Effect of Chemical Mutagens (Sodium Azide and Diethyl Sulphate) on *Amaranthus caudatus* L. and *A. hypochondriacus* L. II- Induction of Variations, Cytological Aberrations, Acid Phosphatase Enzyme and RAPD Analysis

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ABSTRACT

The experiments were carried out during two successive seasons, i.e. 2001/2002 and 2002/2003, in the Flowers and Ornamental Plants Research Gardens, Faculty of Agriculture, Alexandria University, to study the effects of different concentrations of sodium azide (SA) and diethyl sulphate (DES) on the induction of variations, cytological and biological characteristics of *Amaranthus caudatus* L. and *A. hypochondriacus* L.The concentrations used were: 0 (distilled water), 1000, 2000, 3000, 4000 and 5000 ppm. Experiments were carried out in the form of a split – split plot design using three replicates and twenty four treatments in each replicate (2 species X2 mutagens X6 concentrations = 24 treatments). In both seasons, there were morphological changes in the plants according to the different concentrations from both mutagens.The changes contained leaf form, compact growth, inflorescence shape, form and colour. In pollen mother cells (PMCs) meiotic chromosome aberrations were found according to the chemical mutagens treatments. A substantial percentage of chromosome aberrations were induced in M1-generation in the two species. Three types of chromosomal aberrations were observed most frequently including univalents, bivalents and lagging dyads. Species, chemical mutagens and concentrations effects on the total soluble protein and acid phosphatase enzyme fractions of the leaves of the two week old seedlings showed significant effects. Genetic diversity and relationships detected by random amplified polymorphic DNA (RAPD) analysis of *Amaranthus caudatus* and *A. hypochondriacus* showed the relationshap between the two species and the two new mutants

Key words: Sodium azide, diethyl sulphate, A. tmaranthus, variations, RAPD

INTRODUCTION

Amaranthus is commonly called the cockscomb. The genus *Amaranthus* (Family: *Amaranthaceae)* contains about 60 species of mostly weedy plants widely distributed (Transue *et al.*, 1994). *Amaranthus* are usually planted as open -field annuals and they require no special treatment. They thrive in a hot and sunny places. They are coarse annual plants, grown for coloured foliage and showy flower-clusters (Bailey, 1941). *Amaranthus* plants are very useful as dwarf and compact varieties, which often have beautiful variegated foliage, may be grown in pots or used for bedding and for the mixed border (Bailey, 1941). Tall varieties are used for bordes and as cut flowers. *A. caudatus* Linn. (Red) "Love – lies–bleeding" is a tall plant, robust and diffuse. Leaves ovate-oblong, stalked green, spikes red, long slender in a long drooping panicle and the terminal one forming a long, cord-like tail, foliage blood-red. *A. hypochondriacus* Linn. "Prince's feather" local name is "Green thumb" which commonly has a tall glabrous leaves, oblong - lanceolate, acute usually purple or purple - green, spikes blunt, aggregated into a thick, lumpy terminal panicle, with the heavy heads variously coloured, but mostly purple - green

. The number of chemical mutagens is very great and is continuously increasing. However, for practical purposes of mutation induction in cultivated plants only few are really useful. Most of these belong to the special class of alkylating agents as ethyl methanesulphonate (EMS) $CH_3SO_2OC_2H_5$, diethyl sulphate (DES) $SO_2(OC_2H_5)_2$ and sodium azide (SA) NaN_3. Both DES and EMS act directly on guanine adding an ethyl or methyl group at carbon 7. This weakens the linkage of guanine to deoxyribose and the guanine is lost from DNA leaving a gap. Depending on which of the four bases fills in the gap transitions or transversions may eventually arise. Sodium azide is an effective mutagen under certain treatment concentrations. It is possible to obtain high mutation frequencies, most of them apparent by gene mutations with negligible frequency of chromosome aberrations. Azide in acid solutions was found to be very effective in inducing chlorophyll–deficient as well as morphological mutation (Kleinhofs, *et al.* 1974, C.F.IAEA Technical Reports Series, 1977).

Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the efficient utilization of plant genetic-resource collections. Several molecular approaches have been employed

to assess genetic diversity and relationships, but isozyme or random amplified polymorphic DNA (RAPD) data can be generated faster and with less labour than other methods, such as RFLP (restriction fragment length polymorphism) and the use of microsatellites. RAPD analysis enables the detection of informative genetic markers at a large number of loci in both coding and non-coding regions of the genome (Williams *et al.*, 1990). However, the dominant nature of RAPD markers requires that more individuals and loci be sampled compared to co-dominant markers such as RFLP and isozymes (Lynch and Milligan, 1994). Thus, for comparative studies of crops and their wild relatives in plant germplasm collections, a complementary approach using both isozymes and RAPDs may be more appropriate for generating-accurate estimates of genetic diversity and relationships than other methods.

The aim of this work was to study the effects of some concentrations of the mutagenic reagents; diethyl sulphate (DES) and sodium azide (SA) on the induction of variations, cytological aberrations, acid phosphatase enzyme and RAPD analysis in *Amaranthus caudatus* L. and *A. hypochondriacus* L.

MATERIALS AND METHODS

Two species of *Amaranthus, A. caudatus* and *A. hypochondriacus* were used in this study. The seeds were obtained from "Kieft seeds"Holland . Two kinds of mutagenic chemicals were used i.e. sodium azide (SA) and diethyl sulphate (DES) each at 0, 1000, 2000, 3000, 4000 and 5000 ppm.. Both mutagenic chemicals were obtained from Merck Co. Germany. The layout of the experiment for the M1– generation was a spilt – spilt plot design, with three replicates (Snedecor and Cochran, 1967). Every replicate contained 12 treatments for each species (2 mutagens X 6 concentrations) The main plot represented the species, while the two chemical mutagens were the sub -plot. The sub – sub plot represented the concentrations. Fifty seeds were used for each treatment in every replicate, the total number of seeds used in the experiment was 3600 divided into 24 parts (12 parts x 150 seeds for each species) and each treatment was put in a bag. Six bags for each species were soaked for one hour in distilled water at 24± 1 °C (laboratory temperature) before being treated with the different chemical concentrations. Water – soaked seeds were put in sodium azide (SA) or diethyl sulphate (DES) solutions for 5 hours on April 3, 2001 and April 1, 2002 in the first and second seasons respectively.

Chemically treated and non – treated seeds were sown on April 3rd, 2001 in the first season and on April 1st, 2002 in the second one. The seeds of each treatment were divided into three equal parts. Every part was sown in a thirty centimeters diameter clay pot filled with a mixture of equal parts of sand and clay (one pot for every replicate). The pots were placed in partial shade according to the experimental layout of the split – split plot design. On May 2nd, 2001and 2002, the seedlings were individually transplanted to 15th cm. pots using clay soil as they reached a height of about 4 cm. in *A. hypochondriacus* and 8cm. in *A. caudatus*. The pots were arranged according to the spilt- spilt plot design in 3replicates. The M1- field experiments were terminated on July 27th, 2001 and August 15th, 2002 in the first and second seasons respectively. Bulked seeds of all selected and selfed M1-plants from each mutagenic treatment were collected. On April 7th, 2002 and April 5th, 2003 respectively, seeds obtained from both M1-generations of the first and second seasons were sown. The M2–plants were individually transplanted to 15cm diameter clay pots using clay soil. The pots were arranged according to the experimental design mentioned before. The M2 – field experiments were terminated on August 3rd, 2002 and August 29th, 2003 in the first and second seasons respectively. Data were collected in both M1and M2- generations of the two successive experimental seasons. All the changes including leaf form, compact growth, inflorescence shape, form and colour were recorded.

Cytological studies

The flower buds of the two *Amaranthus* species were used to study the chromosome behavior at meiotic division. The buds in early stages of development were collected at the mid-morning hours, where the highest meiotic activity was found. A mixture of three parts of absolute alcohol and one part of glacial acetic acid, saturated with iron acetate was used for killing and fixing the buds, according to the methods of Raghuvanshi (1962) and Eid (1963). Fixation for twenty-four hours at room temperature gave good results. The fixed buds were stored in 70% alcohol and kept in refrigerator for future studies. The aceto-carmine smear technique, as described by Smith (1947) was used in these studies.

For smear preparations, the anthers were placed in a drop of the aceto-carmine solution, the contents of anthers were squeezed out, the debris were removed and the pollen mother cells (PMCs) were broken up by stirring with a needle. A cover slip was placed on the top of the drop, and the slide was heated over an alcohol burner until it was warm enough but not boiling.The slide was then pressed vigorously between several layers of paper towels taking full care not to move the cover slip. In case of dark staining, 45 percent acetic acid solution was used to dilute the stain.

The microsporocytes of the two species were examined at late diakinesis and the first metaphase stages of meiosis soon after preparation to determine the chromosome number and the frequencies of different types of chromosomal associations. The oil immersion

objective 100 x and ocular of 10x were used to study the chromosome.

Acid phosphatase enzyme

Acid phosphatase enzyme was assayed in the crude extract of 2 weeks old seedlings of *A. caudatus* and *A. hypochondriacus* as reported by Dos Prazeres *et al.* (2004).

Total soluble protein content

The total protein content was determined after Lowry *et al.* (1951) by taking 20 seedlings + 25 ml of Tris –HCl pH 7.6 from each treatment. Plant tissue (0.5 gm) + small amount of solution were placed in a mortar and crushed till no tissue appear. The mixture was centrifuged at 5000 rpm under freezing for five minutes, then transferred to keep it under freezing to be used.

DNA extraction

Frozen young leaves (500 mg) were ground to powder in a mortar with liquid nitrogen. The powder was poured into tubes containing 9.0 ml of warm (65°C) CTAB extraction buffer. The tubes were incubated at 65°C for 60-90min. 4.5ml chloroform / octanol (24:1) was added and tubes were rocked to mix 10 min., and centrifuged for 10 min. at 3200 rpm. The supernatants were pipetted off into new tubes and 6 ml isopropanol was added. After 60 min., the tubes were centrifuged for 10 min. and the pellets obtained were put in sterile Eppendorf tubes, containing 400 µl of TE buffer of a pH 8.0 (10 mM Tris HCl, pH 8.0+1.0 mM EDTA, pH 8.0). The DNA's from genotypes were, then, extracted and stored at 20°C until use (Sambrook *et al.* 1989).

PCR amplification

15 primers (Table 1), from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, England HP79 NA), were tested in this experiment to amplify the templated DNA.

Amplification reaction volumes were 25µl, each containing 1x PCR buffer with $MgCl_2$ (50 mM KCl, 10 mM Tris HCl (pH=9.0), 2 mM Mg Cl_2 and 1% trition x 100), 200µM each of dATP, dCTP, dGTP and dTTP, 50PM primer, 50mg template DNA and 1.5µ of taq polymerase. Reaction mixtures were overlaid with 15 µl mineral oil and exposed to the following conditions: 94°C for 3 min. followed by 45 cycles of 1min. at 94°C, 1min. at 36°C, 2 min. at 72°C, and a final 7 min. extension at 72°C (Sambrook *et al.* 1989).Amplification products were visualized with DNA marker on 1.6% agarose gel with 1 x TBE buffer and detected by staining with an ethidium bromide solution for 30 min. Gels were then destained in deionized water for 10min. and photographed on Polarid films under UV light.

Table 1: Primer sequences used in the study,

No. of Primer	M. W.*	Mucleotide Sequence 5`to 3`
1	3019	GAC CGC TTG T
2	3044	GGG TAA CGC C
3	2970	GTT TCG CTC C
4	3077	GAG ACG GAC C
5	3019	CAC CGC TTG T
6	3164	CCT GGG TGG A
7	3060	CCT GGG CCT C
8	2965	CCC GCC TCC G
9	3044	CCG GCC TTA C
10	3028	TTC CCC AAG C
11	2942	CAG CAC CCA C
12	2997	AGT GAG CCA C
13	3053	CGG TAA GCC G
14	2964	CAG GCC CTT C
15	3004	GGT CCC TGA C

* M.W. = Molecular weight

Data handling and cluster analysis

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. If a product was present in a genotype, it was designated "1", if absent it was designated "0" after excluding the inreproducible bands. Pair-¬wise comparisons of genotypes based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients. The similarity coefficients were then used to construct dendograms, using the unweighted pair group method with arithmetic averages (UPGMA) employing the SAHN. (Sequential, Agglomerative, Hierarchical, and Nested clustering) from the NTSYS PC (Numerical Taxonomy and Multivariate Analysis System), version 1.80 (Applied Biostatistics) Program (Rohlf, 1993).

RESULTS AND DISCUSSION

Effect of sodium azide and diethyl sulphate on the induction of variations

Effect on leaf changes

Normal leaf of *Amaranthus caudatus* L. is ovate–oblong, stalked, whereas in *A. hypochondriacus* L. is green oblong – lanceolate. All treatments caused a wide range of leaf deformities in the M1- and M2 – generations of the two seasons (Figure 1). The leaf abnormalities were found in the control plants as well as in the different treatments of the chemical mutagens. In general, the number of leaf form changes in the control was less than that of any other treatment. Most of the changed leaf patterns were found in the M2- generation.

A possible explanation of leaf variations is that the alteration in the ontegen of leaf tissues as a result of the mutagens is through the selective destruction of one or more cell layers in the shoot meristem. Other leaf changes, especially those with distorted pattern of development, may be resulted in as induced polyploidy as reported by Love (1966) on *Euphorbia pulcherrima*, L. Also increasing the mutagenic treatment increased the level of biological damage in the M1. A synergistic effect was seen for chromosome aberration as reported by Wang *et al.* (1994) on rice.

Plants with changed leaves were selfed and their seeds were sown. Leaf abnormalities seemed to be just modification, because they did not appear in the progeny, thus, they were considered to be non-genetic changes. However, these changed leaves can be attributed to the layer rearrangement or to chromosomal disturbances as a result of mutation effect (Abd El-Maksoud and El-Mahrouk, 1992).

Effect on inflorescence colour

Only two types of colour changes appeared in the inflorescences of *Amaranthus caudatus* as a result of SA or DES treatments; albino or light- red compared to the normal dark red characteristic of the species (Figure 2). In case of SA treatments, no colour changes were scored in theM1-generation, while two types of variations were found in the M2 : albino (3 plants at 1000 ppm. and 2 plants at 2000 ppm); light – red (4 plants at 1000 ppm., 3 plants at each of 3000 and 4000 ppm). The total number of colour- modified plants was 15 which make a frequency of about 3% of the total M2- population.

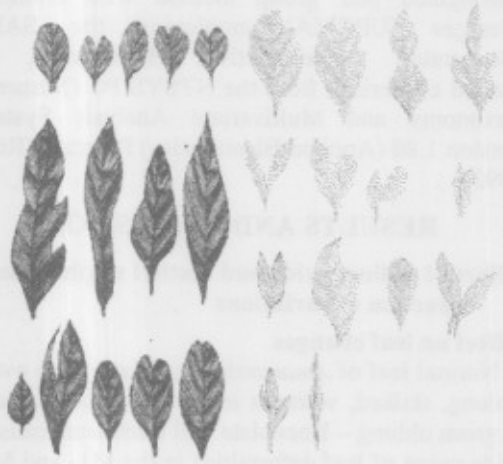


Fig. 1: Drawing showing different types of changed leaf forms of *Amaranthus caudatus* (left) and *Amaranthus hypochondiracus* (right) as induced by the different SA and DES concentrations in both M1- and M2-generations in both seasons.

As for DES treatments, eight light- red variants were found in the M1 (about 3% of the population), while two types of variations were found in the M2: albino (3 plants at 1000 ppm) ; and light – red (15 plants at 4000 ppm. and 8 plants at 5000 ppm.). The total number of colour- modified plants was then 26 which make a frequency of about 7%. This means that most of flower colour variations took place in the M2- populations, and that DES caused higher frequency of variations than SA. No colour changes were detected in *A. hypochondriacus* in all seasons.

It is frequent that SA and DES had changing effect on Inflorescence colour as reported by Drolosom and Smith (1974) on *Sorghum bicolor* using DES; Vandana (1994) on *Vicia faba* using DES; Adamaska *et al.* (1996) on Swede rape using SA; and El-Nashar (1998) on *Tagetes erecta* using SA and DES.

This change in inflorescence colour can be attributed to the effect of the chemical mutagen treatment together with the temperature and light on the development of pigments. The phenotypic expression of the genes concerned should be dependent upon the temperature, since temperature is one of the factors controlling reaction velocity. The amount of an end- product of pigments, as well as rate of formation, should be greater at higher temperature provided, the temperature coefficient of competing processes is not higher than that of the reactions leading to the end product under consideration (Al-Halawany, 1992).

Effect on inflorescence shape

Only one type of Inflorescence shape changes appeared on the inflorescences of *Amaranthus caudatus* as a result of SA or DES treatments; splitted inflorescence compared to the normal non splitting characteristic of the species (Figure 2). In case of SA treatments, this variation was found in the M1-generation: 5 plants at 2000 ppm., 3 plants at 3000 ppm. and 4 plants at 4000 ppm. The total number of plants with splitted inflorescence was 12 which make a frequency of about 3% of the total M1- population; while it was found in the M2 in the frequency of 2 plants at 1000 ppm. 4 plants at 3000 ppm. 3 plants at 4000 ppm. and 1 plant at 5000 ppm. The total number of splitted inflorescence modified plants were 10 which makes a frequency of about 2% of the total M2- population. As for DES treatments, seven splitted variants were found in the M1:2 plants at 3000 ppm., 2 plants at 4000 ppm and 3 plant at 5000 ppm. (about 3% of the population), while the variations were found in the M2: (1 plants at 3000 ppm., 1 plants at 4000 ppm. and 2 plants at 5000 ppm.). The total number of - modified plants with splitted inflorescence was 4 which makes a frequency of about 1% in M2. This means that most of splitted inflorescence took place in the M1- populations, and that SA caused higher

frequency of variations than DES.

Only one type of inflorescence shape changes appeared on the inflorescences of *Amaranthus hypochondriacus* as a result of DES treatments; the compact inflorescence compared to the normal characteristic of the species (Figure 2).

In the first season(2001/2002), no inflorescence changes were scored in the M1-generation, while the variation type was found in the M2 as 2 plants at 5000 ppm. which makes a frequency of about 1% of the total M2-population. In the second season (2002/2003), no inflorescence changes were scored in the M1-generation, while in the M2 only one variant was found at 4000 ppm.which makes a frequency of about 1% of the total M2-population.

Aberrations in inflorescence shape could be attributed to the effect of low and high concentrations of chemical mutagens on the cell number and cell length. Cell number and cell length may be altered in the inflorescence of treated plants. Large inflorescence had larger florets with an increase in cell number and / or cell size. Small inflorescence had smaller florets with a decrease in cell number and / or cell size (Bidwell, 1974).

The presence or absence of variability in the M1-generation was not an indication for predicting variability in the M2- generation. This may indicate that the M1- induced variability was not due to genetical change, but to the regulation of substances controlling growth and differentiation, by the mutagen and its hydrolysis products (Hussein *et al.* 1974).

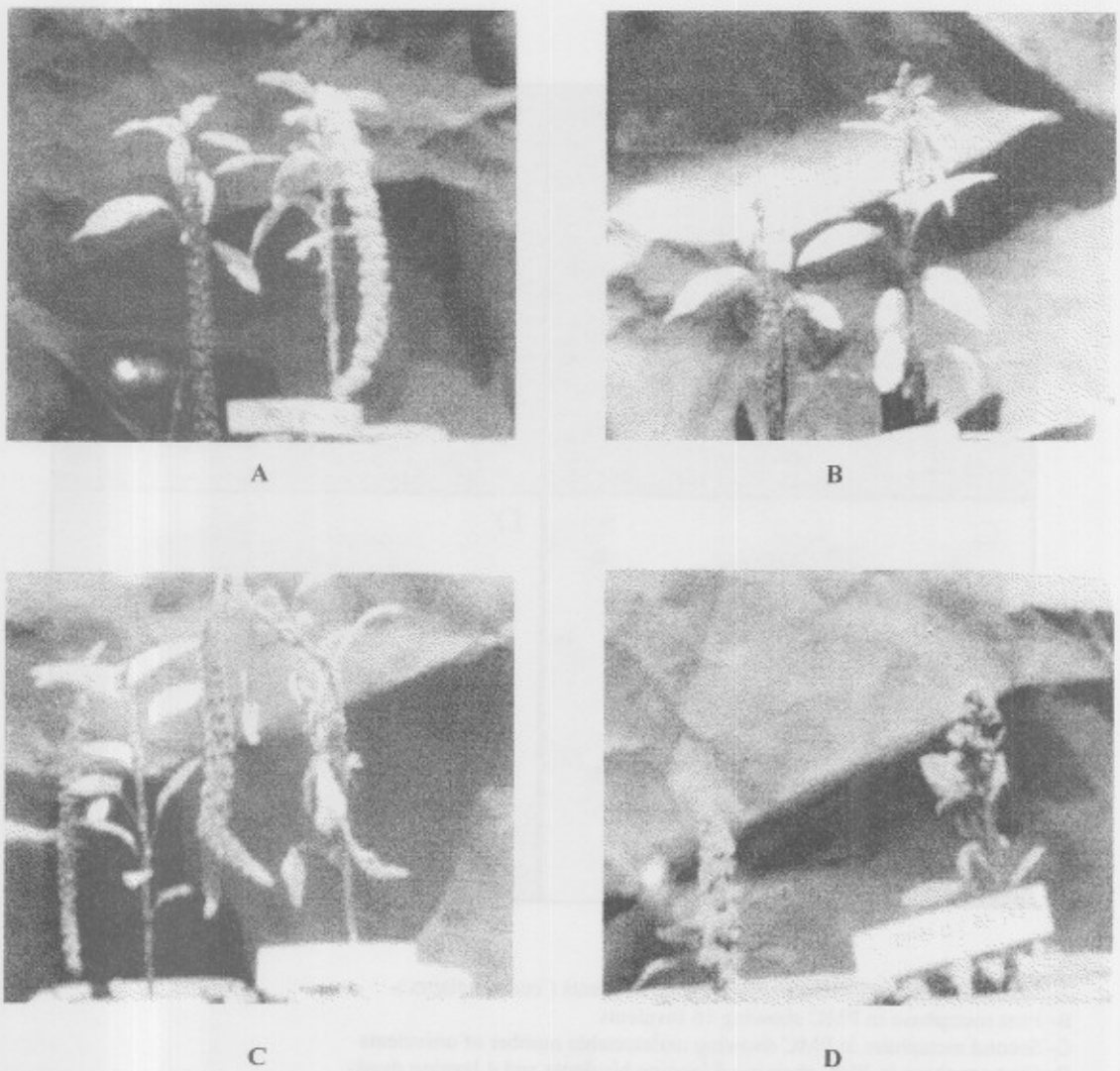


Fig. 2: Photographs showing different types of changed inflorescence of *Amaranthus caudatus* and *Amaranthus hypochondiracus L.* as induced by the different SA and DES concentrations in both M1- and M2-generations of both seasons: (A) albino inflorescence, (B) light-red inflorescence, (C) splitted inflorescence, and (D) compact inflorescence.

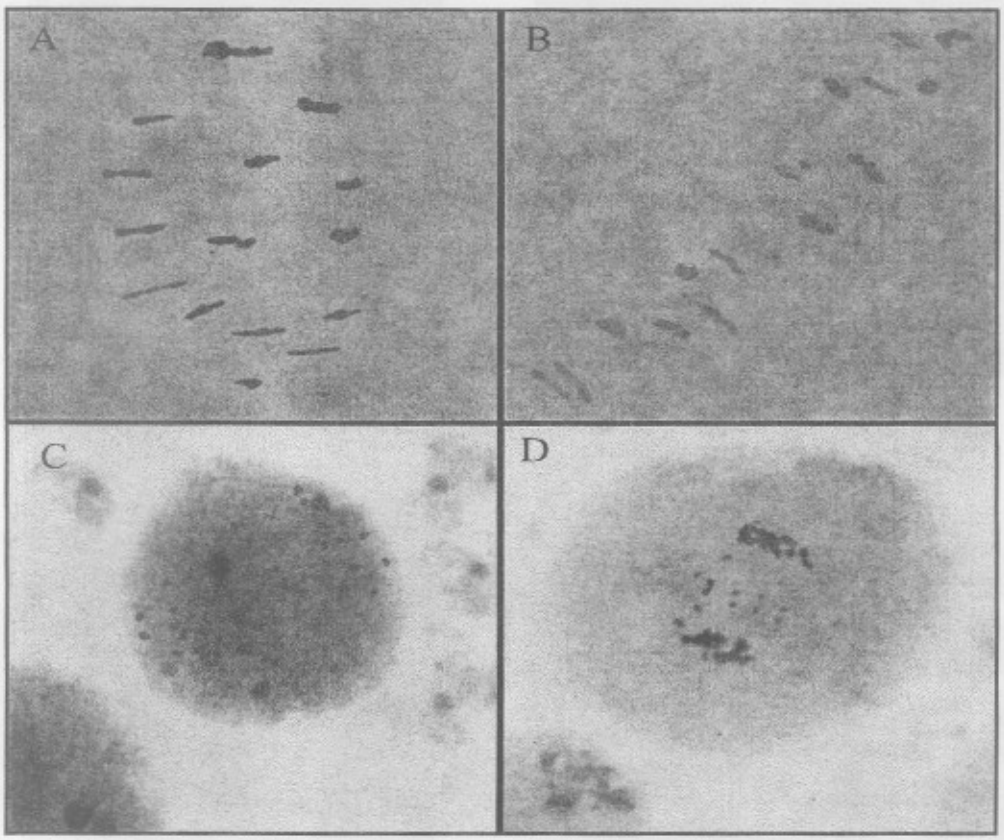
Then, the appearance of morphological variations in the M1-generation of this study (leaf form, leaf size, flower colour and form) may be attributed to the physiological damage, while the disappearance of the same characters in the M2 supports this conclusion. The appearance of other morphological aberration in the M2- was expected due to what previously mentioned by Hussein *et al.*, (1974).

Effect of sodium azide and diethyl sulphate on the cytological aberrations

Amaranthus caudatus and *A. hypochondriacus* species are diploid with 2n = 2x = 32 chromosomes. Figures 3a and b present the different types of chromosomal aberrations induced in pollen mother cells (PMCs) of *A.caudatus* and *A. hypochondriacus* in the M1-generation after chemical mutagen treatments. Six types of chromosomal aberrations were observed, most frequently including lagging bivalent and lagging dyad.

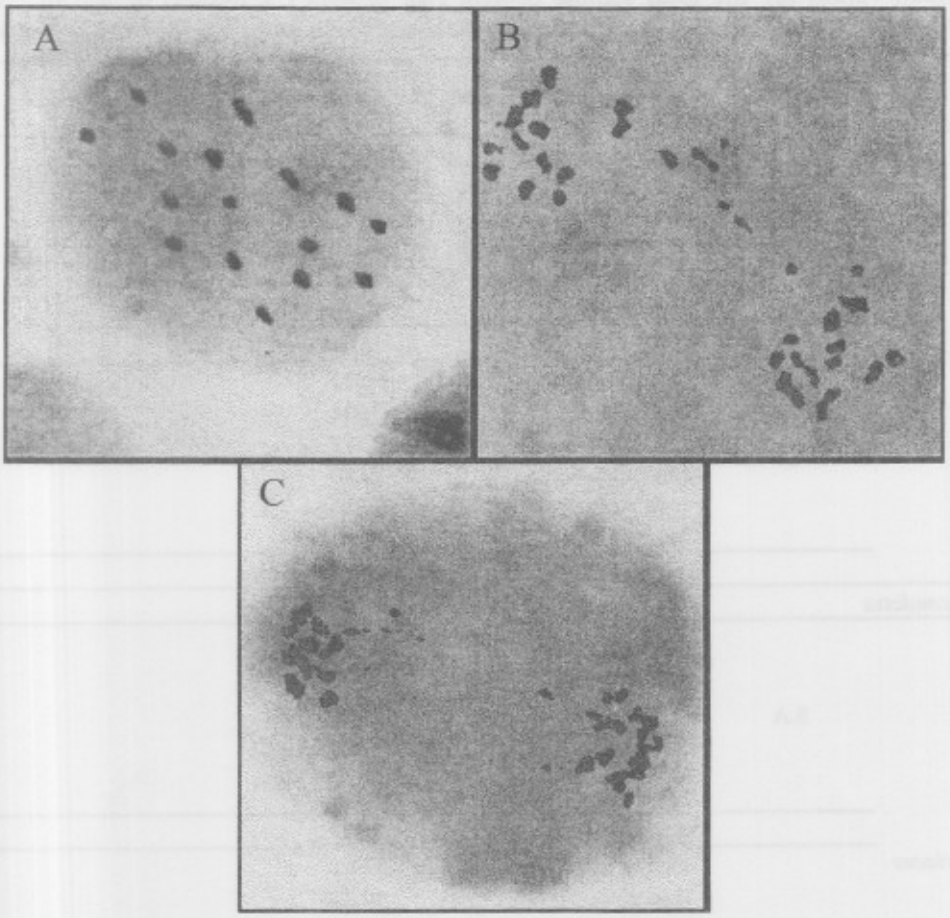
Figure 3a presents some types of chromosomal aberrations detected in *Amaranthus caudatus* such as first metaphase with 16 bivalents, second metaphase with undetectable number of univalents, 4 lagging bivalents and 4 lagging dyads. Figure 3b presents some types of chromosomal aberrations detected in *Amaranthus hypochondriacus* such as first anaphase with lagging bivalents and univalents, early telophase one with lagging univalents.

Lagging bivalents in meiosis may be caused by different factors such as secondary association (Hussein and Abobakr, 1976) or an alteration of the net charge of chromosomal proteins (Seehy, 2003).



A- Late diakinesis in PMC showing normal bivalents (control plants).
B- First metaphase in PMC showing 16 bivalents.
C- Second metaphase in PMC showing undetectable number of univalents
D- First anaphase in PMC showing 4 lagging bivalents and 4 lagging dyads.

Fig. 3.a: Meiotic chromosomal aberrations detected after treating *Amaranthus caudatus* with SA and DES,



A- Late diakinesis in PMC showing 16 normal bivalents.
B- First anaphase in PMC showing lagging bivalents and univalents.
C- Early first telophase in PMC showing lagging univalents.

Fig. 3.b: Meiotic chromosomal aberrations detected after treating *Amaranthus hypochodriacus* with SA and DES,

Effect of sodium azide and diethyl sulphate on the total soluble protein

Data presented in Table 2 showed, for A. caudatus, that the SA control and 3000 ppm. DES treatments had the lowest mean for total soluble protein (SA: 8.913 and DES: 7.840 mg protein / g tissues), while the highest means for total soluble protein were detected at the concentrations of 2000 ppm. SA (18.315 mg protein /g tissues) and 5000 ppm. DES (9.546 mg protein/g tissues). As for *A. hypochondriacus*, the 4000 ppm. treatment had the lowest means for total soluble protein when both mutagens were used (SA: 8.358 and DES: 6.828 mg protein /g tissues). The highest means were detected at the concentrations of 2000 ppm. SA (10.836 mg protein /g tissues) and 1000 ppm. DES (10.023 mg protein/g tissues). In the M2–generation, the comparison between the two species, data indicated that *A. caudatus* had higher average total soluble protein than *A. hypochondriacus*. The comparison between the two mutagens, data indicated that SA had the highest average total soluble protein. Concerning the effects of chemical mutations on the total soluble protein content, drastic changes in protein patterns were obvious at all experimental conditions used in the present study. It must be emphasized that the protein decreases may be a result of the direct physical effects of chemical mutations on molecules and increases may also reflect the repression of gene expression as suggested by Correa *et al.* (1986) on *Amaranthus spp.*; Imeri *et al.* (1987) on A. caudatus; Gorinstein, *et al.* (2001) on Amaranthus hypochondriacus. Drzewiecki *et al.* (2003) on *Amaranthus species* and Medeles *et al.* (2003) on *Amaranthus hypochondriac*

Table 2: Mean values for the total soluble protein (mg protein / g tissues) of *A. caudatus* and *A. hypochondriacus* as affected by the different applications of SA and DES for M2-Generation 2003

Species	Mutagens	Mutagen concentrations	Total soluble protein (mg protein / g tissue)
A. caudatus	S.A	control	8.913
		1ooo p.pm	12.344
		2ooo p.pm	18.315
		3ooo p.pm	12.061
		4ooo p.pm	11.357
		5ooo p.pm	9.288
		Mean of S.A	12.047
	D.E.S	control	9.033
		1ooo p.pm	7.984
		2ooo p.pm	8.573
		3ooo p.pm	7.840
		4ooo p.pm	9.233
		5ooo p.pm	9.546
		Mean of D.E.S	8.701
Mean of A. caudatus			10.374
A. hypochondriacus	S.A	control	8.933
		1ooo p.pm	9.813
		2ooo p.pm	10.836
		3ooo p.pm	9.099
		4ooo p.pm	8.358
		5ooo p.pm	9.345
		Mean of S.A	9.397
	D.E.S	control	8.968
		1ooo p.pm	10.023
		2ooo p.pm	7.726
		3ooo p.pm	9.278
		4ooo p.pm	6.828
		5ooo p.pm	8.762
		Mean of D.E.S	8.598
Mean of A. hypochondriacus			8.997
L.S.D 0.05 for Sp.			1.02
Mean of Mutagens		S.A	10.720
		D.E.S	8.649
L.S.D 0.05 for Mut.			0.48
Mean of concentrations		control	8.962
		1ooo p.pm	10.041
		2ooo p.pm	11.362
		3ooo p.pm	9.570
		4ooo p.pm	8.944
		5ooo p.pm	9.235

Effect of sodium azide and diethyl sulphate on acid phosphatase enzyme

Data presented in Table 3 showed, for *A. caudatus*, that the control SA and DES treatments had the highest means for acid phosphatase enzyme (SA: 5.854 and DES: 5.891 u/mg protein), while the lowest means were detected at the concentrations of 1000ppm. SA (0.000 u/mg protein) and 2000ppm. DES(1.962u/mgprotein). As for *A. hypochondriacus*, the 5000 ppm. SA and 2000 and 3000 ppm. DES treatments had the lowest means for acid phosphatase enzyme (SA: 1.848 and DES: 0.000 u /mg protein), while the highest means were detected at the SA control treatment (12.067 u /mg protein) and the DES control treatment (11.916 u/mg protein).

Table 3: Mean values for the acid phosphatase enzyme (u/mg protein) of *A. caudatus* and *A. hypochondriacus* as affected by the different applications of SA and DES for M2-Generation 2003.

Species	Mutagens	Mutagen concentrations	Acid phosphatase enzyme (u /mg protein)
A. caudatus	S.A	control	5.854
		1ooo ppm	0.000
		2ooo ppm	1.471
		3ooo ppm	1.631
		4ooo ppm	1.227
		5ooo ppm	0.663
		Mean of S.A	1.808
	D.E.S	control	5.891
		1ooo ppm	3.290
		2ooo ppm	1.962
		3ooo ppm	3.832
		4ooo ppm	3.654
		5ooo ppm	3.195
		Mean of D.E.S	4.471
Mean of *A. caudatus*			3.139
A. hypochondriacus	S.A	control	12.067
		1ooo ppm	4.279
		2ooo ppm	3.277
		3ooo ppm	4.471
		4ooo ppm	4.825
		5ooo ppm	1.848
		Mean of S.A	5.128
	D.E.S	control	11.916
		1ooo ppm	1.773
		2ooo ppm	0.000
		3ooo ppm	0.000
		4ooo ppm	6.188
		5ooo ppm	4.511
		Mean of D.E.S	4.065
Mean of *A. hypochondriacus*			4.596
L.S.D 0.05 for Sp.			1.37
Mean of Mutagens		S.A	3.468
		D.E.S	4.268
L.S.D 0.05 for Mut.			0.39
Mean of concentrations		control	8.932
		1ooo ppm	2.336
		2ooo ppm	1.677
		3ooo ppm	2.484
		4ooo ppm	5.224
		5ooo ppm	2.554
L.S.D 0.05 for Conc.			0.82

The comparison between the two species indicated that *A. hypochondriacus* had greater average of acid *phosphatase enzyme* than *A. caudatus*. The comparison between the two mutagens revealed that DES had higher average acid phosphatase enzyme than SA. It may be generally concluded that all treatments resulted in a general reduction of acid phosphatase compared to the corresponding controls except the 4000 ppm. of DES.

Acid phosphatase enzyme was assayed in the crude extract of 2 weeks old seedlings of *A. caudatus* and *A. hypochondriacus*. Drastic decreases in activities were detected for this enzyme. According to repression of gene expression, the present finding can be attributed to the cellular response to chemical mutagens as reported by Dos Prazeres *et al.* (2004).

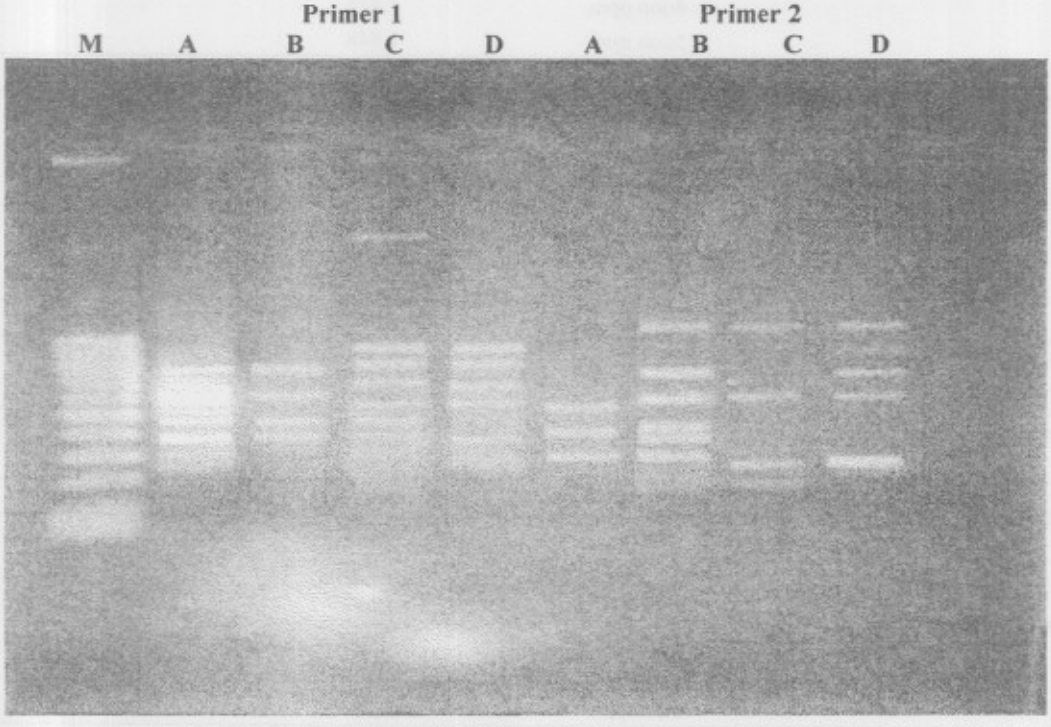
RAPD Analysis of *Amaranthus* species and their lines

Five primers were used (Table 1) to amplify the genome of two *Amaranthus* species *A. caudatus* (A) and *A. hypochondriacus* (C) and the two major variations MOMY1 (B) (1000 ppm. SA white inflorescence, Fig. 2.A) and MOMY2 (D) (4000 ppm. DES compact inflorescence, Fig. 2.D). All of these amplified fragments were polymorphic. The number of bands, amplified per primer, ranged from thirteen in (primer 1) to twenty three in (primer 4) with a mean value of 17 bands per primer (Table 4).

The five primers studied the amplified profiles of a total of 85 DNA fragment (bands) as shown in (Table 4 and Fig. 4 and 5) The comparison between the tested species showed differences in the number and size (molecular weight) of the amplified fragments produced by each *Amaranthus* species. Some bands were common among all tested species, while others were considered specific for some species.

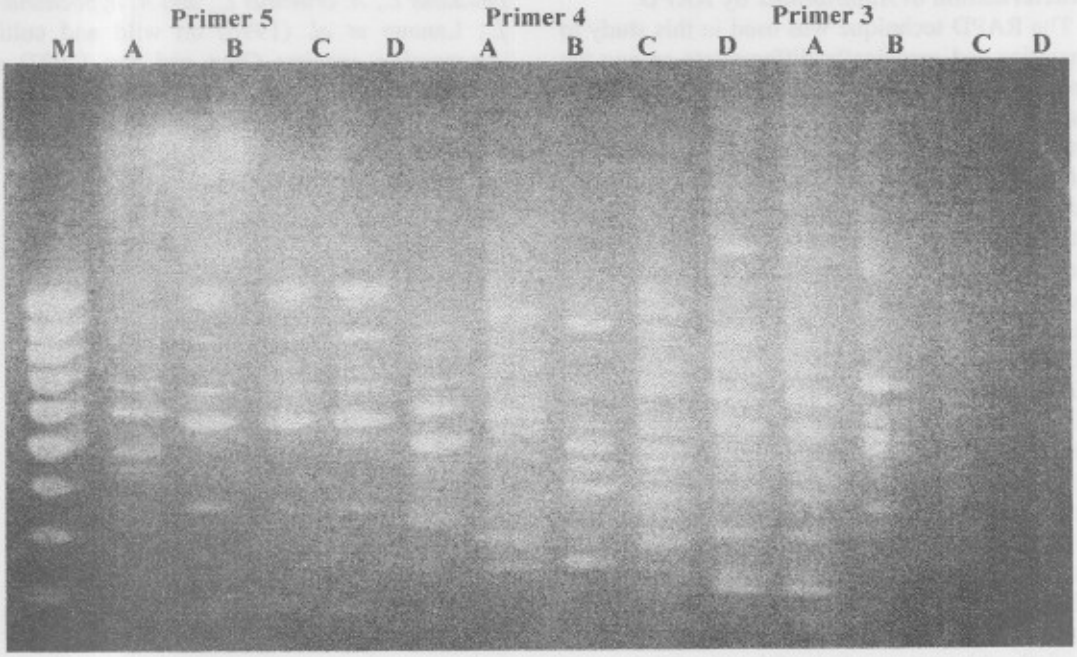
Table 4: Number of amplified bands using five primers, in *Amaranthus* species and their lines.

Primer number	Nucleotide sequence (5`– 3`)	No. of amplified products (a)	No. of polymorphic products (b)	Polymorphism b/a (%)
1	CCT GGG TGG A	13	13	100%
2	CCT GGG CCT C	14	14	100%
3	CCC GCC TCC G	17	17	100%
4	CCG GCC TTA C	23	23	100%
5	TTC CCC AAG C	18	18	100%
Total		85	85	100%



A = *Amaranthus caudatus*
B = *A. caudatus* after treating with 1000 ppm. SA (white inflorescences)
C = *Amaranthus hypochondriacus*
D = *A. hypochondriacus* after treating with 4000 ppm. DES (compact inflorescences)

Fig. 4: RAPD fragments amplified from genomic DNA of the 4 tested *Amaranthus* generated by primer 1(5`-CCT GGG TGG A-3`) and primer 2 (5`-CCT GGG CCT C-3`), M representes the DNA ladder, 1-4 represent the 4 *Amaranthus* plants.



A= *Amaranthus caudatus*
B = *A. caudatus* after treating with 1000 ppm. SA. (white inflorescences) C= *Amaranthus hypochondriacus*
D = *A. hypochondriacus* after treating with 4000 ppm. DES (compact inflorescences)

Fig. 5: RAPD fragments amplified from genomic DNA of the 4 tested Amaranthus generated by primer 3 (5`-CCC GCC TCC G-3`) primer 4 (5`-CCG GCC TTA C-3`and primer 5 (5`-CCG GCC TTA C-3`), M represents the DNA ladder, 1- 4 represent the 4 *Amaranthus* plants.

RAPD- Analysis (Cluster analysis)

One of the goals of the present study was to investigate the efficiency of RAPD markers in determining, accurately, the genetic relationship between *Amaranthus* mutants and their parents. The RAPD markers, produced by five primers, were used to construct a similarity matrix. Simple matching coefficient, ranging from 0.205 to 0.328, suggested a broad genetic base for *Amaranthus* genotypes. The genetic similarity estimates of *A. caudatus* (A) and MOMY1 (B) mutants were 0.275, *A. hypochondriacus* (C) and MOMY2 (D) mutants were 0.328. But the genetic similarity estimates of the two clusters were 0.205 based on the 85 polymorphic bands.

Before reaching the recent advances of molecular genetics, breeders have been improving both qualitative and quantitative inherited traits by conventional breeding methods based on phenotypic evaluation and selection, which are time and resource-consuming. Currently, two main types of molecular markers, biochemical markers and DNA-based markers are available for genetic studies. Listed five properties that distinguish molecular markers from morphological markers. These properties are: (1) genotypes can be determined at the whole plant, tissue or cellular level, (2) a relatively large number of naturally occurring alleles exist at many loci, (3) phenotypic neutrality; deleterious effects are not usually associated with different alleles, (4) alleles at many loci are co-dominant, thus all possible genotypes can be distinguished, and (5) few epistatic or pleiotropic effects are observed. Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the efficient utilization of plant genetics source collections. Among several molecular approaches that have been employed in assessing genetic diversity and relationships is RAPD (Williams *et al*., 1990).

Data can be generated faster and with less labour than other methods such as microsatellites and DNA fingerprints. Although polymorphism at RAPD loci is usually high, the dominant nature of RAPD markers and the low homologies between co-migrating bands from congeneric species have limited their application in population and phylogenetic studies (Lynch and Milligan, 1994). RAPD markers are highly effective in clustering intraspecific accessions of *Amaranthus*, but cannot resolve relationships among the species (Chan and Sun, 1997). These results indicated that RAPD technique can be successfully applied to species with very large genomes like wheat to obtain a proper characterization of genetic relationship.

Characterization of *Amaranthus* by RAPD

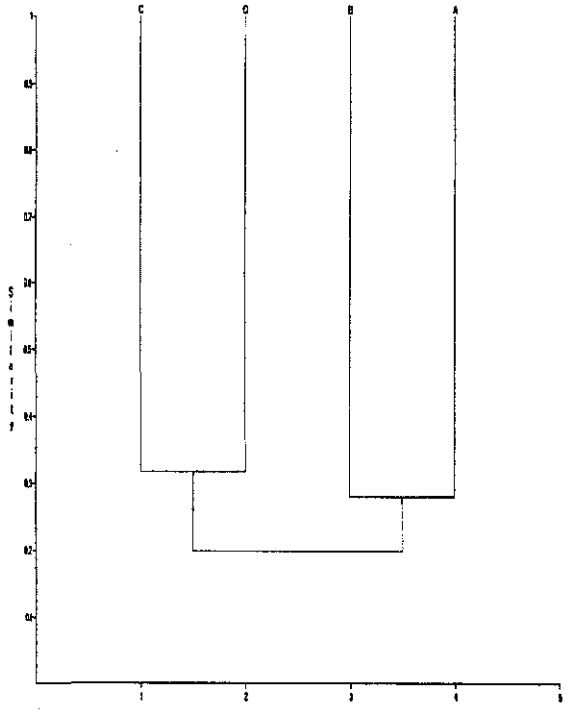
The RAPD technique was used in this study to characterize and genetically differentiate among the 4 species and mutants of *Amaranthus* under investigation. Five arbitrary oligonucleotides (primers) have been used in the PCR-based RAPD (Table 4) to randomly amplify DNA fragments that could display genetic differences among the studied isolates based on the attachment of the random primers to possible complementary sequences in the DNA extracted from the different *Amaranthus* species and mutants.

Collectively, the 5 oligonucleotides amplified 85 fragments (Table 4), high percentage of the amplified fragments showed polymorphism among *Amaranthus* species and mutants.The polymorphism is indicated in the presence or absence of a band that might be a result of various types of mutations that lead to a genetic divergence among individuals or strains (Williams *et al.* 1990).

Primers used in this study showed high variation in the number of amplified bands. Primer 4 showed the highest number of bands (23 bands), whereas primer 1 showed the lowest number of amplified fragments (13 fragments)). Other primers showed various number of bands ranging from 2 to 11. The variation in band number indicates that there are differences between the genomes of these species that can be detected indicating that these species have been genetically diverged.

The RAPD data were analyzed using the PAST program to deduce the similarity matrix and generate the dendogram among the 4 isolates under study (Table 5) which reflects the genetic relationship among the 4 species of *Amaranthus* (Figure 6). The resulted dendogram suggests that the 4 species are divided into two clusters. The dendogram reflects the degree of similarity between the different species. However, it can be observed that the same cluster includes species with different degrees of quality. Since the degrees of quality of a given species are genetically controlled, then it can be concluded that the primers used in this study were unable to differentiate between species. However, it can be observed that the same cluster includes species with different degrees of quality. Since the degrees of quality of a given species are genetically controlled, then it can be concluded that the primers used in this study were unable to differentiate between species according to their degrees of quality. This study indicated that the use of RAPD technique to detect genetic variation at the level of DNA among *Amaranthus* mutants and their parents were sensitive, powerful and can be important in the future when dealing with selection to stress condition.

Using similar techniques was also reported by other workers, i.e. Transue *et al.* (1994) on cultivated grain amaranth species: *Amaranthus caudatus L.*, *A. cruentus L.*, and *A. hypochondriacus L.*; Lanoue *et al.* (1996) on wild and cultivated *Amaranthus* species; Chan and Sun (1997) on 23 cultivated and wild *Amaranthus* species; Sun *et al.* (1999) on 24 cultivated and wild *Amaranthus* accessions using the total low-Cot DNA and five individual repetitive sequences as probes



A=*Amaranthus caudatus*
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D=*A. hypochondriacus* after treating with 4000 ppm. DES (compact inflorescences)

Fig. 6: A dendrogram constructed from similarity coefficient showing the clustering of *Amaranthus* strains using Total Lab Version (1.11)

Table 5: Simple matching coefficient of similarity matrix determined from analysis using 5different primers (RAPD) analyzed by Total Lab Program.

	A	B	C	D
A	1.000			
B	0.275	1.000		
C	0.205	0.205	1.000	
D	0.205	0.205	0.328	1.000

REFERENCES

Abd El-Maksoud, B. and E.M. El-mahrouk **1992.** Effect of ethyl methane sulfonate on the growth and interior quality of *Asparagus densiflorus* (Kunth) Jessop cv. " Sprengeri " Egypt. J. Appl. Sci. 7 (10): 116 -132.

Adamska, E; S. Jezowski; J. Olejniczak and W. Rybinski **1996.** Effect of chemical mutagens on nduced variation in winter Swede rape. Plant Breeding Abst. **66** (9) : 1326 (9656).

Al–Halwany, Iman S.M. **1992.** Effect of EMS on the growth and total alkaloid content in *Catharanthus roseus* L.G. Don. M. Sc. Thesis in Floriculture, Faculty of Agric. Alex. Univ. A.R.E.

Bailey, L.H. **1941.**The Standard Cyclopedia of Horticulture,Vol.I. The Macmillan Company, New York.

Belling, G. **1926.** The Iron-acetocarmine method of fixing chromosomes. Boil. Bull. 160- 172.

Bidwell. R. **1974.** Plant Physiology. Macmillan Publishing Co. Inc. New York.

Chan, K.F. and Sun, M. **1997.** Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus* Theor. Appl. Genet. **95**: 865-873.

Correa, A.D.; L. Jokl, and R. Carlsson **1986.** Amino acid composition of some *Amaranthus sp.* Grain proteins and of its fractions. Arch. Latinoam Nutr, **36** (3): 466- 476 .

Dos Prazeres, J.N.; C.V. Ferreira and H. Aoyama **2004.** Acid phosphatase activities during the germination of *Glycine max* seeds. Plant Physiology and Biochemistry. **42** (1): 15-20.

Drolsom, P.N. and D.C. Smith **1974.** Stability of signle – tiller progenies of *Sorghum bicolor* plants derived after seed treatment with chemical. Plant Breeding Abst. **44**(3): 140 1981

Drzewiecki, J.; E. Delgado-licon; R. Haruenkit; E. Pawelzik; O. martin-belloso; Y. Park; S. Jung; STrakhtenberg and S. Gorinstein **2003.** Identification and differences of total Protein and their soluble fractions in some pseudocereals based on electrophoretic patterns. J. Agric Food Chem. **51**, 7798 -7804.

Eid, S.E. **1963.** A safe method for making squash preparations permanent. Alex. J. Agric. Res. **X 12** :213-215.

El – Nashar, Y. **1998.** Effect of chemical mutagens on *Tagetes erecta.* M. Sc. Thesis Faculty of Agriculture, Alex. Univ. A.R.E.

Gorinstein, S.; E. Delgado-Licon,; E. Pawelzik; H. H. Permady; M. Weisz and S. Trakhtenberg **2001.** Characterization of soluble amaranth and soybean proteins based on fluorescence, hydrophobicity, electrophoresis, ammo Acid analysis, circular dichroism, and differential scanning colorimetry measurements. J. Agric. Food Chem. **49**: (11) 5595-5601.

Hussein, H. A. S.; S. H. Sallam; H. A. Kamel and T. Labib **1974.** The mutagenic effects of EMS on *Salvia splendens*. Egypt. J. Genet. Cytol. **30**: 193 – 203.

Hussein, M.M. and M.A. Abobakr **1976.** Secondary association in *Brassica oleracea* L. Egypt. J. Genet. Cytol. (1) 5: 174 - 183.

Imeri, A.; J.M. Gonzalez; R. Flores; L.G. Elias and R. Bressani. **1987.** Genetic variability and correlation of yield, grain size, chemical composition and protein quality of 25 varieties of amaranth (*Amaranthus caudatus*). Arch. Latinoam Nutr. **37** (1) :132-146.

Lanoue, K.Z.; P.G. Wolf; S. Browning and E.E. Hood **1996.** Phylogenetic analysis of restriction-site variation in wild cultivated *Amaranthus* species (Amaranthaceae). Theor. Appl. Genet. **93**: 722-732.

Love, J. E. **1966.** Some effects of fast neutron irradiation on the somatic tissues of poinsettia .Proc. Amer. Soc. Hort. Sci. **89**: 672–676: 325-333.

Lowry, O. H.; N. J. Rosebruogh; A. L. Farr and R. J. Randall **1951.** Protein measurement with the folinphenol reagent. J. of Bio. Chem. **193**: 265 – 275.

Lynch, M. and B.G. Milligan **1994.** Analysis of population genetic strucuture with RAPD markers. Mol. Ecol. **3**: 91-99.

Medeles, M. C. R.; M.B. Aguilar; R.N. Miguel; V. M. B. Garcia, E.G. Hernandez and M.S. Garcia **2003.** Amino acid sequence, biochemical characterization, and comparative modeling of a nonspecific lipid transfer protein from *Amaranthus hypochondriacus*. Archives of Bioch. Bioph. **415** (1) 24- 33.

Raghuvanshi, S.S. **1962.** Cytological studies in genus *Citrus* IL. C. assomensis. Caryologia. **15**: 143- 149.

Rohlf, F.J. **1993.** NTSYS- pc Numerical Taxonomy and Multivariate System, version 1.80. Applied Biostatistics Inc., New York.

Seehy, M.A. **2003.** Sister chromatid exchanges induced by Lannate. Bull. High. Inst. Public Health. XIX. **4**: 993 -1000

Sambrook, J.; E.F. Fritsch and J. Maniatis **1989.** Molecular Cloning a Laboratory Manual. 2nd edition. Cold spring Harbor Laboratory Press, New york.

Smith, L. 1947. The aceto-carmine smear technic. Stain. Technology. **22** : 17- 31.

Snedecor, G.W. and W.G. Cochran **1967.** Statistical Methods. Sixth Edition. Iowa State

University Press, U.S.A.

Sun, M.; H. Chen and F.C. Leung 1999. Low-cot DNA sequences for fingerprinting analysis of germplasm diversity and relationships in *Amaranthus*. Theor. and Appl. Genet. **99**: 464-472

Transue, D.K.; D.J. Fairbanks; L.R. Robisonand and W.R. Andersen **1994.** Species identification by RAPD analysis of grain Amaranth genetic resources. Crop Sci. **34**: 1385- 1389

Vandana **1994.** Studies on mutations induced by EMS and DES in faba bean II. Vital mutations affecting vegetative organs. Plant Beerding Abst. **64** (6): 842 (6147).

Wang, C.L.; M. Shen; K.N. Zhao and Q.F. Chen **1994.** Mutagenic effects of combined treatment of Cs137, X-rays and sodium azide on alfa *Oryza sativa*, L. Plant Breeding Abst. 64 (10): 1434 (10361).

Williams, J.G.K.; A.R. Kubelik; K.J. Livak; J.A. Rafalski and S.V. Tingey **1990.** DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**: 6531-6535.

الملخص العربي

تأثير المطفران الكيماويان (أزايد الصوديوم وثنائى إيثايل السلفات) على نوعين من الأمرنتس الأمرنتس *Amaranthus caudatus* L. و *A. hypochondriacus* L.

II- إحداث تغيرات شكلية وسيتولوجية وتحليل RAPD

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قسم الزهور ونباتات الزينة وتنسيق الحدائق - كلية الزراعة – جامعة الإسكندرية

أجرى هذا البحث في حدائق أبحاث الزهور ونباتات الزينة بمحطة البحوث الزراعية التابعة لكلية الزراعة جامعة الإسكندرية خلال الموسمين ٢٠٠١/ ٢٠٠٢ و٢٠٠٢/٢٠٠٣.

وقد أختير لهذه الدراسة نوعين من نبات الأمارانتس *Amaranthus* أحدهما النوع *A. caudatus*, L. والثاني *A. hypochondriacus* , L.

كان الهدف الرئيسي من البحث هو دراسة تأثير التركيزات المختلفة من مادتي الصوديوم أزايد (SA) والداى إيثيل سلفيت (DES) المطفرتان على إحداث تغييرات موفولوجية وسيتولوجية وبيولوجية تزيد من القيمة التنسيقية للنبات وكذلك إنتاج طفرات ذات صفات مرغوبة يمكن إكثارها واستخدامها كأصناف جديدة .

وقد تضمن البحث دراسة تأثير المادتين المطفرتين على كل من :

التغييرات المورفولوجية - إحداث الطفرات - السلوك السيتولوجي للكروموسومات - تقدير نسبة البروتين الكلى الذائب للنبات - تقدير نسبة إنزيم Acid phosphatase - إستخدام تكنيك RAPD للتفرقة بين النوعين وبعض الطفرات الجديدة .

وقد عوملت البذور تحت الدراسة بالتركيزات الآتيـــة من المطفران SA و DES : صـــفر (مقـــارنة) و ١٠٠٠ و ٢٠٠٠ و ٣٠٠٠ و ٤٠٠٠ و ٥٠٠٠ جـــزء في المليون ، وذلك فى ٣ / ٤ / ٢٠٠١ بالنسبة للموسم الأول و١ / ٤ / ٢٠٠٢ بالنسبة للموسم الثاني.

وكان تصميم التجربة فى صورة قطع عديدة الإنشقاق فى ثلاث مكررات ضمت كل مكررة نوعين ومطفران وستة تركيزات (٦x٢x٢ = ٢٤ معاملة). وكان عامل القطع الكبير هو نوع النبات وعامل القطع الصغير هو المطفر وعامل القطع تحت الصغير هو التركيز وخصص لكل معاملة ١٥٠ بذرة لكل نوع ومطفر مقسمة على ثلاث مكررات بمعدل ٥٠ بذرة لكل مكررة.

ويمكن تلخيص النتائج التي تم الحصول عليها فيما يلي:.

- الدراسة المورفولوجية تم الحصول على تغيرات مورفولوجية فى كلا الموسمين باستخدام التركيزات المختلفة من المطفرين SAو DES مثل (تغيرات فى شكل الورقة وتغيرات فى شكل النبات وتغيرات فى شكل ولون النورات) .
- أوضحت دراسة الشذوذات الكروموسومية في الإنقسام الميوزي للنوعين بعد المعاملة بالمطفرين الكيماويين ظهور بعض الشذوذات الكروموسومية في الجيل الطفورى الأول .
- أوضحت دراسة تأثير المعاملات بالمطفرين علي البروتينات وإنزيم Acid phosphatase enzyme فى أوراق البادرات عمر أسبوعين من النوعين بالمطفران التأثير المعنوي علي البروتينات الذائبة الكلية والإنزيم.
- إستخدام تكنيك البصمة الوراثية RAPD لبيان العلاقة والتفرقة الوراثية بين نوعى الأمارانتس *A. caudatus*, و *A. hypochondriacus* والطفرات الناتجة مثل التى حدث بها تغيير فى لون النورة من اللون الأحمر إلى اللون الأبيض فى النوع *A. caudatus*, L. أوتكور شكل النورة فى النوع *A. hypochondriacus* .