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Induction of Dowrfism in *Encelia farinosa* by Cycocel and Evaluation of Regenerants Using RAPD and ISSR Markers

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Abstract: The growth retardant cycocel (2–chloroethyl, trimethyl ammonium chloride) was used at concentrations of 0, 1000, 2000, 3000 and 4000 ppm to induce dwarfism in the ornamental plant *Encelia farinosa* in two consecutive seasons 2004/2005 and 2005/2006 at the Ornamental Plants Research Gardens, Faculty of Agriculture, University of Alexandria. Regenerated plants were evaluated using morphological and molecular markers of RAPD and ISSR. The retarding effect of cycocel on the plant was significantly higher than that of the control. The maximum effect at the concentration of 4000ppm, showing the highest reduction in plant height, leaf area, shoot fresh and dry weight. On the other hand, the number of branches per plant and leaf chlorophyll content were increased with increasing cycocel concentration, the maximum mean was recorded with the highest cycocel concentration (4000 ppm). The most important variations obtained were plants with upright growth, dwarfed growth or leaf deformaties at the highest concentration of cycocel. A total of six RAPD and thirteen ISSR primers were used to get a total of 132 RAPD and 159 ISSR bands for overall samples. Results revealed that the highest number of specific bands were 7 RAPD and 20 ISSR markers with the control and fourth treatment (E4), respectively, while E4 has no specific bands with the RAPD primers. ISSR analysis provided more accurate results as it revealed higher degree of polymorphism than that revealed by RAPD. On the other hand, the data with ISSR on Encelia plants treated by 4000 ppm cycocel provide evidence that telomerase activity may be altered. The present study recommends that the 4000ppm of cycocel is useful to produce dwarfism in Encelia plants, which is economically important as a pot plant. Also, using ISSR as rapid and accurate method for identification of altered telomeres and breeding program in Encelia plants.

Key words: *Encelia farinosa*, Cycocel, dwarfism, RAPD markers, ISSR markers

INTRODUCTION

Encelia farinosa, Brittlebush, Incienso, or Encelia is a showy, desert, shrubby plant growing to 1.5 metre. It's herbage is fragrant, with brittle stems arising from a woody trunk. It produces leaves in a dense cluster, which are whitish-gray (silvery). It's flower is bright orange-yellow color typical of a member of the Asteraceae. The plant can be used in borders, as specimen and as low hedges (El-Shennawy, 2005). Most ornamentals receive some dwarfing treatment prior to sale, generaly by using plant growth regulators. Flowering pot plants attract premium prices, so control of floral initiation and development in Encelia is important. Cycocel (2–chloroethyl, trimethyl ammonium chloride) (Chlormequat) is a plant growth regulator used on ornamentals in greenhouses. Cycocel enhances the crops aesthetic appeal and improves durability and handling. Treated crops are more compact with shorter internodes, stronger stems and greener leaves (wasfy, 1995).

Various uses of molecular markers technology in ornamental plants have been described. The marker technology is gradually being accepted in ornamental breeding programs, and is regarded as one of the important breeding tools together with conventional breeding methods. Several molecular technniques are available for detecting genetic differences within and among cultivars (Vos *et al*., 1995). Among these, randomly amplified polymorphic DNAs (RAPD-PCR) is the most widelly used, fast and simple to perform and permits the rapid

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screening of a large number of samples (Welsh and McClelland, 1990; Williams *et al*., 1990; Berville *et al*., 2001). It is used in marker-assisted selection for biotic stress resistance (Michelmore *et al*., 1991; Paran *et al*., 1991). Inter simple sequence repeats (ISSR)-PCR is a method, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate molecular markers. ISSR markers are highly polymorphic, repeatable and useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al*., 2002).

The objectives of this study were to investigate the effect of Cycocel on controlling the growth of Encelia to produce pot plants with the least possible effect on bract size, and to characterize the treated samples at the level of molecular level.

MATERIALS AND METHODS

Plant Materials:

Encelia farinosa was kindly supplied from Floriculture and Ornamental Horticulture Research Garden, El-Shatby.

Field Experiments:

Two field experiments were conducted at the Floriculture and Ornamental Horticulture Research Garden, at El-Shatby, during the two consecutive seasons 2004/2005 and 2005/2006. Seeds of *Encelia farinosa* were sown on September 5, 2004 and 8 September 2005 for the first and second seasons respectively. The seedlings were transplanted to 30 cm pots, (one plant per pot) containing a mixture of sand and clay 1 :1(v/v). Plants were watered every second day (Parletta and Sedgley, 1995). The growth retardants used was cycocel used at the concentrations of: 0, 1000, 2000, 3000 and 4000 ppm prepared and applied as soil drench. The first application was started one month after transpanting on 25thof October 2004 and 2005 in the first and second seasons, respectively. The second application was after 14 days. The drench volume was 100 ml per pot. No watering was applied for two days before and after the drench. The experiments were carried out in the form of a completely randomized block design (RCBD) using three replications and five treatements. Ten plants were used for each treatement in the replicate (Steel and Torrie, 1980). Treatments means were compared using L.S.D. at 0.05 level. The experiment ended on the April 30 2004, 2005 for the first and second seasons; respectively. The following parameters were recorded at the end of each experiment. Plant height (cm) was measued as the distance in centimeters from the surface of the soil to the apex of the tallest branch, number of main branches, branch fresh weight (gm), branch dry weight (gm). Plants were dried in oven at 70ºc for 72 hours then left to cool and weighted in grams, number of leaves, leaves fresh weight (gm), leaves dry weight (gm), leaf area (cm²), using the disk methods (Koller, 1972) five mature leaf blades were taken from the second and third nodes from the base of main stem of each plant. Leaf area was constant area and taking their means then comparing them with leaf weight.

Leaf area = (1cm²) X (FW. Leaf) /(average weight of the disks).

Morphological variations changes in the vegetative growth or inflorescences at each treatment were recorded.

Dna Extraction:

DNA was extracted from 100 mg of young leaves for overall samples, control and the four treatments from the second season plants using Plant Genomic DNA Mini-prep kit (V-gene Biotechnology limited).

RAPD-PCR:

RAPD-PCR was carried out according to Jie Shen *et al*. (2006). Six Random primers were selected as potentially useful. The codes and sequences of the used primers are shown in Table (1).

Polymerase Chain Reactions (PCR) were optimized and mixtures (25µl total volume) were composed of dNTPs (25 pmol), $MgCl_2$ (25mM), 10X Taq DNA polymerase buffer (5µl), primer (25 pmol), genomic DNA (20 ng), Taq DNA polymerase (0.6 unit) and sterile H2O (up to 25 µl). Amplification was carried out in a DNA Thermal Cycler (Perkin Elmer 9700) programmed for 95°C for 5 min (one cycle); followed by 95°C for 1 min, 36°C for 1 min and 72°C for 2 min (35 cycles); 72°C for 10 min (one cycle), then 4°C. Amplification products were separated on 2.0% agarose gel in 0.5X TAE buffer (40mM Tris-acetate, 1mM EDTA) stained with ethidium bromide (EtBr), visualized and photographed by using a gel documentation system (Alpha Imager 1220, Canada). DNA fragments size were determined using the 100bp DNA Ladder marker.

Table 1: Code and sequence of the six different random primers

No.	Oligo Name	SEQUENCE
1	EZ35	5'- AAC TGG AGG AAG GTG GGG -3'
2	EZ351	5'- AGG AGG TGA TCC AAC CGC -3'
3	Chi35	5'- TTR GAT TGG GAA TAY CC -3'
4	Chi 15	5'- GGY GGY TGG AAT GAR GG -3'
5	A2	5'- GAA ACG GGT GGT GAT CGC -3'
6	NAH	5'- GTT TGC AGC TAT GAC GGC TGG GGG TTC GCC -3'

Table 2: Code and sequence of the thirteen different ISSR primers

No.	OligoName	Code	SEQUENCE
1	ISSR 814A	S1	5'-CTC TCT CTC TCT CTC TTG-3'
2	ISSR 844A	S2	5'-CTC TCT CTC TCT CTC TAC-3'
3	ISSR 844B	S3	5'-CTC TCT CTC TCT CTC TGC-3'
4	ISSR 17898A	S4	5'- CAC ACA CAC ACA AC -3'
5	ISSR 17898B	S5	5'- CAC ACA CAC ACA GT -3'
6	ISSR 17899B	S7	5'- CAC ACA CAC ACA GG-3'
7	ISSR HB-8	S8	5'- GAG AGA GAG AGA GG -3'
8	ISSR HB-10	S10	5'- GAG AGA GAG AGA CC -3'
9	ISSR HB-11	S11	5'- GTG TGT GTG TGT CC -3'
10	ISSR HB-12	S12	5'- CAC CAC CAC GC -3'
11	ISSR HB-13	S13	5'- GAG GAG GAG GC -3'
12	ISSR HB-14	S14	5'- CTC CTC CTC GC -3'
13	ISSR HB-15	S15	5'- GTG GTG GTG GC -3'

ISSR:

Fifteen primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were used in ISSR analysis. Thirteen ISSR primers that produced clear and reproducible bands were selected for the amplification of all DNA samples (Table, 2).

The PCR reaction mixture consisted of 20ng genomic DNA, 5X PCR buffer (Promega), 25mM/L $MgCl_2$(Promega), 100µM/L of each dNTP (Promega), 66ng/µl Primer and 5 U/µl Taq polymerase in a 25µl volume. The amplification protocol was 94 ºC for 5min to pre-denature, followed by 5 cycles of 92 ºC for 30 Sec, 35 ºC for 2min and 72 ºC for 90 sec, followed by 35 cycles of 92 ºC for 30 Sec, 40 ºC for 30 Sec and 72 ºC for 90 Sec, with a final extension at 72 ºC for 5 min, and eventually stored at 4 ºC (Jie Shen *et al*., 2006).

The amplified products were electrophoresed in 1% agarose gel with 0.5x TBE buffer. After the gel had been stained with ethidium bromide, band patterns were visualized with a UV transilluminator.

Data Analysis:

ISSR data were scored for presence (1), or absence (0) by using of the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data.

RESULTS AND DISCUSSION

The Plant Height (cm):

Figure (1-A) and Table (3) showed that all cycocel concentrations from 1000 to 4000 ppm were effective in suppressing the plant height as compared to control in the two seasons, respectively. This result may be due to that cycocel prevented cell elongation and inhibited cell division due to its effect as antigibberellins (Hammer *et al*. 1975). Also, it may be at cycocel retarded stem elongation by preventing the formatin of kaurence, a precursor of gibberellins biosynthesis, subsequently inhibiting or reducing only elongation of stem cells (Wasfy, 1995). This results are in agreement with those reported by Kuack *et al.* (1982) on Poinsettias, Sharad *et al*. (2000) on *Chrysanthemum morifolium* and Porwal *et al*. (2002) on *Rosa damascena.*

The Leaf Area (cm^2):

Figure (1-B) and Table (3) showed that cycocel has a retarding effect on the leaf area of the treated plants. These results were attributed to the decrease of leaf area thickness and the leaf mesophyll cells (Tezuka *et al*. 1989), as well as to the higher number of cells obtained per unit area of leaves (Crittendon, 1967), in addition to the inhibitory effect of cycocel on cell siz, cell enlargement and elongation as reported by Holocomb and Gohn (1995) on Poinsettia and Auda *et al.* (2002) on *Barleria cristata*.

Table 3: Means values for the Average plant hight (cm), leaf area (cm^2), and Chlorophyll content in leaves (mg/l) of *Encelia farinosa*, as affected by cycocel concentrations in the two seasons of 2004 and 2005.

Different concentration of Cycocel (ppm)	Average of plant height (cm)		Average of Leaf area (cm^2)		Average of Chlorophyll content in leaves (gm/l)	
	2004	2005	2004	2005	2004	2005
0	78[a]	75[a]	21.73 [a]	22.00 [a]	45.6 [c]	51.5 [b]
1000	50[b]	51[b]	15.10 [b]	16.00 [b]	47.4 b[c]	51.8 [b]
2000	43[c]	45[c]	10.0 [c]	10.50 [c]	54.3 [ab]	54.2 [b]
3000	33[d]	34[d]	7.80 [d]	7.10 [d]	54.8 [ab]	61.3 [a]
4000	16[e]	15[e]	7.00 [d]	5.40 [e]	60.0 [a]	64.1 [a]
LSD at 0.05	2.83	3.87	1.3802	1.3866	8.5	5.2

L.S.D $_{0.05}$ = least significant differences at 0.05 level.

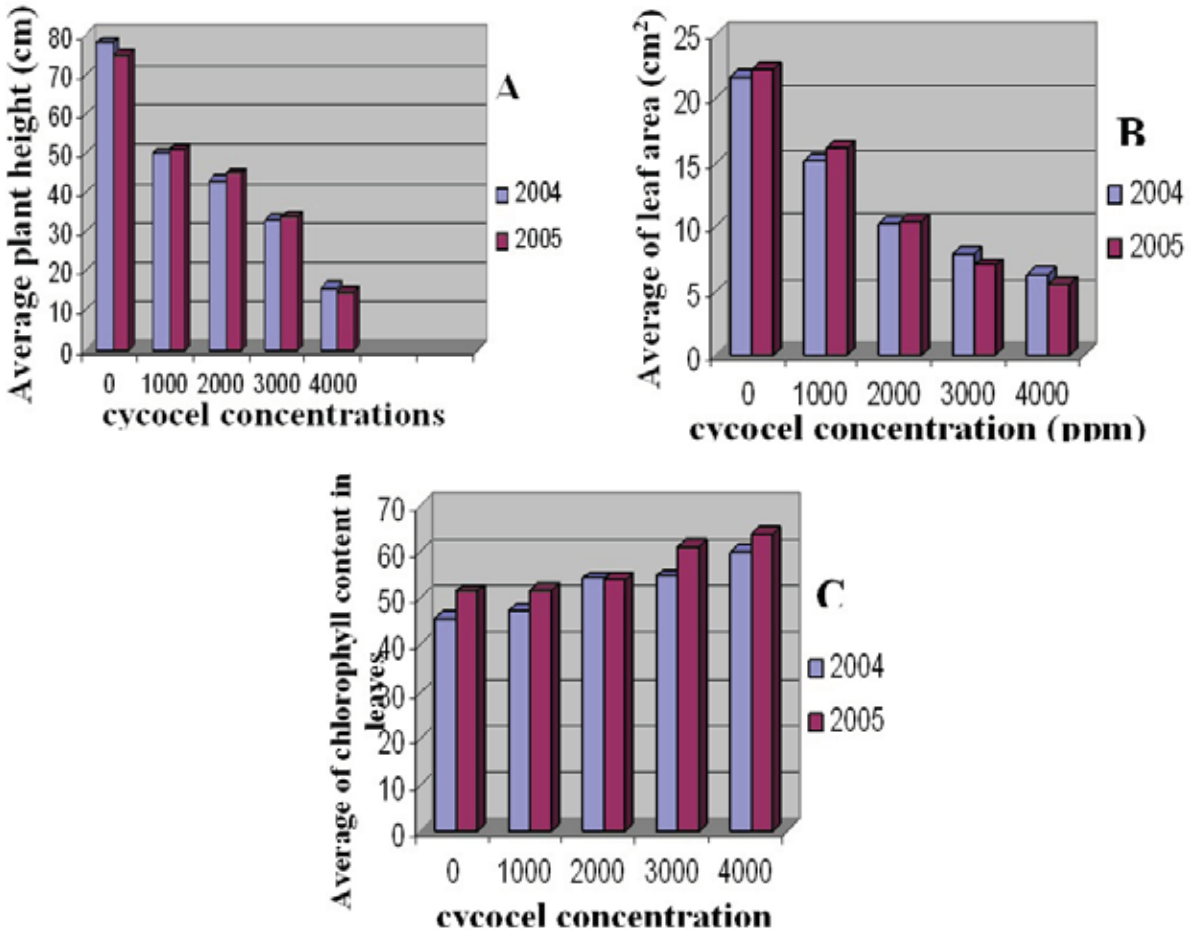

Fig. 1: Effect of different concentrations of cycocel on: (A) plant height, (B) leaf area (cm^2) and (C) leaf chlorophyll content (mg/g F.W.) of Encelia farinosa in the two seasons of 2004 and 2005.

Leaf Chlorophyll Contents:

Figure (1-C) and Table (3) showed that the leaf chlorophyll contents were significantly increased in the treated plants, compared with the control. The highest leaf chlorophyll contents were 60mg and 64.1mg in the first and second seasons, respectively with the highest concentration of cycocel (4000). These results may be due to the influence of the growth retardant on delaying the leaf senescence and hence keeping the green pigment form degradation (Wasfy, 1995). These results are in agreement with those reported by Hosni (1996), Yoon and Lang (1998) and Mostafa (2000).

Table 4: Means values for the Average Number of branches and Average Number of leaves of *Encelia farinosa*, as affected by cycocel concentrations in the two seasons of 2004 and 2005.

Different concentration of Cycocel (ppm)	Average Number of branches		Average Numer of leaves	
	2004	2005	2004	2005
0	2.1[c]	1.8[e]	175.73 [a]	175.33 [a]
1000	3.4[c]	2.8[d]	64.20 [b]	63.70 [b]
2000	5.6[b]	6.1[c]	39.43 [c]	40.33 [c]
3000	6.3[b]	7.2[b]	29.53 [cd]	29.33 [d]
4000	8.9[a]	9.5[a]	20.70 [d]	18.66 [e]
LSD at 0.05	1.97	0.63	14.108	6.426

L.S.D $_{0.05}$ = least significant differences at 0.05 level.

Table 5: Means values for the Average branches fresh weight (gm), Branch dry weight (gm), Leaf fresh weight (gm) and Leaf dry weight (gm) of *Encelia farinosa*, as affected by cycocel concentrations in the two seasons of 2004 and 2005.

Different concentration of Cycocel (ppm)	Average of Branch fresh weight (gm)		Average of Branch dry weight (gm)		Average Leaf fresh weight (gm)		Average Leaf dry weight (gm)	
	2004	2005	2004	2005	2004	2005	2004	2005
0	46.63[a]	47.67[a]	19.57[a]	20.13[a]	0.90 [a]	0.90 [a]	0.22[a]	0.17[a]
1000	34.03[b]	33.80[b]	12.53[b]	14.90[b]	0.60 [b]	0.64 [b]	0.12[b]	0.12[b]
2000	21.83[c]	22.83[c]	5.60[c]	5.83[c]	0.43 [c]	0.43 [c]	0.12[b]	0.12[b]
3000	13.73[d]	13.77[d]	3.43[d]	3.93[d]	0.32 [d]	0.32 [d]	0.07[bc]	0.07[c]
4000	7.36[e]	7.43[e]	1.50[e]	1.60[e]	0.21 [e]	0.21 [e]	0.03[c]	0.03[d]
LSD at 0.05	1.0019	2.7023	1.8403	0.7756	0.0433	0.0791	0.0699	0.0247

L.S.D $_{0.05}$ = least significant differences at 0.05 level.

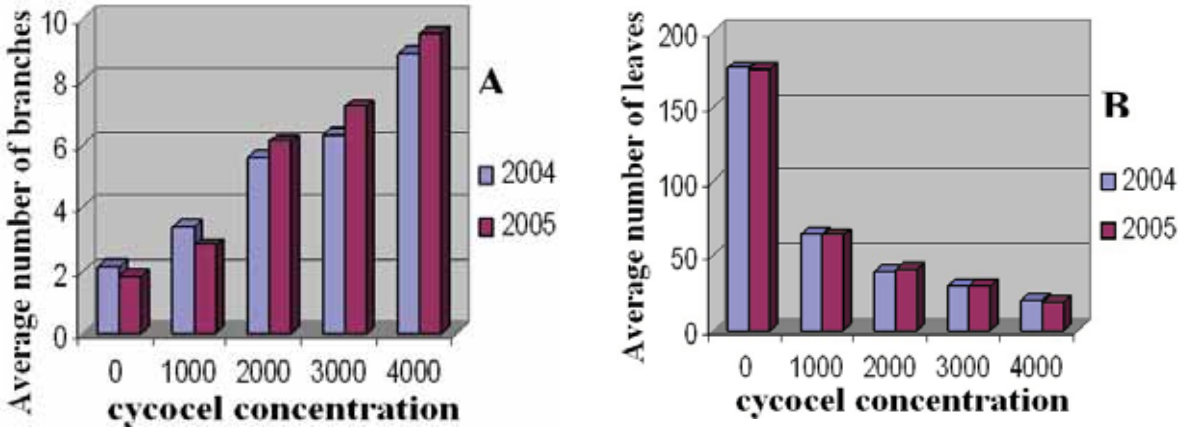

Fig. 2: Effect of different concentrations of cycocel on: (A) number of branches and (B) number of leaves of Encelia farinosa in the two seasons of 2004 and 2005.

The Number of Branches per Plant:

Figure (2-A) and Table (4) presented the mean values for the number of branches per plant. Data showed that there was a significant increase in the number of branches with increasing cycocel concentrations as compared to control in the first and second seasons. The maximum number of branches have been scored with the concentration of 4000 ppm. These results were probably due to that Cycocyle concentrations inhibit the stem elongation and stimulate growth of the lateral branches as a result of breaking the apical dominance of treated plants. These results are in agrrement with those reported by Holcomb (1979) on *Elatior Begonia*, Bachthaler and Jansen (1982) on *Pelargoniums*, Gad et al. (1997) on *Fuchsia magellanica*.

The Number of Leaves per Plant:

Figure (2-B) and Table (4) showed that, the number of leaves per plant were decreased due to the application of the Cycocel. These finding may be related to the influence of cycocel on reducing the initiation of leaves primordias leading to a reduction of the produced number of leaves. These results were in agreement with those obtained by Bhattacharjee and Gupta (1981) on Sunflower, Rusch *et al.* (1987) on *Hydrangea macrophylla*, EL-Kheir *et al.* (1994) on Soy bean and Auda *et al* (2002) on *Barleria cristata*.

The Branches Fresh Weight (gm):

Figure (3-A) and Table (5) showed that the highest concentration of cycocel (4000 ppm) caused a significant reduction in the shoot fresh weight compared with the control. This results were probably due to the reduction in plant height and branch length and thickness, which were similar to those mentioned by Bhandari and Sen (1975) on *Citrullus lanatus* and *Citrullus colocynthis*, Hassanein (1985) on *Pelargonium graveolens*, EL-Khteeb *et a*l. (1991) on *Ruta graveolens* and Auda *et al*. (2002) on *Barleria cristata*.

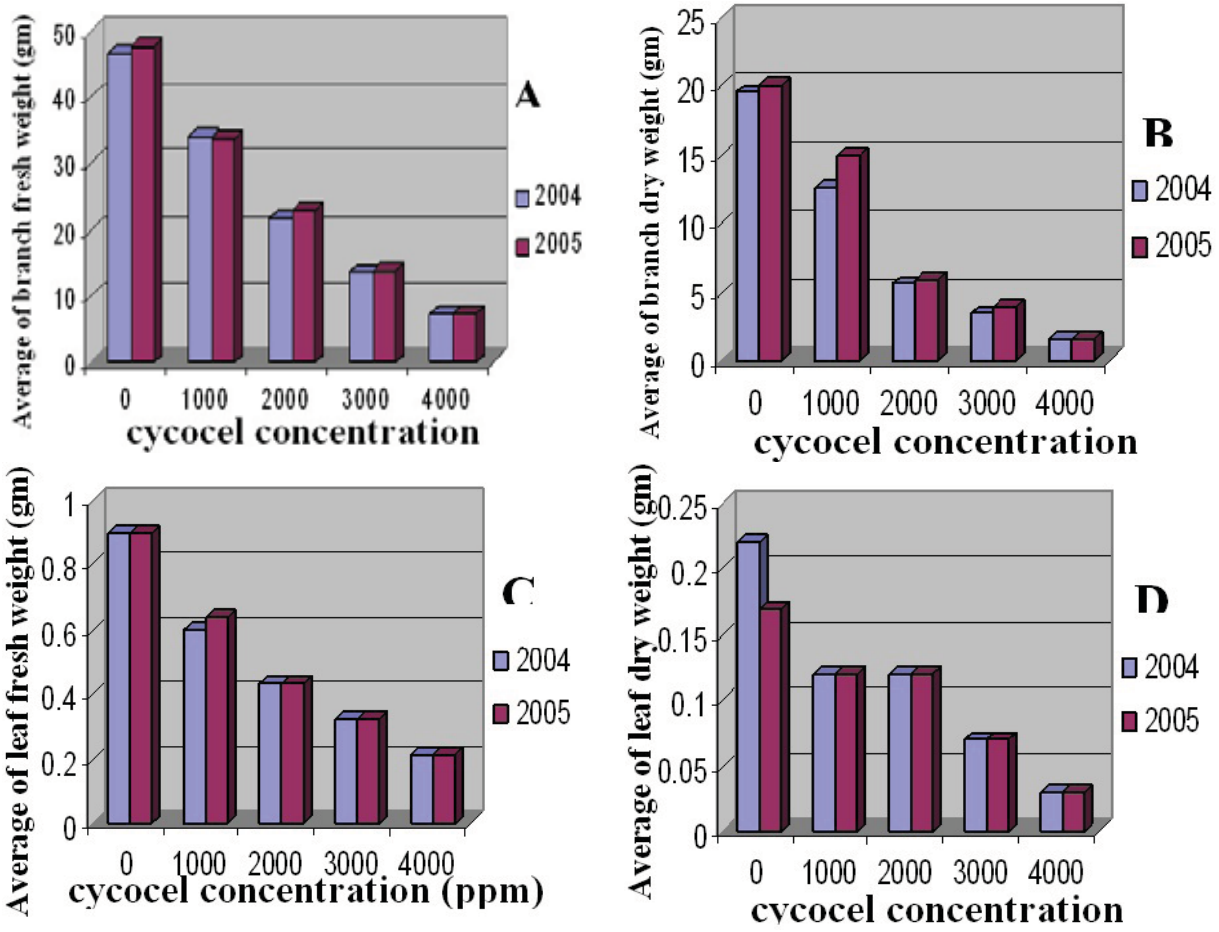

Fig. 3: Effect of different concentrations of cycocel on: (A) branch fresh weight, (B) branche dry weight, (C) leaves fresh weight and (D) leaves dry weight of Encelia farinosa in the two seasons of 2004 and 2005.

The Branch Dry Weights (gm):

Shoot dry weight of *Encelia farinosa* was affected by the different concentrations of cycocel (Figure, 3-B and Table, 5), since it had a significant effect on decreasing the branch dry weight . These results may be attributed to the decreases in internode length, plant height, and shoot length as mentioned by Mostafa (2000) on Senecio. However, EL- Torky and EL- Shennawy (1992) reported that the increase of cycocel dose suppresses gibberelic acid synthesis leading to clear reduction in dry weight which is a reflection for the effect of cycocel on shortening the internodes of Poinsettia plant.

The Leaves Fresh Weight (gm):

Figure (3-C) and Table (5) showed that there were significant decreases in the leaf fresh weight compared with the control after using different concentrations of cycocel. The previous results may be due to the influence of cycocel on increasing the generated stomata number on the upper leaves surfaces, leading to increasing the evaporation amount of water via enhancing transpiration process, consequently the fresh weight of the leaves would be reduced (Ahn and Yeam, 1977). Also, cycocel retarded cell division and /or cell expansion in lamina tissues which resulted in depressed leaf fresh weight.

Leaves Dry Weight (gm):

Figure (3-D) and Table (5) indicated that cycocel has a retarding effect on the leaf dry weight of the treated plants. These results were attributed to the decreased of leaf thickness, leaf number and/or leaf area. The mentioned results are in accordance with those reported by Hassan and Agina (1980) on *Polianthus tuberosa*, Aphalo et al. (1997) on *Betula pendula*, Mostafa (2000) on *Senecio cruentus* and EL- Kateeb et al. (1991) on *Ruta graveolens* and Khimani *et al.* (1994) on Gaillardia.

Morphological Variations:

All plants in the different treatments were examined in both seasons for the abnormal morphological changes in the vegetative growth and flowering. These changes included; 1) Habit of growth and 2) Leaf form.

Habit of Growth: *Encelia farinosa***,** is a shrubby plant growing to 1.5 metre. It's herbage is fragrant, with brittle stems arising from a woody trunk. It produces leaves in adense cluster, which are a whitish-gray (silvery). The treatments caused changes in growth habit some plants in both seasons such as plants with shape Rosset, which detected with the 2000 ppm cycocel concentration, Plants with upright growth with the 3000 ppm of cycocel and dwarfed plants with the 4000 ppm of cycocel (Figure 4).

Fig. 4: Control of *Enecilia farinosa*, 1000 ppm cycocel, 2000ppm (formed Rosset shape), 3000 ppm (formed upright growth) and 4000 ppm (formed dwarf plant).

Leaf Form:

Normal leaf of *Encelia farinosa* is triangular, simple, alternate, silvery, gray or whitish, top is uniform, underside more pubescent. All treatments caused a wide range of leaf deformities in the two seasons (Figure 5).

RAPD-PCR Analysis:

Agarose gel electrophoresis for the six RAPD-PCR amplified DNA products of overall Encelia samples, control and four treatments used in the present study was shown in Figure (6). DNA patterns were scored as presence (1) or absence (0). The results revealed that, the six primers generated a total of 198 fragments ranging from 641 to 48 bp, 132 of them were polymorphic (66.66%) as presented in Table (6). Primer PC5 had the highest total number of bands (49 bands). Twenty two (for primer A9B7) to 49 (for primer PC5) amplified fragments per primer were observed. However, 11 bands were common (monomorphic) for overall control and four treatments ranged from one band for primers A9B7, Chi15 and EZ351 to three bands for primers A4 and PC5. The lowest number of polymorphic bands [17 out of 22 (77.27% and out of 26 (65.38) amplified bands], was detected for primers Chi15 and EZ351, respectively, while the highest number of polymorphic bands (31 out of 49 amplified bands) was detected for primer PC5 (63.26%).

Specific markers generated from RAPD-PCR analysis are shown in Table (7). Fourteen out of 198 RAPD-PCR bands were found to be useful as specific markers. The largest number of RAPD-PCR specific marker was scored

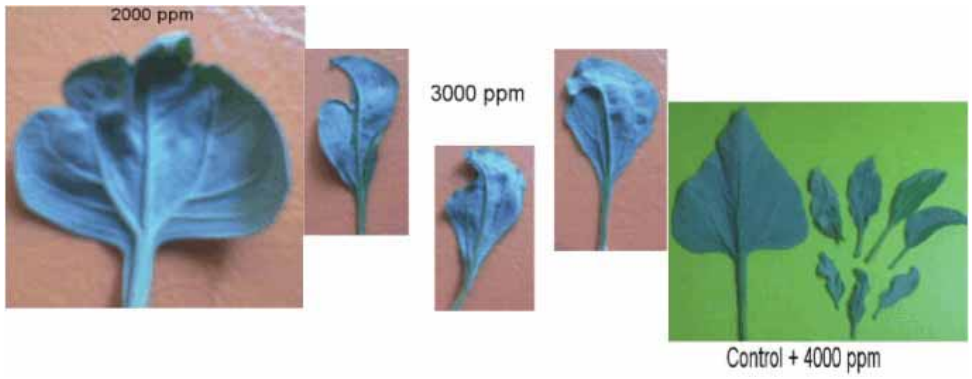

Fig. 5: Leaf deformities in the two seasons, as a result of cycocel Treatments.

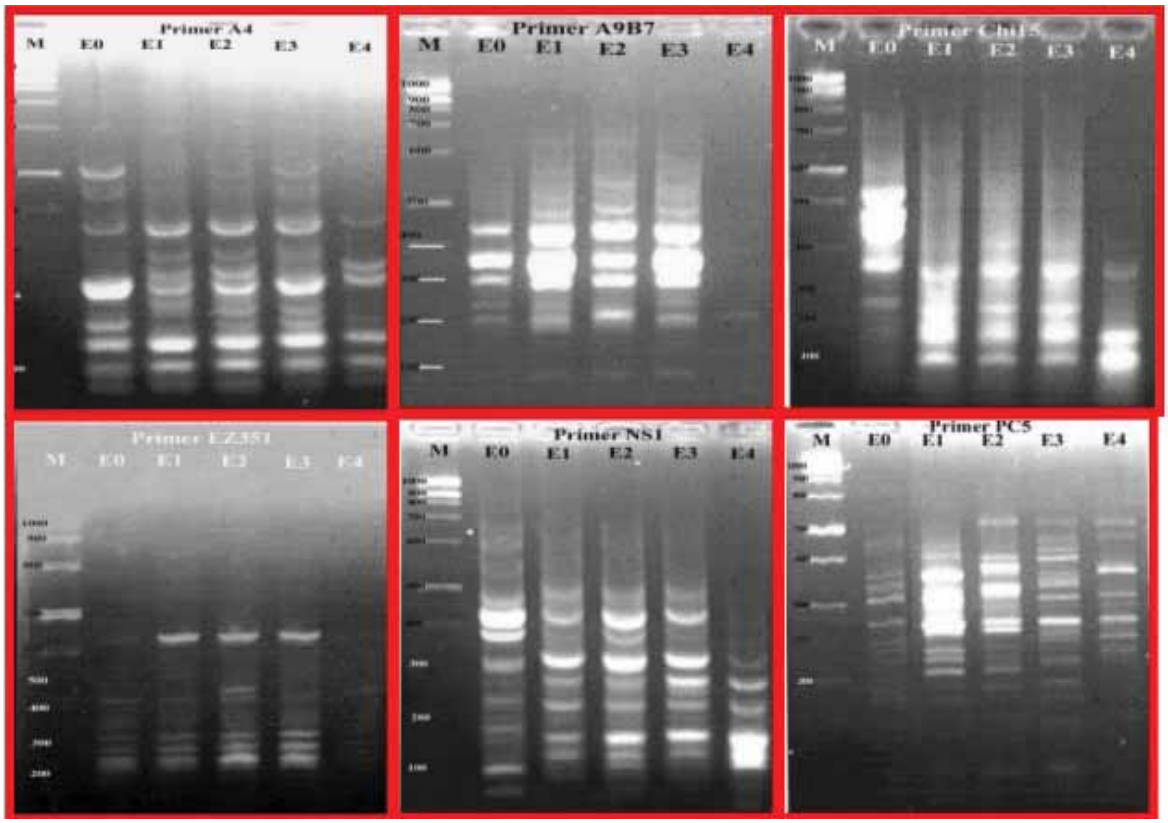

Fig. 6: Agarose gel (2%) in TAE buffer stained with Ethidium Bromide showing RAPD-PCR polymorphism of DNA for overall Encelia samples (E0: control, E1, E2, E3 and E4 are 1000ppm, 2000ppm, 3000ppm and 4000ppm cycocel treated samples, respectively) using RAPD primers. M refers to 100bp DNA Ladder.

for control (7 markers), while the lowest specific number was scored for treatments 2 and 3 (2 markers). On the other hand there was no specific markers scored with the fourth treatment. In the meantime, the highest total number of specific bands was four bands using primer Chi15. On the other hand, primer A4 did not produce any specific markers. The primers A9B7 and EZ351 generated the least total number of RAPD-PCR specific markers (2 markers for each of them) (Table, 7). In conclusion, the six primers used allowed enough distinction among the samples under study. These specific markers could be used in subsequent experiments to detect polymorphic genes with economic importance among these and other samples.

Table 6: The total number of polymporphic and common bands for each of the treatments, using six RAPD primers.

Primers	E0	E1	E2	E3	E4	Total	Total number of Common bands	Polymorphic bands	% polymorphism
Pr. A4	7	5	8	7	5	32	3	18	56.25
Pr. A9B7	3	5	6	6	2	22	1	17	77.27
Pr. Chi 15	6	6	5	6	3	26	1	17	65.38
Pr. EZ351	6	6	7	5	2	26	1	19	73.1
Pr. NS1	11	8	9	10	5	43	2	30	69.77
Pr. PC5	8	12	9	13	7	49	3	31	63.26
Total	41	42	44	47	24	198	11	132	66.66

E0 = Control;
E1= Treated samples with 1000 ppm cycocel;
E2= Treated samples with 2000ppm cycocel;
E3= Treated samples with 3000ppm cycocel and
E4= Treated samples with 4000ppm cycocel.

Table 7: The molecular weight and total number of specific polymorphic bands in each of the Encelia treated, using six RAPD primers

Primers	Molecular weight of specific bands (bp)					Total specific bands in each primer
	E0	E1	E2	E3	E4	
Pr. A4	-	-	-	-	-	-
Pr. A9B7	-	-	565	270	-	2
Pr. Chi 15	603,543,484	216	-	-	-	4
Pr. EZ351	52	-	161	-	-	2
Pr. NS1	490, 412, 123	-	-	-	-	3
Pr. PC5	-	399, 390	-	448	-	3
Total	7	3	2	2	-	14

E0 = Control;
E1= Treated samples with 1000 ppm cycocel;
E2= Treated samples with 2000ppm cycocel;
E3= Treated samples with 3000ppm cycocel and
E4= Treated samples with 4000ppm cycocel.

Table 8: The summation number of common and polymorphic PCR banding patterns using 13 ISSR primers with the all treatments.

Primers	E0	E1	E2	E3	E4	Total	Total number of Common bands	Polymorphic bands	% polymorphism
Pr.S1	5	3	3	3	7	21	2	9	42.86
Pr.S2	5	5	4	7	9	30	1	24	80
Pr.S3	4	5	6	6	5	26	4	5	19.23
Pr.S4	6	2	3	3	5	19	1	11	57.9
Pr.S5	4	6	6	6	6	28	1	22	78.57
Pr.S7	5	6	6	8	8	33	2	22	66.67
Pr.S8	6	4	5	5	5	25	3	10	40
Pr.S10	5	3	3	3	5	19	2	8	42.11
Pr.S11	3	3	3	3	8	20	3	0	0
Pr.S12	3	4	5	4	9	25	3	6	24
Pr.S13	3	4	3	4	7	21	2	7	33.33
Pr.S14	2	5	8	6	5	26	0	23	88.46
Pr.S15	4	4	2	5	3	18	1	12	66.67
Total	55	54	57	63	82	311	25	159	51.13

E0 = Control;
E1= Treated samples with 1000 ppm cycocel;
E2= Treated samples with 2000ppm cycocel;
E3= Treated samples with 3000ppm cycocel and
E4= Treated samples with 4000ppm cycocel.

ISSR Analysis:

ISSR markers are useful for gene tagging and can be used for finding markers linked to the gene of interest. Moreover, unlike SSR which are species-specific; ISSR markers are considered as universal, which could be used in any species (Reddy *et al*., 2002). Thirteen primers (Table, 8) from the fifteen succeeded to anneal with overall Encelia samples (control and four treatments) used in the present study. These primers gave a total of 311 reproducible ISSR bands. The number of amplified fragments ranged from 18 (primer S15) to 33 (primer S7) as shown in Table (8) and Figure (7). The resulted banding patterns using the primer S11 showed no polymorphism. The highest percentage of polymorphism (88.46%) was recorded using the primer S14, while the lowest percentage (19.23%) was recorded using primer S3.

Table 9: The molecular weight and total number of specific polymorphic bands in each of the Encelia treated, using thirteen ISSR primers

Primers	Molecular weight of specific bands (bp)					Total specific bands in each primer
	E0	E1	E2	E3	E4	
Pr.S1	-	-	458	-	325	2
Pr.S2	-	-	-	-	286	1
Pr.S3	-	-	-	-	415	1
Pr.S4	290	-	-	505	575	3
Pr.S5	-	-	-	-	-	-
Pr.S7	-	-	-	-	155	1
Pr.S8	-	-	-	-	-	-
Pr.S10	-	-	-	-	1079	1
Pr.S11	-	-	-	-	1097, 776, 631, 516, 328	5
Pr.S12	-	-	-	-	1075, 750, 586, 498	4
Pr.S13	-	-	-	-	968, 777, 646, 335	4
Pr.S14	-	516	687	-	50	3
Pr.S15	-	-	-	300	-	1
Total	1	1	2	2	20	26

E0 = Control;
E1= Treated samples with 1000 ppm cycocel;
E2= Treated samples with 2000ppm cycocel;
E3= Treated samples with 3000ppm cycocel and
E4= Treated samples with 4000ppm cycocel.

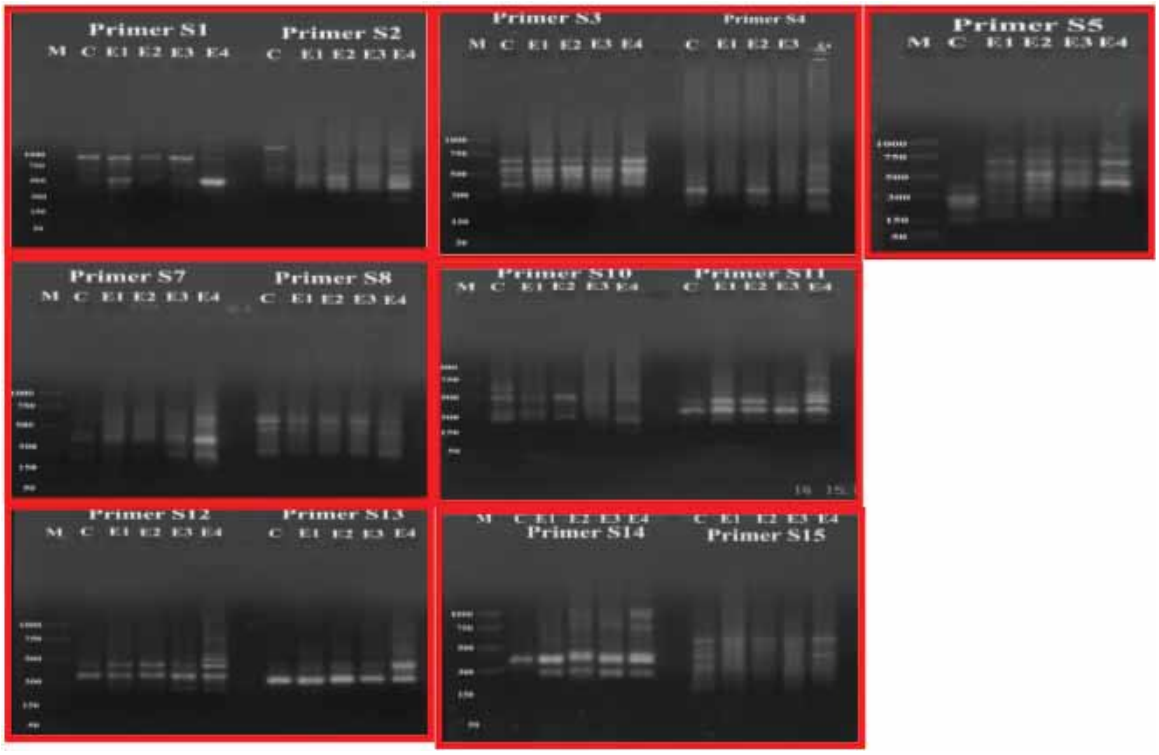

Fig. 7: Agarose gel (1%) in TBE buffer stained with ethidium bromide showing ISSR-PCR polymorphism of DNA for overall Encelia samples (C: control, E1, E2, E3 and E4 are 1000ppm, 2000ppm, 3000ppm and 4000ppm cycocel treated samples, respectively) using ISSR primers. M refers to 100bp DNA Ladder.

Resultes presented in Table (9) reveal a total of 26 specific ISSR markers for the overall Encelia samples. The control and the first treatment were discriminated by one (S4-290bp and S14-516bp, respectively) marker, while the second and third treatments were discriminated by two markers (S1-458bp, S14-687bp and S4-505bp, S15-300bp, respectively). On the other hand, the fourth treatment was characterized by twenty markers. We can concluded that, some of the specific fragments obtained in the fourth treatment (4000 ppm) could be specific markers for polymorphism and leaf blade deformaties.

The results indicated that the most visible changes occurred in the fourth treatment (4000ppm cycocel), where bands with molecular weights 608, 496, 408 and 284 bp were absent while it appeared in the other treatments and control. These modificatins may provide evidence that the telomerase activity was altered, since the Inter simple sequence repeats (ISSR-PCR) method identifies the genetic differences between repeated motives of microsatellite sequences occurring within coding regions, both centromeric and telomeric which, in the opinion of numerous authors, are highly polymorphic (Zietkiewicz et al. 1994). Telomerase is an enzyme essential for the synthesis and maintenance of telomeric DNA and the long-term stability of the genome. The plant hormone auxin increased telomerase activity in tobacco and *Arabidopsis*. Potentiation by indole-3-acetic acid of the induction of telomerase activity was dependent (Tamura *et al* 1999 and Ren *et al* 2007).

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