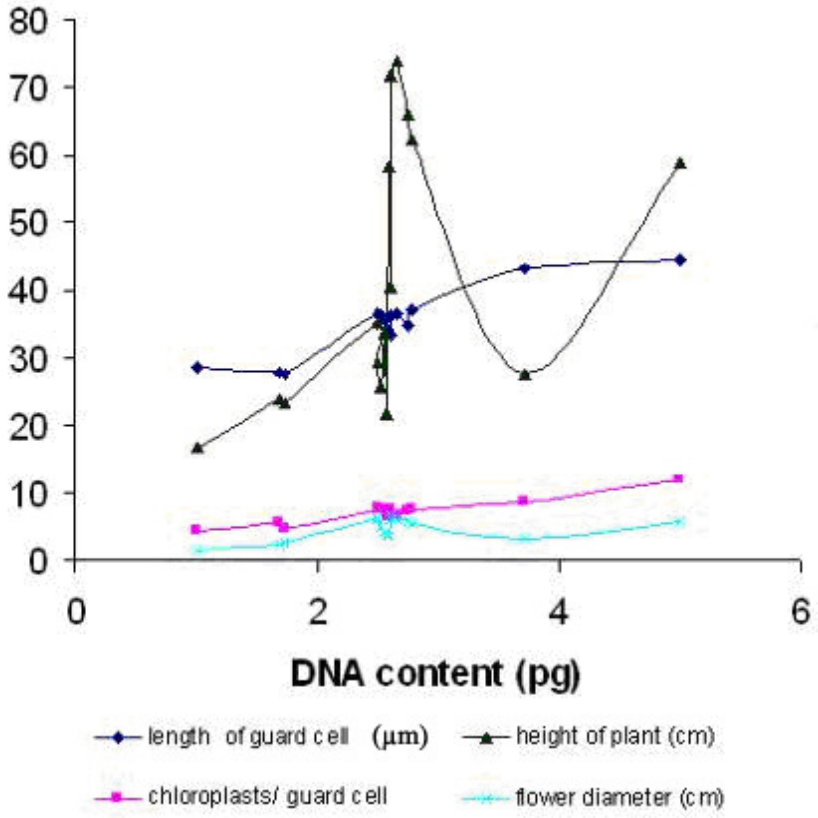

Figure 3.8 Correlations between DNA content and some characteristics of zinnia

3.4 Chromosome numbers

Chromosome numbers of *Zinnia* species were examined and the results obtained were listed in Table 3.7. The chromosome number of *Z. angustifolia* (2n = 22) was found to be lower than other species. The chromosome numbers of *Z. haageana* and *Z. elegans* were the same (2n = 24). However, *Z. elegans* (2n = 48) cv. 'Jungle' was tetraploid.

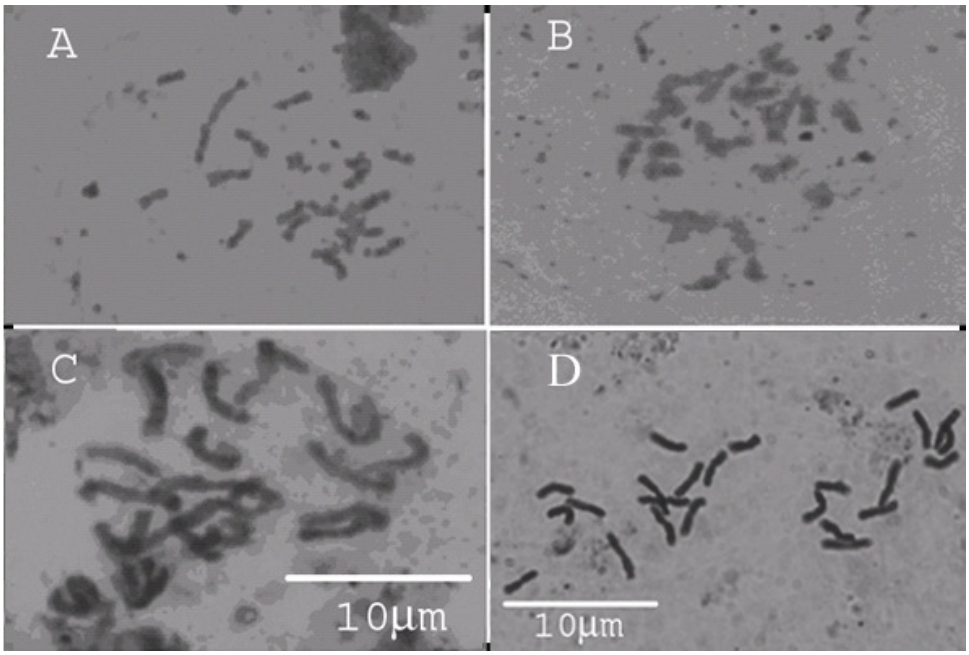

Figure 3.9 Chromosomes of *Z. angustifolia* cv. 'Starbright' (A), *Z haageana* cv. 'Persian carpet' (B), *Z. elegans* cv. 'Peter pan' (C) and cv. 'Dreamland' (D).

Table 3.7 Chromosome numbers of zinnia species and cultivars

Species	Cultivars	Chromosome number (2n)
Z. angustifolia	'Starbright'	22
Z. haageana	'Persian carpet'	24
Z. elegans	'Dreamland'	24
	'Peter pan'	24
	'Jungle'	48

3.5 *In vitro* culture

A. Effect of media formula

The effects of several culture media namely KS (Kohlenbach and Schmidt, 1975), FK (Fukuda and Komamine, 1980), MS (Murashige and Skoog, 1962) and ½MS on shoot propagation were investigated. The response of number of shoot, and root induction on all media were shown on Table 3.8. When explants were cultured for 30 days, a significantly greater number of shoots of *Z. angustifolia* were observed on FK and KS media than MS and ½ MS media (Figure 3.1). While more shoots and length of shoots of *Z. elegans* and *Z. haageana* explants were induced on KS medium greater than on MS medium; however it was not significant different (Figure 3.11-3.12).

Considering root induction, MS medium induced more roots than other media in all species. For *Z. angustifolia* and *Z. haageana,* FK and KS media did not induce any roots to rank 3. Therefore the rate of rooting depended on the species (Table 3.8)

For callus induction, it was found that MS medium induced large callus (more than 1 cm. in diameter) while other media did not induce large callus. (Table 3.9) Callus induction depended on the species i.e. *Z. haageana* was induced easily (42.1%) while in *Z. elegans* was lower (30.2%) (Table 3.9).

Table 3.8 Effect of media types on shoot and root formation in zinnia cultivars after 4 weeks in culture

Zinnia cultivars	Types of media	No. of shoots (mean±SD)	Shoot length (cm.) (mean±SD)	Root ranking (%)		
				1	2	3
Z. angustifolia cv. 'Starbright'	MS	1.96 ± 1.80 [b]	1.35 ± 0.94 [b]	0	0	2.0
	½MS	1.72 ± 1.45 [b]	1.20 ± 0.78 [b]	0	2.0	0
	FK	4.49 ± 3.07 [a]	1.52 ± 0.56 [ab]	6.8	1.7	0
	KS	4.73 ± 2.93 [a]	1.85 ± 1.03 [a]	3.6	0	0
Z. haageana cv. 'Persian carpet'	MS	2.00 ± 0.93 [c]	3.54 ± 1.99 [c]	9.1	40.9	9.1
	½MS	1.73 ± 1.42 [c]	3.21 ± 1.95 [c]	4.8	52.4	4.8
	FK	3.15 ± 0.62 [c]	2.79 ± 1.36 [c]	14.8	0	0
	KS	3.33 ± 0.94 [c]	2.66 ± 1.34 [c]	20.0	4.0	0
Z. elegans cv. 'Dreamland'	MS	1.83 ± 1.34 [d]	5.63 ± 1.68 [d]	0	21.7	17.4
	½MS	1.66 ± 0.86 [d]	1.27 ± 0.50 [d]	12.5	15.6	12.5
	FK	1.50 ± 0.83 [d]	1.10 ± 0.29 [d]	4.2	12.5	0
	KS	2.13 ± 1.14 [d]	1.50 ± 0.81 [d]	20.0	6.7	3.3

Note: Number followed by the same letter in the column was not significantly different at $p \leq 0.05$

Table 3.9 Effect of media types on callus induction in zinnia cultivars after 4 weeks in culture

Species	Medium	Callus diameter (%)		
		0.1-0.5 cm.	0.5-1.0 cm.	> 1.0 cm.
Z. angustifolia cv. 'Starbright'	MS	41.2	7.8	3.9
	½MS	24.0	26.0	4.0
	FK	25.4	18.6	0
	KS	36.4	9.1	0
Z. haageana cv. 'Persian carpet'	MS	31.8	4.5	0
	½MS	28.6	9.5	0
	FK	25.9	14.8	0
	KS	44.0	8.0	0
Z. elegans cv.'Dreamland'	MS	21.7	13.0	4.3
	½MS	18.8	12.5	0
	FK	33.3	0	0
	KS	16.7	3.3	0

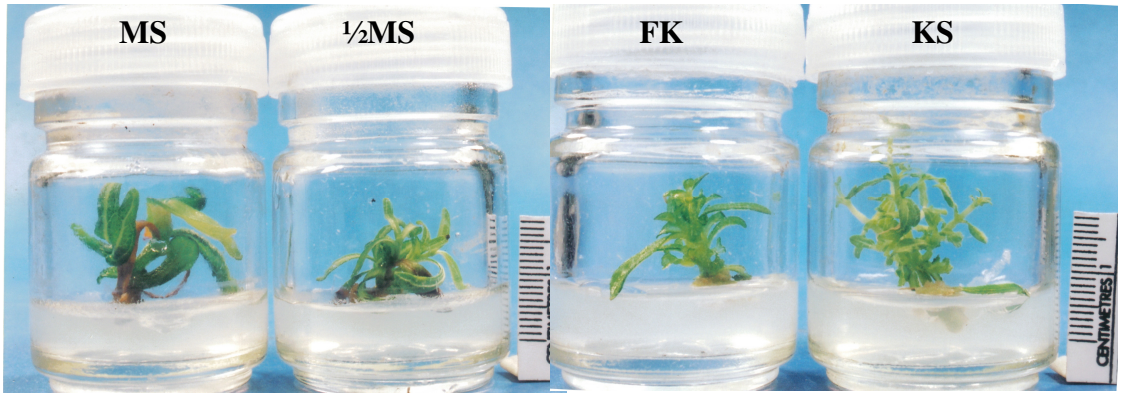

Figure 3.10 *Z. angustifolia* cultured on MS, ½ MS, FK and KS media after 4 weeks of culture

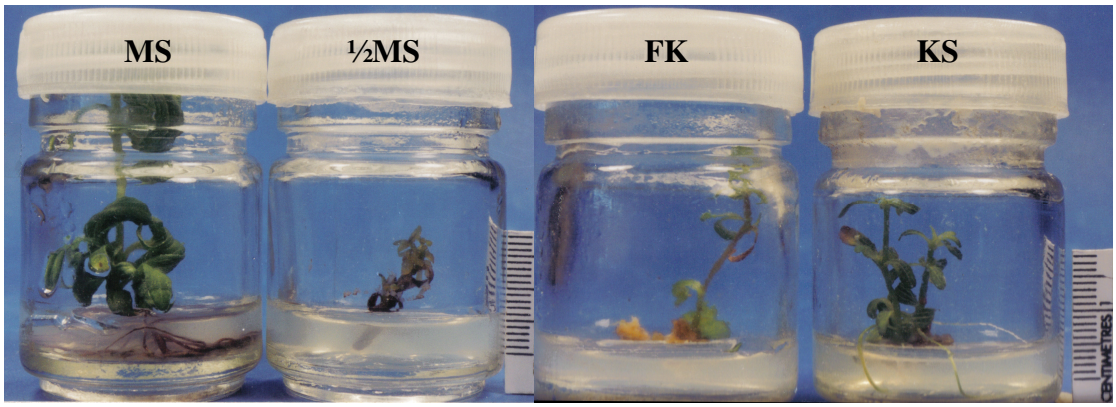

Figure 3.11 *Z. haageana* cultured on MS, ½ MS, FK and KS media after 4 weeks of culture

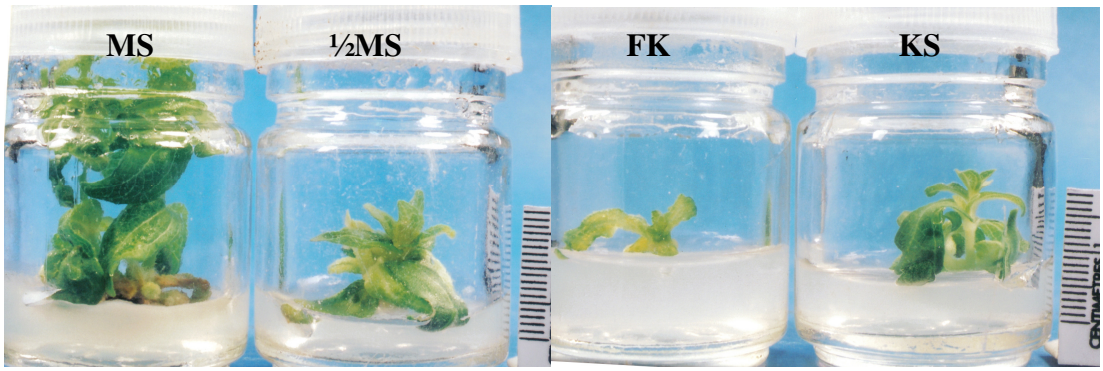

Figure 3.12 *Z. elegans* cultured on MS, ½ MS, FK and KS media after 4 weeks of culture

B. Effect of plant growth regulators

When shoots of *Z. elegans, Z. haageana* and *Z. angustifolia* were cultured on KS medium supplemented with BA and kinetin but without auxin, results revealed that more shoots were induced on kinetin containing media than BA. In contrast, more shoots of *Z. angustifolia* were induced on BA containing media than kinetin without auxin (Table 3.10). More shoots were induced on auxin free medium than auxin containing media in all cultivars tested without any significant difference. When shoots were cultured on different types of auxins with the same cytokinin, it was found that shoots were induced easier on medium supplemented with IBA than other auxins (Table 3.10). The percentage of root ranking and greater numbers of shoots in *Z. angustifolia* and *Z. haageana* (but not in *Z. elegans*) was high when cultured on media containing kinetin in combination with IBA. (Table 3.10). Hence, Kinetin and IBA were the appropriate growth regulators for shoot and root induction in this study.

It was observed that callus could be initiated when media were supplemented with auxins. 2, 4-D induced callus of *Z. angustifolia* and *Z. haageana* better than *Z. elegans*. Both IBA and NAA were less effective than 2, 4-D meanwhile IBA was superior to NAA (Table 3.11). With respect to the used cytokinins, callus of *Z. haageana* was induced by BA better than kinetin (Table 3.11, Figure 3.15-3.16) whereas callus of *Z. angustifolia* and *Z. elegans* was induced better by kinetin (Table 3.11, Figure 3.13-3.14, 3.17-3.18).

Since the previous data revealed that kinetin and IBA were effective for the induction of shoot and root in the three zinnia cultivars hence further experiment to clarify the effect of kinetin and IBA was performed as shown in Table 3.12. It was found that kinetin and IBA at the concentrations tested had no effect on number of

shoots and roots in three zinnia cultivars. In contrast, these concentrations affected shoot length in *Z. angustifolia* since the highest shoot length (10.63±5.03, $p \leq 0.05$) was found at 1 µM kinetin and 5 µM IBA. In *Z. haageana*, kinetin and IBA seemed to have no effect on shoot length since KS medium devoid growth regulators produced better shoot length. In *Z. elegans*, however, there was no significant difference in shoot length caused by kinetin and IBA. The combination of kinetin and IBA at all concentrations promoted root formation. For callus induction, it seemed that KS media containing kinetin and IBA were able to induce callus while KS media without kinetin and IBA could not produce callus at all (Table 3.13)

Table 3.10 Effect of types of growth regulators on shoot and root formation in zinnia cultivars after 4 weeks in culture

Species	Cytokinin (1 µM)	Auxin (0.1µM)	No of shoots (mean±SD)	Shoot length (mean ±SD)	Root ranking (%)		
					1	2	3
Z. angustifolia cv. 'Starbright'	BA	No auxin	4.55 ± 2.50 [a]	1.53 ± 0.37 [b]	5.0	0	0
		2,4-D	2.35 ± 1.60 [a]	1.06 ± 0.33 [b]	4.2	0	0
		IBA	3.00 ± 2.52 [a]	1.38 ± 0.65 [b]	0	5.6	0
		NAA	4.00 ± 2.55 [a]	1.53 ± 0.50 [b]	4.2	0	0
	Kinetin	No auxin	3.91 ± 2.50 [a]	1.93 ± 0.84 [b]	8.7	8.7	4.3
		2,4-D	1.43 ± 0.76 [a]	1.26 ± 0.76 [b]	0	0	0
		IBA	5.61 ± 2.69 [a]	1.70 ± 1.29 [b]	21.7	0	0
		NAA	2.81 ± 1.80 [a]	1.52 ± 0.81 [b]	9.5	4.8	0
Z. haageana cv. 'Persian carpet'	BA	No auxin	1.60 ± 0.70 [c]	4.03 ± 3.52 [d]	40.0	40.0	20.0
		2,4-D	1.42 ± 0.67 [c]	2.25 ± 0.85 [d]	0	0	0
		IBA	1.20 ± 0.45 [c]	1.64 ± 1.32 [d]	0	0	0
		NAA	2.11 ± 1.76 [c]	2.65 ± 2.12 [d]	22.2	44.4	22.2
	Kinetin	No auxin	2.64 ± 1.12 [c]	4.92 ± 3.48 [e]	18.2	54.5	0
		2,4-D	1.00 ± 0.00 [c]	1.53 ± 0.33 [e]	0	16.7	0
		IBA	3.08 ± 1.17 [c]	5.11 ± 2.18 [e]	33.3	50.0	0
		NAA	1.42 ± 0.52 [c]	2.93 ± 3.18 [e]	0	33.3	0.3
Z. elegans cv. 'Dreamland'	BA	No auxin	2.11 ± 1.41 [f]	1.67 ± 0.85 [g]	21.1	21.1	0
		2,4-D	1.10 ± 0.32 [f]	0.95 ± 0.37 [g]	0	0	0
		IBA	1.81 ± 0.98 [f]	1.52 ± 0.62 [g]	25.0	6.3	0
		NAA	1.53 ± 0.92 [f]	1.11 ± 0.55 [g]	13.3	6.7	6.7
	Kinetin	No auxin	2.35 ± 1.46 [f]	2.31 ± 1.18 [g]	35.3	11.8	5.9
		2,4-D	0.88 ± 0.35 [f]	0.74 ± 0.39 [g]	0	0	0
		IBA	1.60 ± 0.74 [f]	2.42 ± 1.42 [g]	40.0	0	0
		NAA	1.25 ± 0.58 [f]	1.77 ± 1.35 [g]	31.3	18.8	31.3

Note: number followed by the same letter in the column was not significantly different at $p \leq 0.05$

Table 3.11 Effect of types of growth regulators on callus induction in zinnia cultivars after 4 weeks in culture

Species	Cytokinin (1 µM)	Auxin (0.1µM)	Callus diameter (%)		
			0.1-0.5 cm.	0.5-1.0 cm.	> 1.0 cm.
Z. angustifolia cv. 'Starbright'	BA	No auxin	10.0	35.0	35.0
		2,4-D	30.0	35.0	20.0
		IBA	22.2	50.0	11.1
		NAA	20.8	33.3	29.2
	Kinetin	No auxin	26.1	17.4	39.1
		2,4-D	28.6	42.9	14.3
		IBA	8.7	34.8	21.7
		NAA	23.8	19.0	33.3
Z. haageana cv. 'Persian carpet'	BA	No auxin	40	10	0
		2,4-D	83.3	16.7	8.3
		IBA	40	40	0
		NAA	55.6	0	0
	Kinetin	No auxin	9.1	0	0
		2,4-D	50.0	0	0
		IBA	41.7	0	0
		NAA	0	0	0
Z. elegans cv. 'Dreamland'	BA	No auxin	10.5	15.8	0
		2,4-D	30.0	0	0
		IBA	50.0	0	0
		NAA	40.0	0	0
	Kinetin	No auxin	17.6	5.9	0
		2,4-D	12.5	50.0	0
		IBA	66.7	13.3	0
		NAA	6.3	0	0

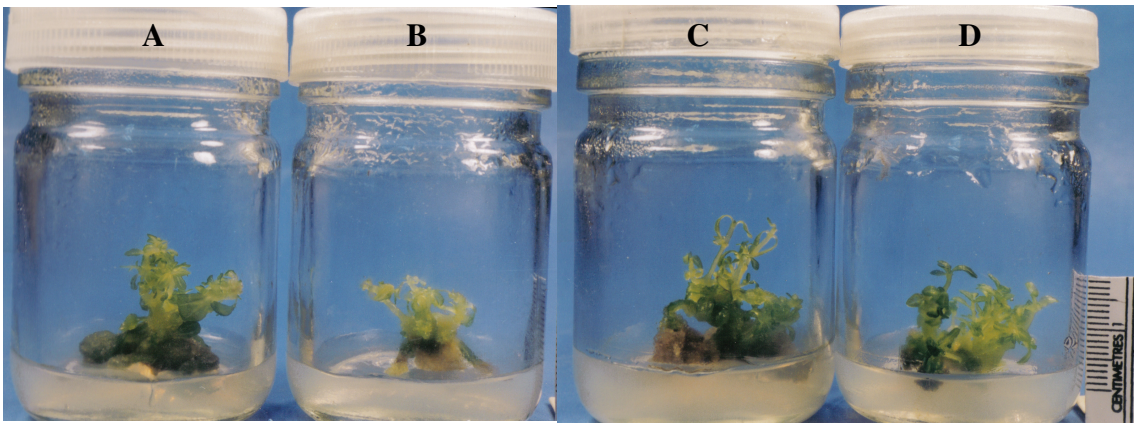

Figure 3.13 *Z. angustofolia* cultured on KS medium plus 1 µM BA (A), 1 µM BA + 0.1 µM 2,4-D (B),1 µM BA + 0.1 µM IBA (C) and 1 µM BA + 0.1 µM NAA (D).

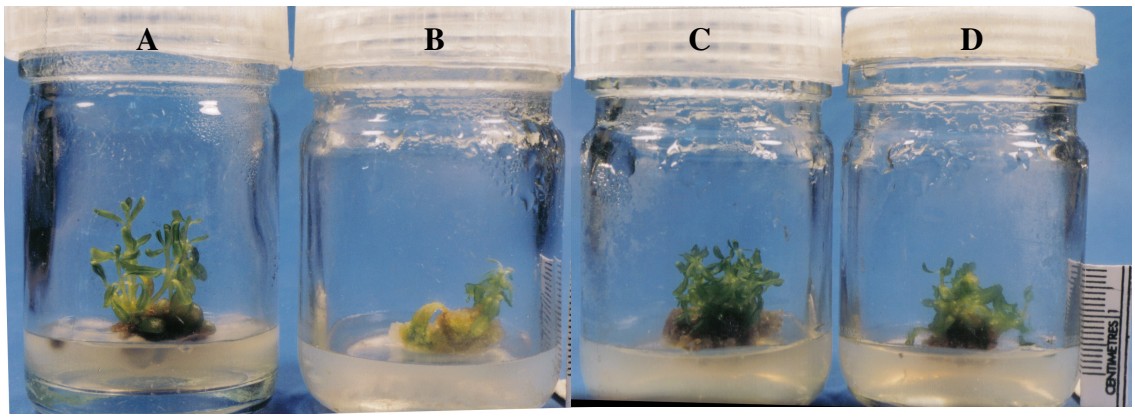

Figure 3.14 *Z. angustofolia* cultured on KS medium plus 1 µM Kinetin (A), 1 µM Kinetin + 0.1 µM 2,4-D (B), 1 µM Kinetin + 0.1 µM IBA (C) and 1 µM Kinetin + 0.1 µM NAA (D).

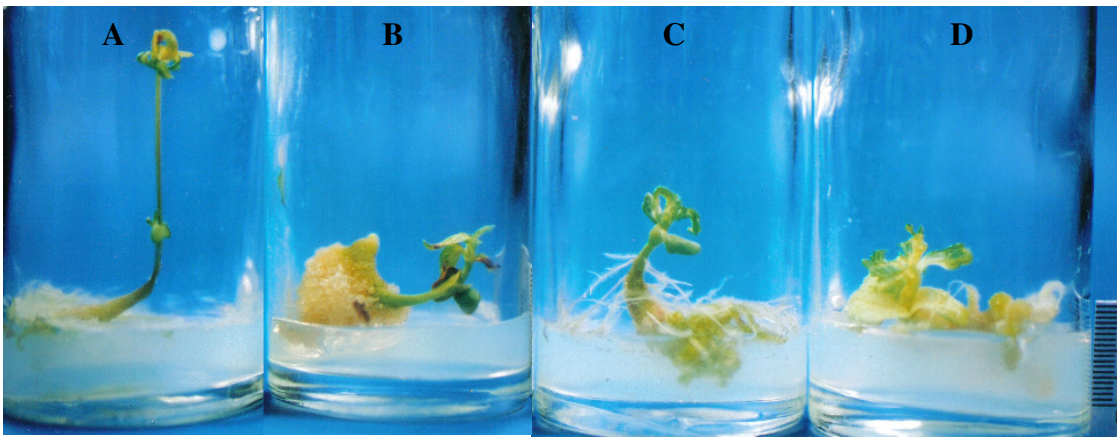

Figure 3.15 *Z. haageana* cultured on KS medium plus 1 µM BA (A), 1 µM BA + 0.1 µM 2,4-D (B), 1 µM BA + 0.1 µM IBA (C) and 1 µM BA + 0.1µM NAA (D).

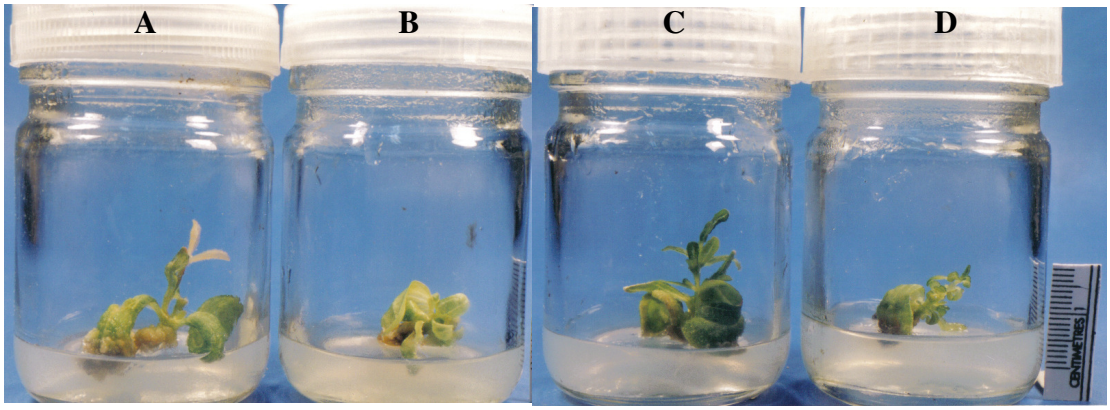

Figure 3.16 *Z. haageana* cultured on KS medium plus 1 µM Kinetin (A), 1 µM Kinetin + 0.1 µM 2,4-D (B), 1 µM Kinetin + 0.1 µM IBA (C) and 1 µM Kinetin + 0.1 µM NAA (D).

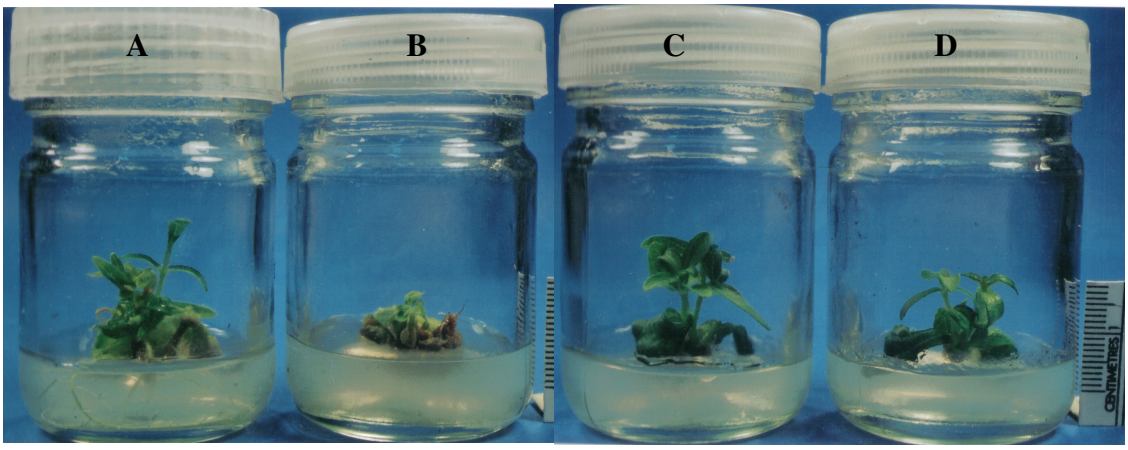

Figure 3.17 *Z. elegans* cultured on KS medium plus 1 µM BA (A), 1 µM BA + 0.1µM 2,4-D (B), 1 µM BA + 0.1 µM IBA (C) and 1 µM BA + 0.1 µM NAA (D).

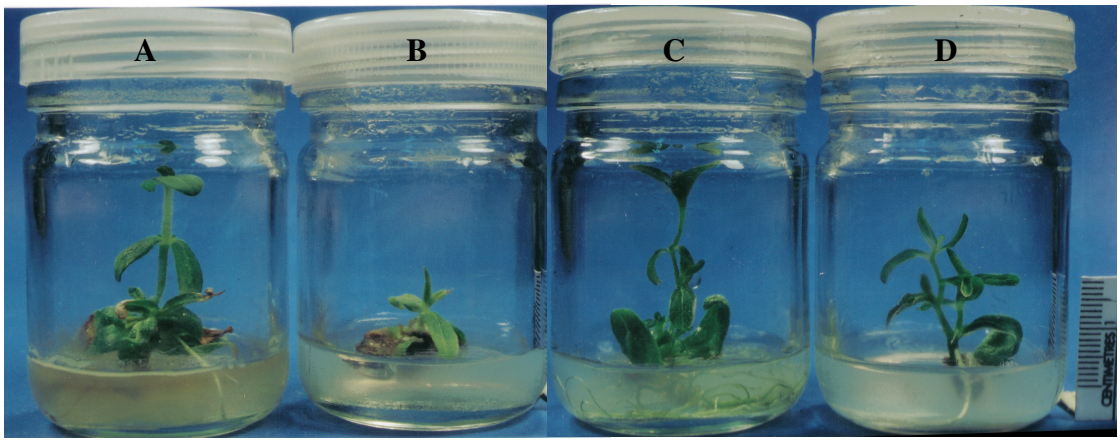

Figure 3.18 *Z. elegans* cultured on KS medium plus 1 µM Kinetin (A), 1 µM Kinetin + 0.1 µM 2,4-D (B), 1 µM Kinetin + 0.1 µM IBA (C) and 1 µM Kinetin + 0.1 µM NAA (D).

Table 3.12 Effect of kinetin and IBA concentrations on shoot and root formation in zinnia cultivars after 4 weeks in culture

Species	Kinetin (μM)	IBA (μM)	No. of shoots (mean±SD)	Shoot length (mean±SD)	Root ranking (%)		
					1	2	3
Z. angustifolia cv. 'Starbright'	0	0	2.60 ± 1.51 ^{c}	5.98 ± 2.98 ^{ab}	60.0	20.0	0
	1	0.05	2.65 ± 1.46 ^{c}	7.67 ± 4.86 ^{ab}	50.0	30.0	0
		0.5	2.60 ± 1.93 ^{c}	7.27 ± 4.64 ^{ab}	50.0	30.0	5.0
		5	2.30 ± 1.34 ^{c}	10.63± 5.03 ^{b}	5.0	30.0	60.0
	5	0.05	2.57 ± 1.66 ^{c}	4.59 ± 2.51 ^{a}	23.8	9.5	0
		0.5	2.25 ± 1.71 ^{c}	4.01 ± 3.29 ^{a}	25.0	10.0	10.0
		5	2.25 ± 1.41 ^{c}	5.44 ± 4.38 ^{ab}	25.0	20.0	20.0
	10	0.05	3.30 ± 1.49 ^{c}	6.11 ± 3.43 ^{ab}	40.0	5.0	15.0
		0.5	2.90 ± 1.37 ^{c}	5.03 ± 3.29 ^{ab}	15.0	15.0	0
		5	3.06 ± 1.73 ^{c}	5.32 ± 3.44 ^{ab}	18.8	31.3	6.3
Z. haageana cv. 'Persian carpet'	0	0	2.38 ± 1.77 ^{d}	8.51 ± 4.82 ^{h}	12.5	75.0	12.5
	1	0.05	1.67 ± 1.23 ^{d}	2.29 ± 1.43 ^{g}	33.3	16.7	0
		0.5	1.69 ± 1.03 ^{d}	4.46 ± 2.57 ^{gh}	30.8	38.5	15.4
		5	1.88 ± 2.21 ^{d}	3.68 ± 2.27 ^{gh}	11.8	47.1	41.2
	5	0.05	2.46 ± 1.33 ^{d}	6.71 ± 3.56 ^{gh}	61.5	15.4	15.4
		0.5	1.40 ± 0.52 ^{d}	4.16 ± 3.79 ^{gh}	30.0	40.0	20.0
		5	1.71 ± 0.99 ^{d}	5.84 ± 5.37 ^{gh}	28.6	28.6	7.1
	10	0.05	2.43 ± 1.65 ^{d}	5.13 ± 4.05 ^{gh}	50.0	21.4	14.3
		0.5	1.92 ± 1.98 ^{d}	3.39 ± 1.94 ^{gh}	16.7	41.7	25.0
		5	1.95 ± 0.97 ^{d}	2.69 ± 1.66 ^{g}	52.6	21.1	5.3
Z. elegans cv. 'Dreamland'	0	0	2.20 ± 1.19 ^{e}	3.13 ± 1.90 ^{f}	28.0	20.0	20.0
	1	0.05	2.23 ± 1.42 ^{e}	3.47 ± 2.17 ^{f}	30.8	11.5	30.8
		0.5	1.78 ± 1.25 ^{e}	3.01 ± 2.89 ^{f}	11.1	25.0	22.2
		5	1.65 ± 1.09 ^{e}	3.38 ± 2.58 ^{f}	15.4	19.2	23.1
	5	0.05	1.69 ± 1.05 ^{e}	3.13 ± 2.69 ^{f}	12.8	10.3	33.3
		0.5	1.81 ± 0.97 ^{e}	4.22 ± 3.41 ^{f}	13.5	24.3	13.5
		5	1.63 ± 0.75 ^{e}	3.27 ± 2.45 ^{f}	23.5	23.5	38.2
	10	0.05	1.87 ± 0.97 ^{e}	2.31 ± 1.22 ^{f}	19.4	13.9	27.8
		0.5	1.69 ± 0.86 ^{e}	2.47 ± 1.94 ^{f}	28.1	28.1	06.3
		5	1.62 ± 0.64 ^{e}	2.22 ± 1.77 ^{f}	26.5	14.7	11.8

Note: number followed by the same letter in the column was not significantly different at $p \leq 0.05$

Table 3.13 Effect of kinetin and IBA concentrations on callus induction in zinnia cultivars after 4 weeks in culture

Species	Kinetin (µM)	IBA (µM)	Callus diameter (%)		
			0.1-0.5 cm.	0.1-0.5 cm.	0.1-0.5 cm.
Z. angustifolia cv. 'Starbright'	0	0	0	0	0
	1	0.05	5.0	0	0
		0.5	10.0	0	0
		5	5.0	0	0
	5	0.05	9.5	0	0
		0.5	40.0	0	0
		5	35.0	20.0	0
	10	0.05	35.0	20.0	0
		0.5	25.0	0	0
		5	31.3	12.5	0
Z. haageana cv. 'Persian carpet'	0	0	0	0	0
	1	0.05	8.3	0	0
		0.5	15.4	7.7	0
		5	5.9	5.9	0
	5	0.05	0	0	0
		0.5	0	0	0
		5	35.7	0	0
	10	0.05	21.4	0	0
		0.5	16.7	16.7	0
		5	26.3	10.5	10.5
Z. elegans cv.'Dreamland'	0	0	0	0	0
	1	0.05	23.1	3.8	0
		0.5	8.3	2.8	0
		5	3.8	0	0
	5	0.05	18.0	10.3	0
		0.5	8.1	10.8	0
		5	20.6	14.7	2.9
	10	0.05	19.4	27.8	0
		0.5	9.4	21.9	3.1
		5	26.5	5.9	2.9

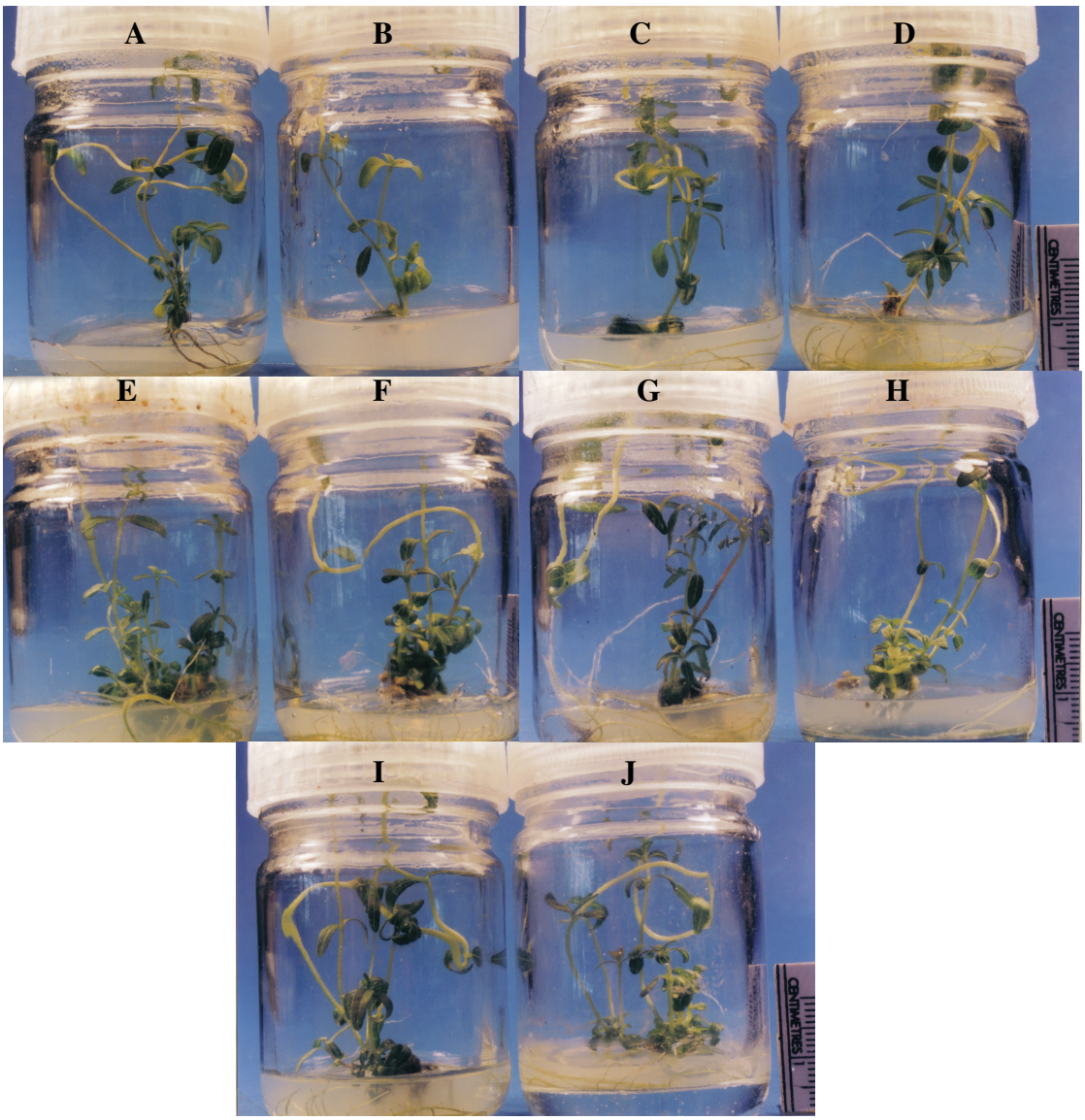

Figure 3.19 *Z. angustifolia* cultured on KS medium supplemented with various concentrations of kinetin and IBA

A) No growth regulator, B) 1 µM kinetin + 0.05 µM IBA
C) 1 µM kinetin + 0.50 µM IBA, D) 1 µM kinetin + 5.00 µM IBA
E) 5 µM kinetin + 0.05 µM IBA, F) 5 µM kinetin + 0.50 µM IBA
G) 5 µM kinetin + 5.00 µM IBA, H) 10 µM kinetin + 0.05 µM IBA
I) 10 µM kinetin + 0.50 µM IBA, J) 10 µM kinetin + 5.00 µM IBA

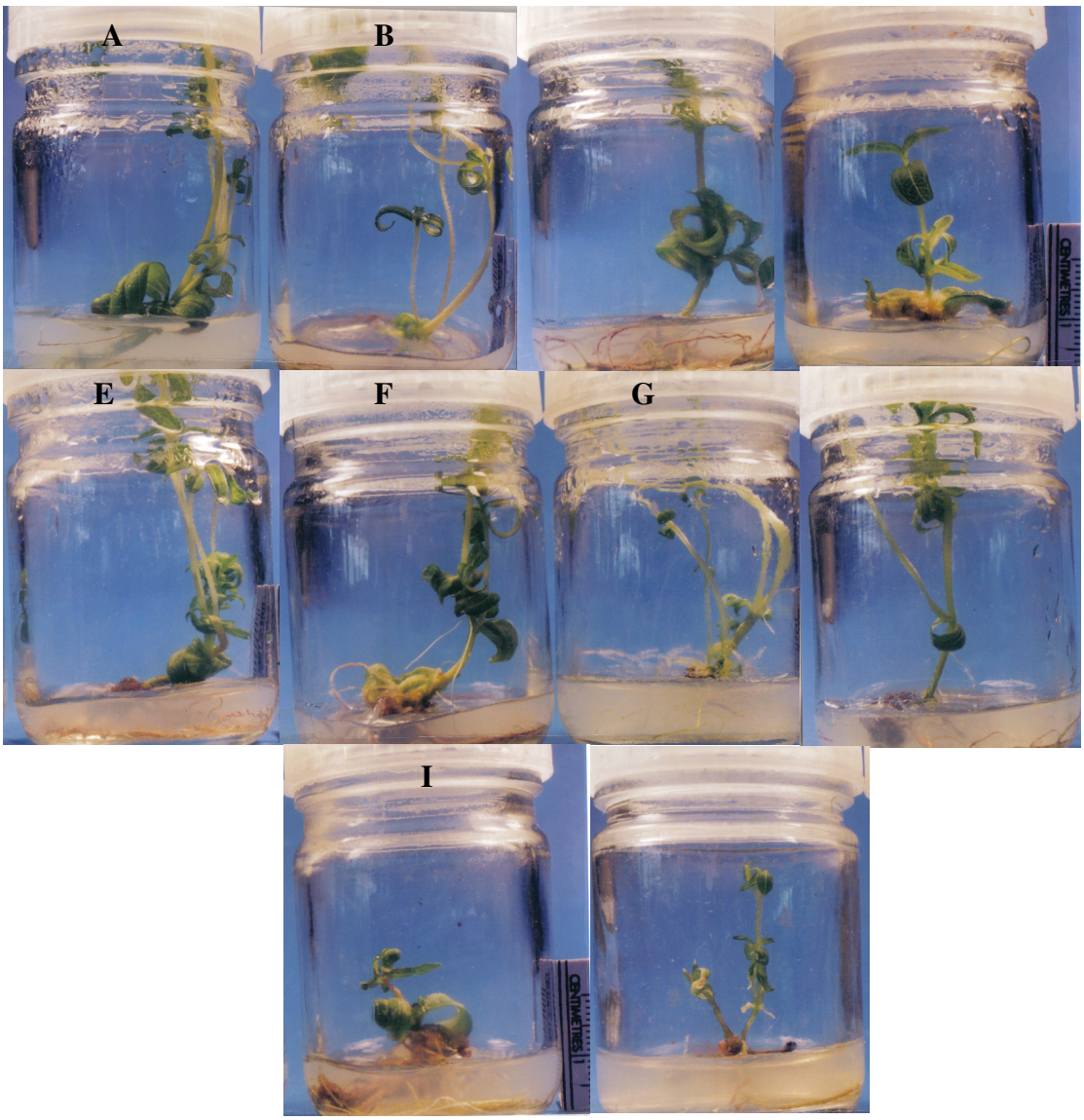

Figure 3.20 *Z. haageana* cultured on KS medium supplemented with various concentrations of kinetin and IBA

A) No growth regulator, B) 1 µM kinetin + 0.05 µM IBA
C) 1 µM kinetin + 0.50 µM IBA, D) 1 µM kinetin + 5.00 µM IBA
E) 5 µM kinetin + 0.05 µM IBA, F) 5 µM kinetin + 0.50 µM IBA
G) 5 µM kinetin + 5.00 µM IBA, H) 10 µM kinetin + 0.05 µM IBA
I) 10 µM kinetin + 0.50 µM IBA, J) 10 µM kinetin + 5.00 µM IBA

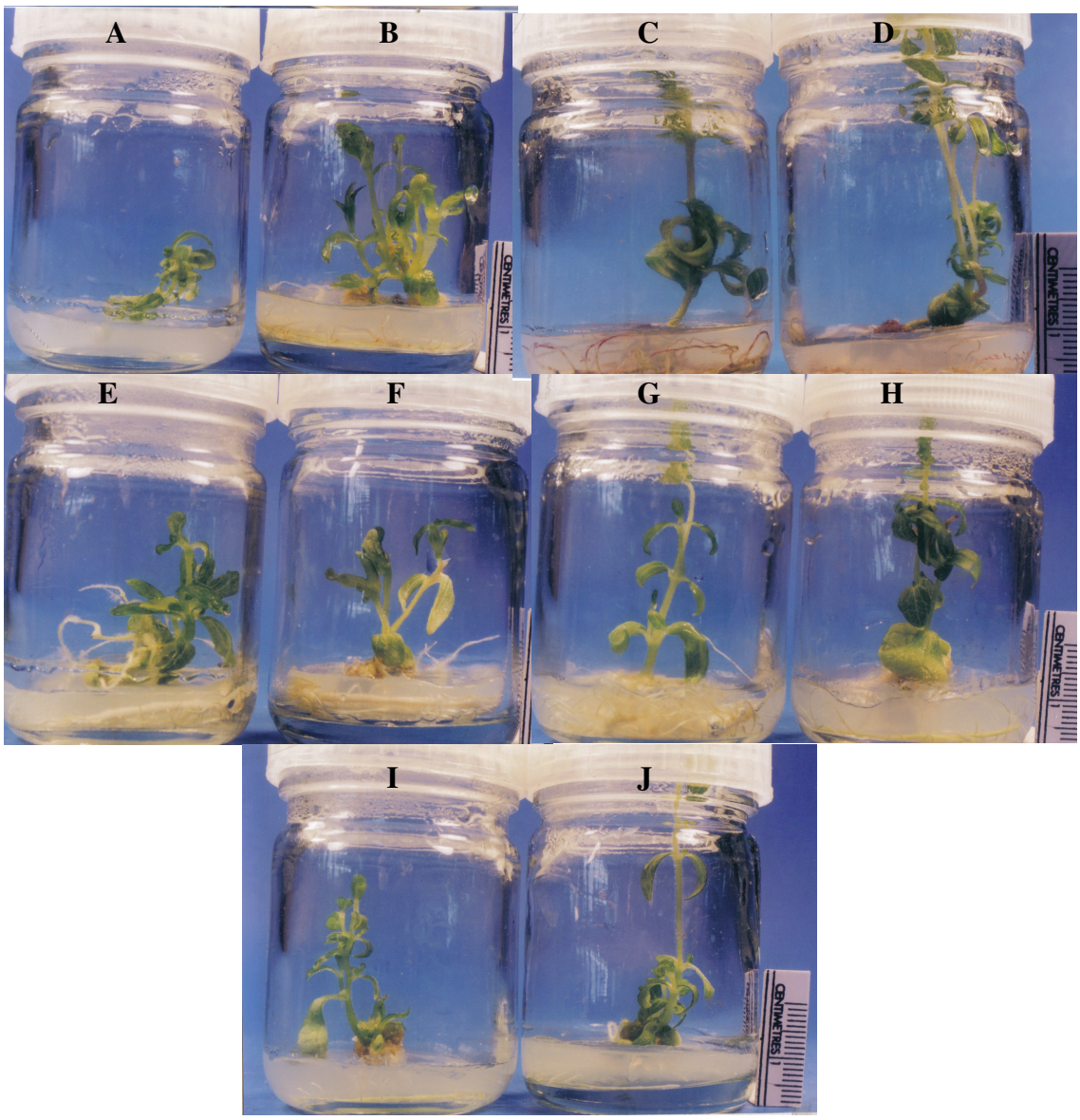

Figure 3.21 *Z. elegans* cultured on KS medium supplemented with various concentrations of kinetin and IBA

A) No growth regulator, B) 1 µM kinetin + 0.05 µM IBA
C) 1 µM kinetin + 0.50 µM IBA, D) 1 µM kinetin + 5.00 µM IBA
E) 5 µM kinetin + 0.05 µM IBA, F) 5 µM kinetin + 0.50 µM IBA
G) 5 µM kinetin + 5.00 µM IBA, H) 10 µM kinetin + 0.05 µM IBA
I) 10 µM kinetin + 0.50 µM IBA, J) 10 µM kinetin + 5.00 µM IBA

C. Effect of $AgNO_3$

It is generally known that plant growth regulators play a significant role in the morphogenesis of plant tissue culture. Investigations on the manipulation of several cytokinins and auxins in the previous section have lead to the development of zinnia regeneration system. Ethylene is produced during *in vitro* culture and this associated with poor regeneration of culture materials. Addition of some chemicals such as cobalt chloride or silver nitrate can inhibit ethylene production or its function. Therefore this part describes the effect of silver nitrate, an ethylene inhibitor, on the frequency of number of shoots per explant of *in vitro* culture.

Of the three cultivars, *Z. angustifolia* cv. 'Starbright' produced the highest number of shoots (5.58) at the concentration of 2 mg/l $AgNO_3$ ($p \leq 0.05$) (Table 3.14, Figure 3.22). No statistically significant difference in the number of shoot regeneration between *Z. haageana* cv. 'Persian carpet' and *Z. elegans* cv. 'Dreamland' were found. It seemed that $AgNO_3$ inhibited shoot length of *Z. angustifolia* cv. 'Starbright' and *Z. elegans* cv. 'Dreamland' whilst enhanced shoot length of *Z. haageana* cv. 'Persian carpet' at the concentration of 2 mg/l. ($p \leq 0.05$) (Table 3.14). In the presence of $AgNO_3$, root formation was promoted in *Z. haageana* cv. 'Persian carpet' at all concentrations tested than *Z. angustifolia* cv. 'Starbright' and *Z. elegans* cv. 'Dreamland' (Table 3.14). Callus also could be induced in three zinnia cultivars and the best result was found in *Z. elegans* cv. 'Dreamland' at the concentration of 4 mg/l $AgNO_3$ (Table 3.15)

Table 3.14 Effect of $AgNO_3$ on shoot number, shoot height and root formation in three zinnia cultivars

Cultivars	$AgNO_3$ (mg/l)	No. of shoots (mean±SD)	Shoot length (cm) (mean±SD)	Root ranking (%)		
				1	2	3
Z. angustifolia cv. 'Starbright'	1	2.57 ± 1.34 [a]	1.87 ± 0.87 [c]	7.1	0	0
	2	5.58 ± 2.61 [b]	1.71 ± 0.27 [c]	0	0	0
	4	3.15 ± 2.36 [ab]	1.63 ± 0.55 [c]	8.8	0	2.9
	8	3.27 ± 2.57 [ab]	1.71 ± 0.54 [c]	10.0	0	0
	16	3.59 ± 2.29 [ab]	1.32 ± 0.48 [c]	0	0	0
Z. haageana cv. 'Persian Carpet'	1	1.92 ± 1.08 [c]	3.33 ± 1.30 [a]	16.7	0	0
	2	1.75 ± 0.71 [c]	7.06 ± 2.81 [b]	50.0	0	0
	4	2.27 ± 1.74 [c]	5.28 ± 2.92 [ab]	36.4	9.1	0
	8	2.08 ± 0.76 [c]	4.76 ± 2.27 [ab]	69.2	7.7	0
	16	2.25 ± 1.04 [c]	3.88 ± 2.04 [ab]	37.5	12.5	12.5
Z. elegans cv. 'Dreamland'	1	2.19 ± 1.25 [d]	1.73 ± 1.08 [e]	14.3	14.3	42.9
	2	1.88 ± 1.11 [d]	1.45 ± 0.64 [e]	0	0	0
	4	1.90 ± 1.21 [d]	1.70 ± 0.53 [e]	3.3	10.0	6.7
	8	1.62 ± 0.78 [d]	1.43 ± 0.45 [e]	11.8	2.9	23.5
	16	1.47 ± 0.83 [d]	1.70 ± 0.65 [e]	0	0	0

Note: number followed by the same letter in the column was not significantly different at $p \leq 0.05$

Table 3.15 Effect of $AgNO_3$ on callus induction in zinnia cultivars after 4 weeks in culture

Species	AgNO3 (mg/l)	Callus diameter (%)		
		0.1-0.5 cm.	0.5-1.0 cm.	> 1.0 cm.
Z. angustifolia cv. 'Starbright'	1	21.4	14.3	14.3
	2	47.4	0	0
	4	44.1	11.8	0
	8	46.6	13.3	0
	16	76.5	5.9	0
Z. haageana cv. 'Persian Carpet'	1	66.7	8.3	0
	2	37.5	12.5	0
	4	72.7	0	0
	8	30.8	0	0
	16	50.0	0	0
Z. elegans cv.'Dreamland'	1	14.3	14.3	0
	2	29.4	11.8	0
	4	23.3	23.3	6.7
	8	44.1	5.9	0
	16	40.0	13.3	0

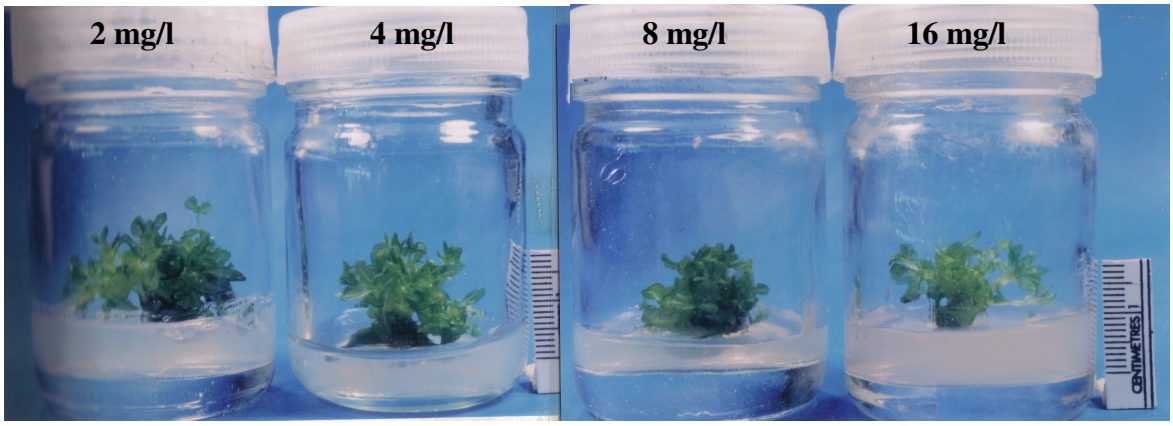

Figure 3.22 *Z. angustifolia* in KS with 5 µM kinetin + 0.5 µM IBA adding 2, 4, 8 and 16 mg/l $AgNO_3$

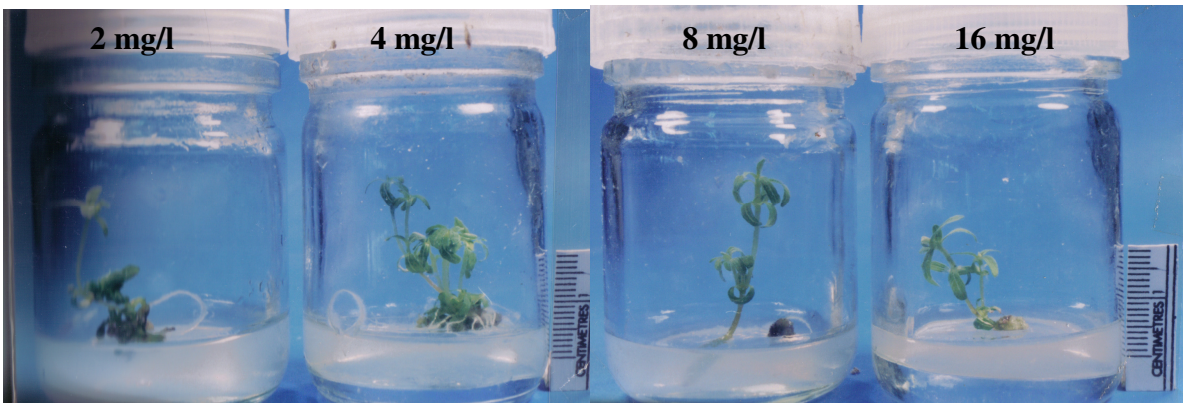

Figure 3.23 *Z. haageana* in KS with 5 µM kinetin + 0.5 µM IBA adding 2, 4, 8 and 16 mg/l $AgNO_3$

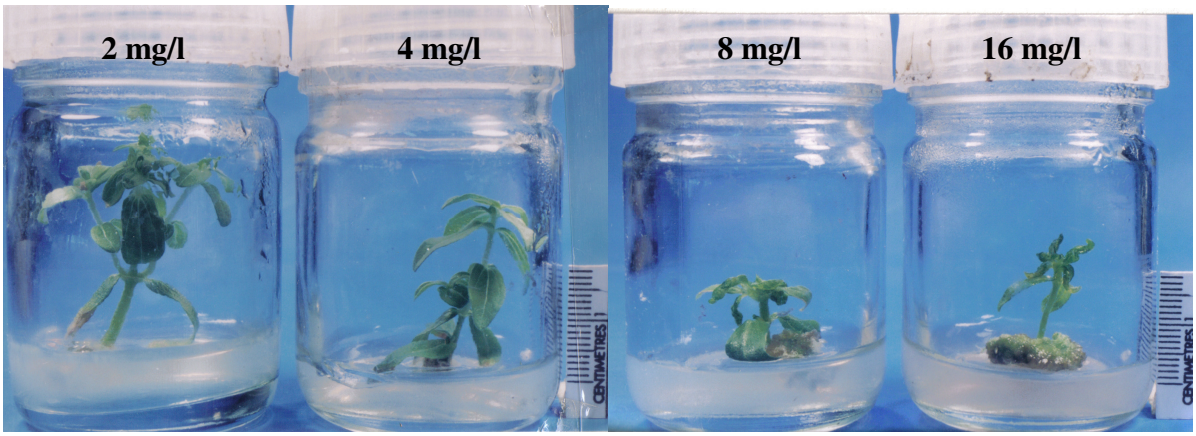

Figure 3.24 *Z. elegans* in KS with 5 µM kinetin + 0.5 µM IBA adding 2, 4, 8 and 16 mg/l $AgNO_3$

D. Organogenesis

From part A of the *in vitro* study, it was noted that explants cultured on MS medium induced large amount of callus and growth rate of callus was high(Table 3.9). Hence MS medium supplemented with TDZ, BA, NAA 2, 4-D, and IBA at several concentrations were tried. Results revealed that among the growth regulators tested, MS medium fortified with 0.1-1 µM TDZ produced the best callus initiation. The texture of call us was friable and white or pale green in color (Figure 3.25) whilst 2, 4-D, IBA and NAA containing medium gave poorer results than TDZ both in texture and amount of the callus (Figure 3.26-3.27).

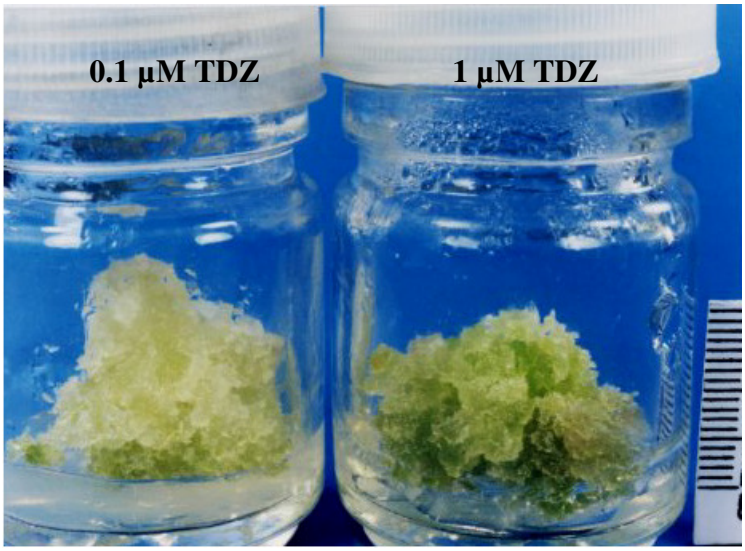

Figure 3.25 Callus induced on MS medium supplemented with 0.1 µM TDZ and 1 µM TDZ

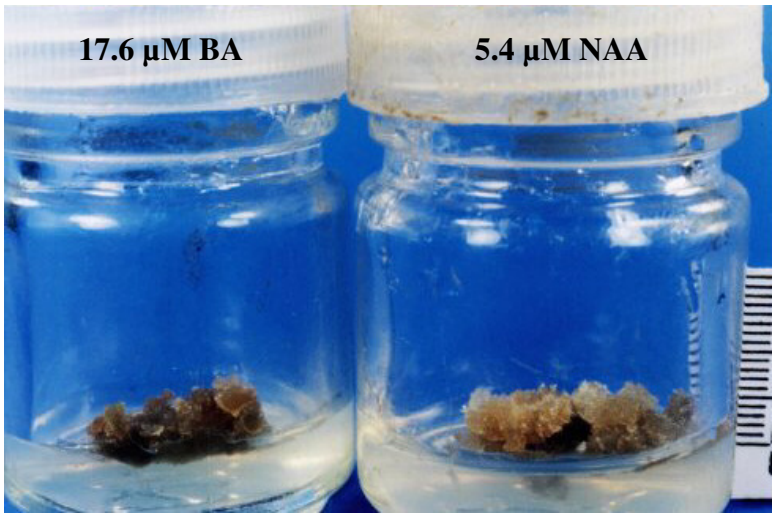

Figure 3.26 Callus induced on MS medium supplemented with 17.6 µM BA and 5.4 µM NAA

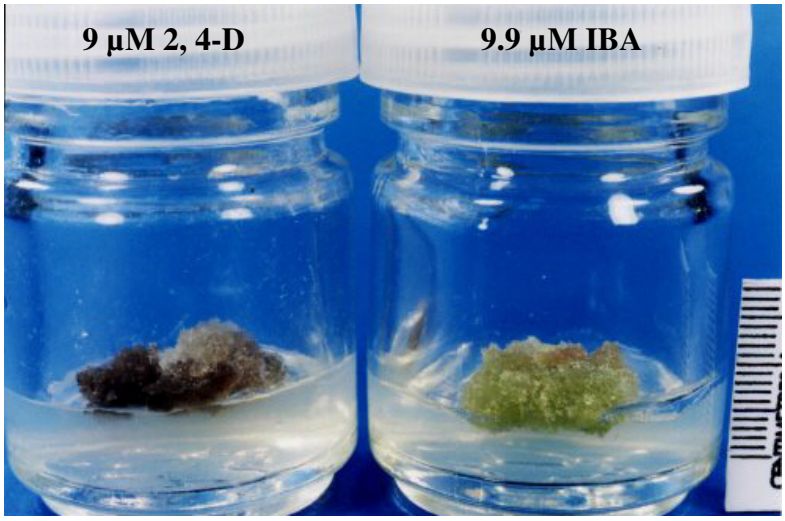

Figure 3.27 Callus induced on MS medium supplemented with 9 µM 2, 4-D and 9.9 µM IBA

When calluses from three cultivars were cultured for more than 4 weeks, roots formed at the base of the calluses except 2, 4-D, BA and NAA containing media. In contrast, green spots were formed on the TDZ derived calluses and developed further (Figure 3.28a). Calluses from three cultivars remained meristematic after 4-8 weeks

since these calluses underwent shoot and root development (Figure 3.28b, c). Lower concentration of TDZ (0.1µM) stimulated the formation of shoots but they elongated while the higher concentration (1 µM) had fewer shoots and were slow to elongate. It is interesting to note that the long-term culture of these callus derived plants produced *in vitro* flowering (Figure 3.28 d).

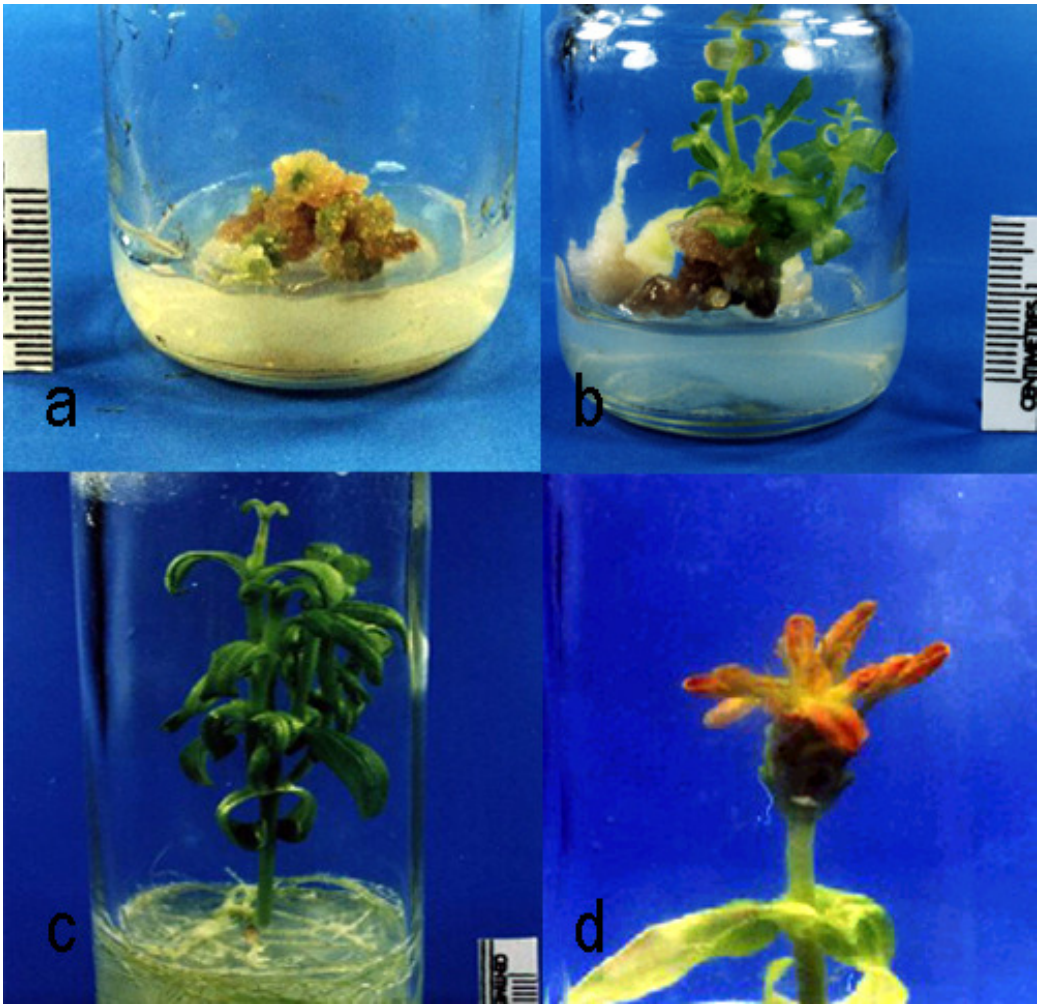

Figure 3.28 Organogenesis in *Z. elegans* cv. 'Dreamland' (a) friable callus cultured on MS medium supplemented with 0.1 µM TDZ (b) shoot regeneration from callus (c) complete plantlet and (d) *in vitro* flowering of callus derived plant.