Cytogenetic and molecular variation on *Vicia faba* treated with creatine monohydrate

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Abstract: The cytogenotoxicity of creatine monohydrate conc. (1.5, 2, 2.5 and 3g/100 ml) were examined on *Vicia faba* plant, i:e: meiotic division behavior, leaf protein by using SDS-PAGE protein electrophoresis and changes in DNA of M_2 faba bean plant by using RAPD analysis. All creatine monohydrate treatments showed abnormal pollen mother cells (PMCs) which increased as the concentration and treatment period increased. The most common abnormalities were stickines, disturbed, laggard, bridges and micro-multi nuclei. The electrophoretic study of leaf proteins showed alteration of some minor protein bands after creatine treatments. The highest concentration of creatine showed a polymorphic number of genetic bands by using RAPD-PCR product comparing with control. Results strongly suggested that creatine monohydrate is clastogenic.
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1. Introduction

Creatine monohydrate has become one of the most popular sports supplements among professional and a mateur atheletes. In human body, creatine is converted to creatine phosphate which helps to fuel skeletal muscles, provides support for immediate energy production, high intensity workout. Over the past several years, numerous studies have shown that creatine supplementation may help improvement atheletic performance (Henein, 2004). On the other hand, many plant products were widely used by human for increasing activity and human capacity to physical work; improve the general body function; enhance the natural body resistance; help to restore the immune system mechanism, help to maintain activity and fitness from these plant products such as ginseng, nigella, pollen grains, garlic oil, beta carotene, selenium, and others (Henein, 2004).

However, the use of these compounds may lead to many complications and side effects. Many investigators have suggested that the study of chromosomal aberration in mitotic and meiotic division, total DNA or RNA contents, changes in storage protein banding patterns and RAPD-PCR profile changes are suitable systems to detect the potential cytological and molecular effects caused by these compounds. Chromosomal aberrations, alterations in protein banding pattern and DNA alteration by using RAPD analysis were observed in several reports such as Chen *et al., (*2000) caffeinein in *Vicia faba*, Mohamed (2000) some biofertilizers on many plants, Polit *et al.,(*2000) BAP and lAA on *Vicia faba*), Abd EL-Hamied (2001) extracts of some umbelliferrous plants on *Vicia faba*, Maslam (2004) some medicinal extracts on *Allium cepa*, Mohamed–(2004)some medicinal plant extracts *on Allium cepa*, Usciati, *et al.,* (2004) 6- benzl aminopurine on *Cicer arietinum*,Baeshin *et al.,* (2009), leaf extract of Rhaza stricta (Decne) on *Allium cepa*,Priti *et al.,* (2009)antibiotics in *Pisum sativum*, Gabriele *et al.,* (2010) paraquat in *Hordeum vulgare* and human Lymphocytes, Ganguly *et al.,(*2010) organophate and zadirctinbase, insecticides on *Lathyrus sativus L.*, Guzin *et al, (*2010) boron on wheat *(Triticum aestivum L.)* and bean (*Phaseolus vulgaris L.*), Haroun (2010) *kockia indica* extract on *Vicia faba L.)*, Korpe (2010) copper induced stress on egg plant *(Solanum melongena L*), Min et al., (2010) (Aluminum on *Vicai faba)*, Pinho *et al., (*2010)(*Baccharis trimera* (Less) DC using the *Allium cepa)*, (energy drink:bison and ovulation iducer drug :clomid on *Vicai faba*)

The aims of the present study were to detect the mutagenic effects of creatine monohydrate on *Vicia faba* by estimating meiotic aberrations, changes in leaf protein banding patterns and determine the genetic alteration using RAPD analysis.

2. Material and Methods

2.1. Materials:

2.1.1. Samples:

Plants of *Vicia faba* (Giza 40 variety) were used in this study, fresh leaves and the plants in flower stage.

2.1.2..Reaction Mixture:

Amplification of DNA was performed in 10 µl reaction mix containing 20 ng genomic DNA, 0.5 U *Taq* polymerase (Promega, USA), 200 µM of each of

dNTPs, 10 pmole random primer (Operon,Tech., Inc., USA), 10 mM Tris-HCl and 1.5 mM $MgCl_2$, pH 9.0).

2.1.3.RAPD primers :

10-mer random primers used are illustrated in table (1).

Table 1. RAPD primers and their sequences

Primers	Name	Sequence 5`- 3`
Produce variations	A 20	GTTGCGATCC
	B 12	CCTTGACGCA
	C 11	AAAGCTGCGG
	C 16	CACACTCCAG
	G 17	ACGACCGACA
Unable to produce variation	A 3	AGTGAGCCAC
	A 18	AGGTGACCGT
	B16	TTTGCCCGCA
	E18	GGACTGCAGA
	G18	GGCTCATGTG

2.2. Methods:

2.2.1. Cytogenetic analysis

At the flowering stage, plants of *Vicia faba* (Giza 40 variety) were sprayed with four concentrations of creatine monohydrate 1.50, 2, 2.50 and 3g/100 ml (dietar supplement produced by General Nutrition Corporation, pittsburgh, PA15222, USA). Control plants were sprayed with distilled water. Ten flower buds from ten different plants were gathered through duration of 24, 48h and 15 days. For meiotic studies the appropriate flower buds were collected and fixed in carnoy's solution (ethyl alcohol absolute and glacial acetic acid in the ratio 3:1) for 24 h and then transferred to 70% ethyl alcohol and kept in refrigerator. The cytological analysis was carried out by using 2% acetocarmine stain as described by Darlington and La Cour (1976). The data recorded for different treatments were statistically analyzed using t-test to determine significant differences between the treatments.

2.2.2. SDS-PAGE protein analysis

Fresh leaves were taken from *Vicia faba* plants after sprayed with four creatine concentrations and distilled water after 15 days and then decoated and milled to fine powder. Soluble water proteins were extracted over night using Tris-HCl buffer (pH 6.8) according to Laemmli (1970). Centrifugation was performed at 10,000 rpm for 10 min and 40 µl supernatant of soluble proteins were loaded in SDS-slab gel of 15% acrylamide containing 10% SDS. Gel was run at a current of 15 m A for 1 h followed by 25mA for 4-5 h. Molecular weights of different bands were calibrated using the wide range protein marker ranged from 25-230 kDa according to Matta *et al.*, (1981).

2.2.3. RAPD-PCR analysis

DNA extraction and RAPD amplification conditions

DNA was extracted from *Vicia faba* leaves sprayed with the highest creatine concentration (3g/100 ml) using CTAB method (Doyle and Doyle 1990). DNA concentration was determined by comparing with serial dilutions of Lambda DNA, electrophorsed in 0.8% agarose gel, stained in 0.2 µg/ml ethidium bromide and photographed under UV illumination. RAPD analysis was performed using UNO thermal cycler (Perkin Elmer, Germany) 10-mer random primers Table (1).

Amplification was performed in 10 µl reaction mix [containing 20 ng genomic DNA, 0.5 U *Taq* polymerase (Promega, USA), 200 µM of each of dNTPs, 10 pmole random primer (Operon,Tech., Inc., USA), 10 mM Tris-HCl and 1.5 mM $MgCl_2$, pH 9.0)]. Amplification was performed for 45 cycles, using UNO thermal cycler (Biometra, Germany) as follows: One cycle at 92°C for 3 min., 45 cycles at 92°C for 30 sec., 35°C for 60 sec. and a final extension of 10 min at 72°C. PCR products were analyzed using 2% agarose gel electrophoresis and visualized with 0.2 µg/ml ethidium bromide staining. The fragments were photographed with Gel Doc 2000 (Bio RAD). The sizes of the fragments were estimated based on a DNA ladder of Perkin Elmer (Germany).

3. Results and Discussion

3.1.Meiotic abnormalities of *Vicia faba* treated with creatine monohydrate

A wide spectrum of meiotic abnormalities was recorded in ten flower buds from different plant after different treatments with creatine monohydrate. Data in Table (2) shows that the total abnormal PMCs% was increased by the increasing of creatine concentration in the most treatments. Also, this trait was increased by increasing the period duration from 24 to 28 h in all treatments; however this trait was decreased in 15 days period duration in the most treatments as a result of recovery in this period. On the other hand, the abnormal PMCs% in the second division was lower than those recorded in the first division in the most creatine treatments as a result of recovery in this cell age. The most frequent types of abnormalities were: stickiness, disturbed, (micro-multi) nuclei, laggards, bridge and multipolar after creatine treatments.

Table 2. Numbers and percentages of abnormal PMCs in 1st & 2nd meiotic divisions and total mean of meiotic abnormalities after spraying of *Vicia faba* plants with creatine monohydrate for (24, 48 h & 15 days)

Time	Con.g/100ml	Abnormality % in 1st division			Abnormality % in 2nd division			PMCs meiotic divisions		
		dividing cell No.	Abnormal cell.	%	dividing cell No.	Abnormal cell	%	Total dividing cell No.	Abnormal cell	Mean of % abnormal PMCs±se.
Control		276	6	2.17	234	12	5.13	510	18	3.44 ± 1.07
24h.	1.5	45	8	17.78	96	28	29.17	141	36	25.26 ± 2.18**
	2	238	98	41.18	332	114	34.34	570	212	37.10 ± 2.96**
	2.5	414	222	53.62	356	176	49.44	770	398	51.5 ± 2.32**
	3	306	154	50.33	294	124	42.18	600	278	45.95 ± 2.99**
48h.	1.5	90	40	44.44	98	54	55.10	188	94	49.86 ± 0.95**
	2	290	158	54.48	328	112	34.10	618	270	43.66 ± 1.05**
	2.5	440	266	60.45	312	154	49.36	752	420	55.92 ± 3.18**
	3	286	168	58.74	344	168	48.84	630	336	53.39 ± 0.61**
15days	1.5	154	48	31.17	68	8	11.76	222	56	20.09 ± 2.18**
	2	274	118	43.07	318	118	37.11	592	236	39.82 ± 2.19**
	2.5	380	200	52.63	308	138	44.81	688	338	48.82 ± 1.76**
	3	356	188	52.81	326	190	58.28	682	378	55.58 ± 0.78**

The results in Table (3) and Figure (1) revealed that the abnormalities were present in metaphase, anaphase and telophase stages of the meiosis with all treatments. The induction of meiotic abnormalities appears to be a common effect of most chemicals (Shehata *et al*., 2008; Fisun and Goc Rasgele 2009).

The stickiness and disturbed stages were the most common abnormalities. The first type of abnormalities is the stickiness found in most phases of meiosis after different creatine treatments. (Fig. 1a, b, c, d, e, f, g and k). The number of sticky cells increased in all stages of meiotic division as the creatine concentration increased in the most treatments. Also, this trait was increased by the increasing of duration period from 24 to 48h then decreased in the 15 days period duration in most treatments, Table (3). The obtained results are in agreement with many reports, for instance (Laemmli 1970; Matta *et al*., 1981; Shehata *et al*., 2008; Fisun and Goc Rasgele 2009). They suggested that the chromosome stickiness may results from breakage and exchange between chromatin fibers on the surface of adjoining chromosomes.

The second type of abnormalities is the disturbed which observed in metaphase and anaphase in all treatments and the percentage of this trait was not depended of the creatine concentration or period duration (Fig. 1g, h, I and j). This abnormality was observed by other reports such as: Polit *et al*., 2000 (BAP and lAA on *Vicia faba*), Maslam 2004 (some medicinal extracts on *Allium cepa*), Usciati, *et al*., 2004 (6- benzlaminopurine on *Cicer arietinum)* after many chemicals treatments they suggested that the chromosomes disturbed may results from the effect of the chemical treatment on proteins constituting the spindle apparatus.

Laggard chromosomes were observed in some creatine treatments in metaphase anaphase and telophase (Fig. 1f, k, l and n). Laggard at metaphase could by attributed to the failure of the spindle apparatus to organize and function in a normal way (Atef *et al*. 2011).

Table 3.Types and Percentages of abnormalities in 1st & 2nd meiotic divisions after sparaying of *Vicia faba* plants with creatine monohydrate for (24, 48 ,hours & 15 days).

Time	Cont.	%abnormal in 1st division				%abnormal in 2nd division					
		stickiness	Disturbed	Laggards	Bridge	stickiness	Disturbed	Laggards	Bridge	Multipolar	(Mico-Multi) Nuclei
Control		__	2.17	__	__	.85	4.27	__	__	__	__
24h.	1.5	__	17.78	__	__	12.50	16.67	__	__	__	__
	2	26.05	10.13	__	__	14.46	19.88	__	__	__	__
	2.5	29.95	23.67	__	__	32.02	15.17	1.12	__	__	1.12
	3	42.48	7.84	__	__	27.21	11.56	.68	1.36	.68	.68
48h.	1.5	20.00	24.44	__	__	24.49	20.41	__	__	__	10.20
	2	28.97	20.69	3.45	1.38	17.68	15.24	__	__	__	1.21
	2.5	39.09	17.73	2.27	1.36	29.49	14.74	__	1.28	__	3.85
	3	44.06	11.19	2.10	1.40	28.49	8.72	2.33	2.33	__	6.98
15days	1.5	11.69	19.48	__	__	__	11.76	__	__	__	__
	2	25.55	16.79	__	.73	19.50	13.84	1.26	__	__	2.50
	2.5	33.16	14.74	2.11	2.63	22.08	13.64	1.95	1.30	.65	5.19
	3	30.90	16.85	3.37	1.69	33.74	13.50	2.45	1.23	__	7.36

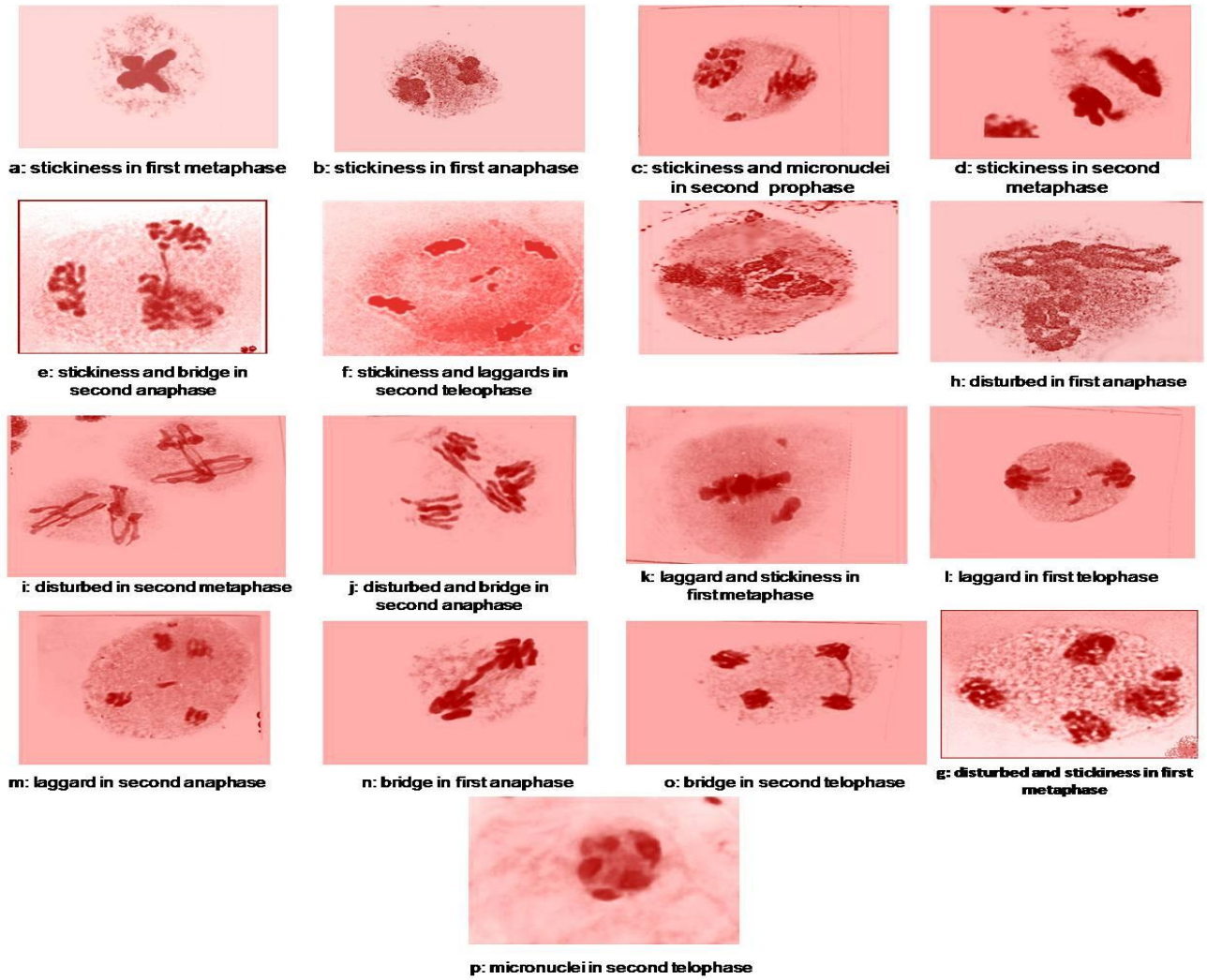

Fig 1.:Meiotic abnormalities produced after different treatments with creatine monohydrate in *Vicia faba* plant.

These laggerd may be distributed randomly to either poles at both anaphase and telophase I and II which result ultimately in aneupliody, Amer and Ali (1988). Or may they give micronuclei at telophase, Ozturk (2008). The induction of laggard chromosomes could be attributed to irregular orientation of chromosomes, Patil and Bhat (1992). In addition to previous common abnormalities, it was observed more on meiotic division including bridges, micronuclei and multinucleate. Bridges were induced in some treatments (Fig. 1e, j, m and o) and they could be due to the breakage and reunion, Asita and Makhalemele (2009) or due to the general stickness of chromosomes, Ozturk (2008). While, micronuclei and multinucleate were also recorded in some treatments in the second meiotic division (Fig. 1c, p and q) and our results are in agreement with the results of Srivastsva and Singh (2009). Finally, the inductions of these chromosomal abnormalities were pointed to the mutagenic potential of creatine monohydrate.

3.2. SDS-PAGE protein analysis

Water soluble proteins in Fig. (2) presented the mutagenic effect of different creatine concentrations of treated *Vicia faba*. All creatine concentration caused disappearance of two bands with molecular weights 60 and 90 kDa. The disappearance of some bands in soluble proteins of *V. faba* to the inherited effects of creatine, which it could be explained on the basis of mutational event at the regulatory genes that prevent or attenuate transcription (Muller and Gottschalk 1993). Induction of laggards, bridges and micronuclei by creatine treatments may lead to the loss of genetic materials. Therefore, some bands were disappeared due to the loss of their corresponding genes (Abd el salam *et al*. 1993).

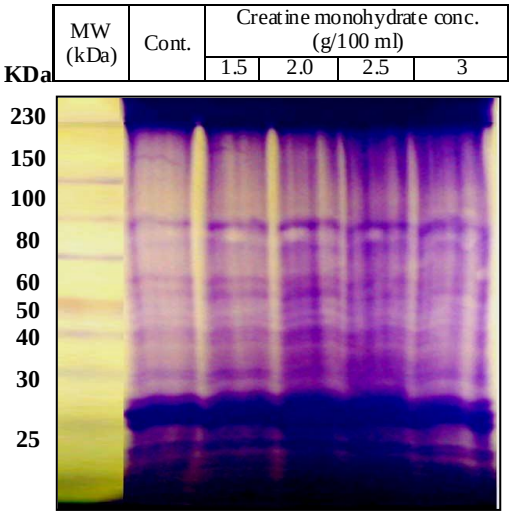

Fig. 2. SDS-PAGE banding patterns of water soluble proteins for *V .faba* plants after sprayed with four concentrations of creatine monohydrate.
(omit)

3.3. RAPD-PCR analysis

RAPD reaction was performed in M_2 treated *Vicia faba* plants with the highest creatine concentrations (3g/100ml) by using 10 primers and five primers (A20, B12, C11, C16, and G17) only exhibited variation. RAPD analysis using the five random primers showed uniquely four new fragments that disappeared in the control, while existing under creatine treatment. However, five other fragments existed in the control and disappeared under treatment (Table 4 and Fig. 3). This observation gives good evidence to the ability of creatine to induce point mutations as a result of deletion compromising at least one nucleotide as revealed by the disappearance or appearance of many genetic bands as compared with control (Rong and Yin 2004, Baeshin *et al*.2009, Korpe and Aras 2010).

In conclusion, RAPD-PCR is more sensitive technique for detecting genetic alteration than SDS-PAGE protein profiles and can be used as an investigational tool to detect the genotoxic effects of pollutions. From the obvious results, we concluded that creatine monohydrate have a cytogenotoxic effects and should be avoid taking this product

Table 4. RAPD profile using 5 primers in M^2 treated *Vicia faba* plants treated with 3 g /100 ml creatine.

Primer	Ms (bp)	Control	Creatine
A 20	2644	-	+
	562	-	+
B 12	448	+	-
	378	+	-
C 11	1012	+	-
	839	+	-
	674	-	+
C 16	264	+	-
G 17	778	-	+

+ and - = appearance and disappearance of fragments

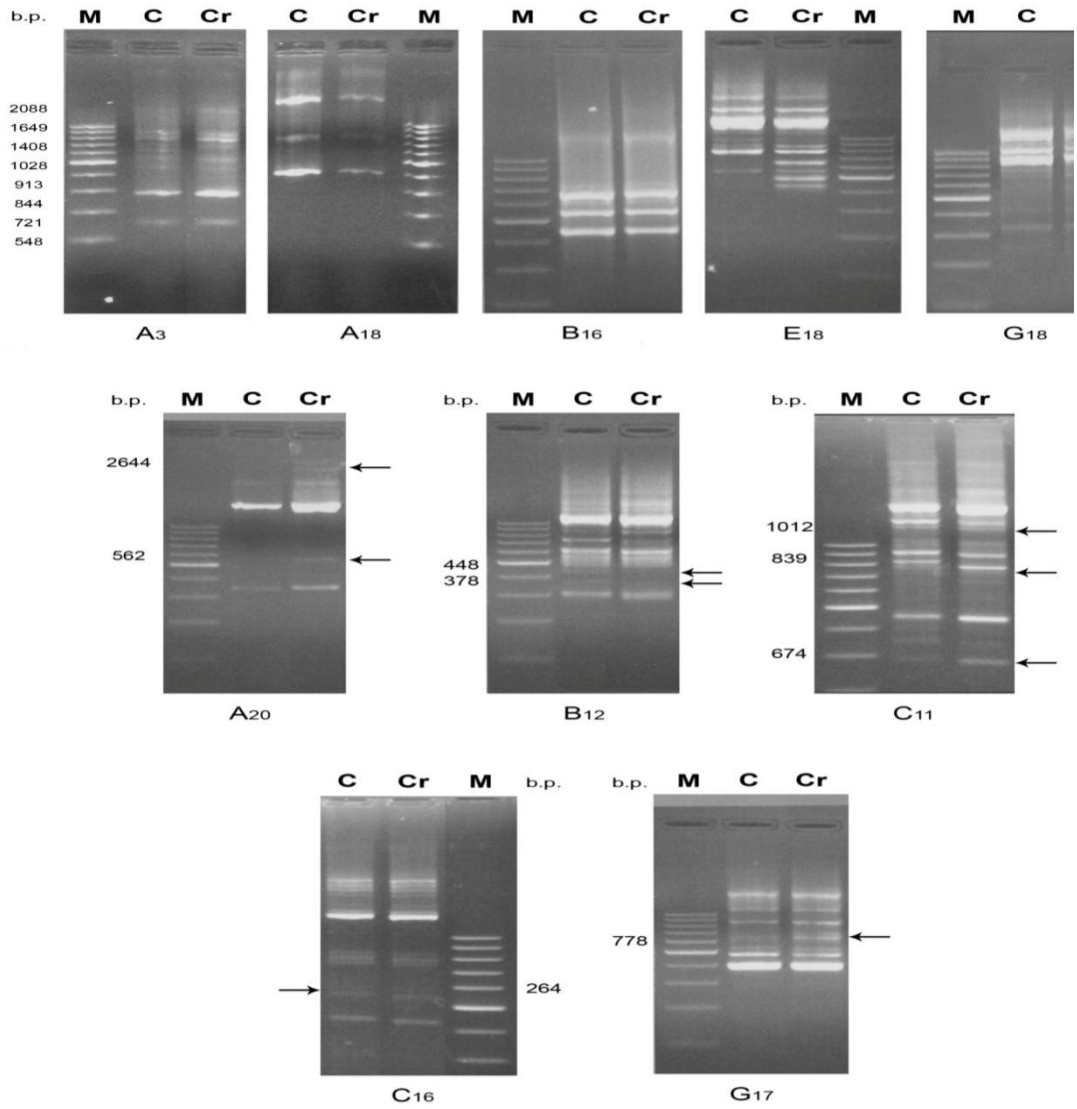

Fig.(3): RAPD profiles of genomic DNA from M_2 *Vicia faba* plants treated with 3g/100 ml creatine monohydrate by using 10 primer.

(M: DNA maker, C: control, Cr: creatine)

4.References

1. Abd EL-Hamied, N.R. (2001): Cytogenetic effects of water extracts of some umbelli – ferrous plants on *Vicia faba* M.Sc. Thesis of Cytogenetics, Fac. Of Girls, Ain Shams Univ.
2. Amer,S. M. and Ali, E.M. (1988): Cytological effects of pesticides. VXII: Effects of insecticide diclorvos in root mitosis of *Vica faba*. *Cytologia*, 51:21-25.
3. Atef, A.A.H.; Abd EL-Hamid N.R., Abd EL-Hady, E.A. and AL-Ansary, A.M. (2011): Cytogenetic effect of insecticide Tellition and Fungicide Dithane M 45 on meiotic cells and seed storage proteins of *Vicia faba*. *Journal of American Science*, 7 (1): 19-25.
4. Asita, A.O. and Makhalemele, R. (2009): Genotoxic effects of dithane, malathion and garden ripcord on onion root tip cells.AJFAND online, 9(4):1191-1209.
5. Baeshin, N.A.; Sabir, J.S.M. and Qari, S,H. (2009): Cytogenetic and molecular evaluations of genetic effects of leaf extract of *Rhaza stricta* (Decne) on *Allium cepa* root tip- meristems. *Egypt J. Genet. Cytol*., 38: 73-83.
6. Chen, Y.; Zhang, L.; Zhou, Y. and Chen, Z. (2000): Inducing somatic meiosis like reduction

at high frequency by caffeine in root tip of *Vicia faba*. *Mut. Res*., 20, 452 (1): 67-72.
7. Darlington C.D. and La Cour, E. (1976): The handling of chromomes. Sixth edition, George Allan and Unwin Lid., London.
8. Doyle, J. J. and Doyle, J. L. (1990): Isolation of DNA from fresh tissue. *Focus*, 12: 13-15
9. Fisun, K.and Goc Rasgele, P. (2009): Genotoxic effects of raxil on root tips and anthers of *Allium cepaL*. *Caryologia*, 62(1):1-9.
10. Gabriele, J.; Svetla, G.; Mila, S and Stanislava, K. (2010): Cytotoxic and genotxic effects of paraquat in *Hordeum vulgare* and human Lymphocytes in vitro. *Environ. Toxicol*. 25:294-303.
11. Ganguly, S.; Bhattacharya, S.; Mandi, S. and Tarafdar, J. (2010): Biological detection and analysis of toxicity of organophate and zadirctinbase, insecticides in *Lathyrus sativus L. Ecotox-icology* 19: 85-95.
12. Guzin, K.M.; Serdal, S. and Irem, U. (2010): Assessment of genotoxic effects of boron on wheat *(Triticum aestivum L.)* and bean (*Phaseolus vulgaris L.*) by using RAPD Analysis. *Bull. Environ. Contam. Toxicol,* 84: 759-764.
13. Haroun, S.A. (2010): Mutgeneic effects of *kockia indica* extract on *Vicia faba L. Journal of American Science*, 6 (7): 292-299.
14. Henein, W.H. (2004): Atlas 1: Everything about Drugs from A to Z. First Edition. EL-Nasr Modern Library, Cairo, Egypt.
15. Korpe, A.D. and Aras, S. (2010): Evaluation of copper induced stress on egg plant *(Solanum melongena L.)* seedlings at the moleculr and population levels by use of various biomarkers. *Mutation Research/Genetic Toxicology and Environmenal Mutagenesis*, 550 (1): 45-55.
16. Laemmli, U.K. (1970): Cleavage of structurl proteins during the assemble of the head of bacteriophage T4. *Nature*, 227:680-685.
17. Matta, N; Gatehouse, J. A. and Boulter, A. (1981): The structure of legumin of *Vicia faba L. A reapraisal. J. EXP. Bot*., 32 (126): 183-197.
18. Maslam, S.S.M. (2004): Molecular, ultra structual and cytological characteristics of *Allium cepa* meristems treated with some medicinal extracts. MS.c. (Cytology) Faculty of Girls, Ain Shams Uni., Cairo, Egypt.
19. Min, Yi; Huilan, Yi; Honghai, Li and Lihua, Wu. (2010): Aluminum induces chromosome aberrations, micronuclei, and cell cycle dysfunction in root cells of *Vicai faba*. *Environ. Toxicol*., 25: 124-129.
20. Mohamed, O.N. (2000): Mutation al and cytogentic effect of some biofertilizers. Ph.D. Thesis of cytogenetics, Fac. of Girls, Ain Shams Univ.
21. Mohamed, S.S. (2004): Molecular, ultrastructural and cytological characteristics of *Allium cepa* Meristems treated with some medicinal plant extracts. M.Sc. Thesis of cytology, Fc. of Girls, Ain Shams Uinv.
22. Ozturk,I. (2008): Effect of fungicide on meiosis of tomato (*Lycopersicon esculantum* mill). *Bangldesh J.Bot*.,37(2):121-125.
23. Patil, B.C. and Bhat, G.I. (1992): Acomparative study of MH and EMS in the induction of chromosomal aberration on lateral root meristem in *Clitoria ternatea L. Cytologia*, 57:295-226.
24. Pinho, D.; Sturbelle, R.T; Roth, M. M. and Garcias G. L. (2010): Evalution of mutagenic activity resulting from the infusion *Baccharis trimera* (Less) DC using the *Allium cepa* test and a chromosomal test for aberrations in human lymphocytes test. *Revista Breaileira de farmcognosia*, 20(2): 20-25.
25. Polit, J. T.; Maszewki, J and Kozmierezak, A. (2000): Effect of BAP and lAA on the expression of G_1, and G_2 control points and G_1-S and G_2- M transitions in root meristem cells of *Vicia faba*. *Cell Biol. Int*., 27 (7): 559-66.
26. Priti, K.; Lalit, M.T.; Tapan, K.N.; Tarun, K.B.; Lelit, S. and Bibhesh, K.S. (2009): Chromosomal abnormalities arising under the action of antibiotics in *Pisum sativum*. *Nature and Science*, 7 (3): 104-112.
27. Shehata, A.I.; Al-Ghethar, H.; Al-homaidan, A. A. and Arif, I. A. (2008): Comparative cytotoxicity of the herbicide artazine to four inbred maiz lines *(Zea maysL.)*. *Saudi Jof Bio. Sci*., 15(1):9-23.
28. Srivastava, A.K. and Singh, A.K. (2009): Effects of insecticide profenophos on, early growth, meiotic behavior and chlorophyll mutation of barley. *Acta. Physiol. Plant*, 31:537-544.
29. Usciati, M.; Codaccioni, M. And Guern, J. (2004): Early cytological and biochemical events induced by 6- benzlaminopurine application on inhibited axillary buds of *Cicer arietinum* plants. *Journal of Experimental Botany*, 23: 1009-1020.

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