Stimulation of chiasmata frequency and mitosis of *Vicia faba* L. after treatment with three PGPB lead to genetic variation assessed by ISSR.

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Abstract: Recombination process occurs during meiosis **I** of cell division results in gene diversity and stability between generations. Biological control relevance:nitrogen fixing, potassium dissolving bacteria and plant growth promoting bacteria have been reported to be much effective and economic than synthetic chemical fertilizers in stimulating plant growth. The promoting effect of biofertilizers has been extensively studied as a way to facilitate in absorbing the required essential minerals or modulating plant hormone levels or for analyzing the inhibitory effects of various pathogens on plant growth and development. However, the stimulatory effect of biofertilizers on genetic variability of cultivated crops as a consequence of meiotic recombination was not extensively studied. The present work aims to study the stimulation effect of biofertilizers on meiotic recombination in diplotene and metaphase **I** and its extended effect on the first generation. Three study groups are designed with three different biofertilizers (Biogene, Potassiomag and spore suspension of *Actinomycetes chiabensis*). Each study group includes three concentrations (X, X1, X2) and a control for each group. For every treatment, about 3700 cells were examined and analyzed by statistical methods. Chiasmata frequency were examined per 100 cells in diplotene and metaphase **I**. The mitosis of first generation were analyzed. Total and types of abnormalities in mitosis were compared by their corresponding percentage in meiotic division. Genetic variation of the seeds of the first generation were studied by ISSR-PCR. The application of biofertilizers resulted in a stimulatory effect in all meiotic and mitotic processes. The application in total protein content essential for growth was dose-dependent. The conclusion is that the use of biofertilizers is safer than chemical fertilizers although some abnormalities were induced by the former.
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Key words: Chiasmata frequency; Mitosis; ISSR-PCR.

1. Introduction

Meiosis is a specialized cell division process reproducing haploid gametes from diploid pollen mother cells. The main step in meiotic division is exchange of homologous recombination of chromosome arms generating crossing over that permit their proper alignment and segregation during the first meiotic division. Crossing over is initiated by the formation of programmed double strand breaks (DSBs) all along the genome at places called hotspots (Serrentino and Borde, 2012 and Brachet *et al*., 2012). Most DSBs occur in the loop-axis module in open chromatin regions (Panizza *et al*., 2011), where a series of meiosis-specific proteins are anchored and detected as DNAseI or MNase hypersensitive regions (Brachet *et al*., 2012). A chiasma is the technical term for the point where two chromatids are intertwined in a cell. The chiasmata are the points where two nonsister chromatids exchange genetic material during chromosomal crossover at meiosis. They become visible during the diplonema stage of meiosis, but the actual crossing over of genetic material is thought to occur during the previous pachytene stage. At the population and species levels, meiotic recombination, along with independent assortment of chromosomes, facilitates the redistribution of genetic material between generations, and increases genetic diversity among individuals in a population.

In order to increase crop production for fulfillment of malnutrition requirements, the system is relying exclusively on the use of chemical fertilizers. Whereas, it is also proved that use of chemical fertilizers and other pesticides has caused tremendous harm to the environment by pollution and contamination in water and soil (Badr, 1982 *et al*. and Badr and Ibrahim, 1987). The biofertilizers are candidate to be totally safe, they becomes wildly used because of their environmental friendly fertilizer properties. Biofertilizers are composed of organisms that help to provide nutrients and microorganisms required for the benefits of the plants. The bacteria, fungi and blue-green algae are the main sources of biofertilizers. However, it is the most advanced biotechnology that can increase the output, and contain a variety of enzymes which accelerates and improves plant growth and protect plants from pests and improve resistance to disease as well as its roll in protection from frost due to stimulating protein synthesis in the plant (Limpens and Bisseling, 2014). The stimulation of protein synthesis was reported after

using phosphorus solubilizing microorganisms in vegetable pea (Kumar *et al*., 2005; Nirmal *et al*., 2006, and Nanjappan*et al*., 2007).

The present studies has intended for the evaluation of the potential of biofertilizer to induce variations in a plant model system *Vicia faba* L. in the course of meiosis and mitosis of the offspring. The biofertilizer used are Biogene as nitrogen fixer bacteria which produce growth regulators in root absorption area, Potassiomag as potassium solubilizing bacteria is recommended for all crops and spore suspension of *Streptomysis chiabensis* as filamentous bacteria and plant growth promoting bacteria (PGPB). The aim of our study is to explore the cytological changes during the development of pollen mother cells and its direct influences on chromosomes behavior, analysis of different parameters and the potential of inducing genetic variability in the first generation.

2. Material and Methods:

Plant material: *Vicia faba* L. seeds (2n=12) var. Giza 843, provided by Agricultural Research Center, Giza, Egypt.

Biofertilizers Biogene and Potassiomag are provided by the Biofertilizers Institute, Agriculture Research Center, Giza, Egypt (the recommended doses were considered). Spore suspension (Ssp) of *Streptomyces chiabensis* was prepared according to Hewedy, *et.al*.)

Three study groups of 100 gm seeds each were treated with 3 concentrations:

First group of seeds was mixed with Biogene with different concentrations X=0.83gm, X1=1.66gm and X2=3.32 gm then sown in field and allowed to germinate. A control group was soaked in distilled water and allowed to undergo the experimental protocol.

Second group of seeds was mixed with Potassiomag: X=0.4gm, X1=0.8gm and X2=1.6 gm, then sown in field and allowed to germinate. A control group was soaked in distilled water and allowed to undergo the experimental protocol.

Third group of seeds were mixed with spore suspension (Ssp.) of *Streptomyces chiabensis* with different concentrations: X= 0.025gm, X1= 0.05gm and X2 = 0.1gm, then sown in field and allowed to germinate. A control group was soaked in saline solution and allowed to undergo the experimental protocol (0.85%).

Methods:

Meiotic study:

The flower buds with anthers in immature pollen stages were harvested 25-30 days after planting, the right size of flower buds were randomly collected (20 flower buds for each treatment), fixed in Carnoy's solution (ethanol: acetic acid 3:1 v/v) for 24 hours and stored in 70% ethanol at 4°C. The anther were then transferred to 1% Iron Aceto Carmine stain (1gm carmine in 100ml 45% acetic acid) on a slide, smeared with cover glass (after eliminating anther debris) according to Belling, 1926 and modified technique of Arzani *et al*., 2000. The numbers of normal and abnormal cells are tabulated. Types and frequency of aberrations were scored at the first and second meiotic divisions (using Olympus microscope BX 10, 40x). The most common type of abnormality was photographed.

The chiasma frequency

The chiasma frequency formed by bivalent chromosomes in PMCs of *Vicia faba* L. plants at diplotene and metaphase I stages was determined according to Singh *et al*. (1979). The total number of chiasmata in bivalent chromosomes for 100 PMCs were scored, their mean were used to represent chiasma frequency per PMCs for each dose in all treatments of the three study groups: biogene, potassiomag and Ssp. of *S. chibensis*as well as control groups.

Mitotic study

Dried seeds (5 for each treatment) representing the first generation harvested from the three study groups of Biogene, Potassiomag and Ssp. of *Streptomyces chiabensis* and their control were germinated in tap water for cytological study to access the use of the three stimulatory agents in inducing genotoxic effect. Radicals of 1.5-2cm were detached, fixed in Carnoy's fixative (ethyl alcohol: glacial acetic acid 3:1 (v/v)) for 24 hours (Carnoy, 1886), repeatedly washed in water, then Feulgen's squash technique was carried out (Colman 1938). Six temporarily slides were prepared for each treatment and control. At least 1000 cells per slide were examined under 40x magnification for mitotic analysis. The number of total cells in the mitotic division was scored and the percentage of cell division was calculated (MI), percentage of mitotic phases, percentage and type of abnormalities in each mitotic phases was tabulated. The significance between the mean results and control was determined by ANOVA (graph pad prism ver. 4).

Statistical Analysis

The results obtained were determined using the statistical analytical system (SPSS) software (Graph pad prism ver.4). One-way analysis of variance test (ANOVA) was used for comparing means of more than two independent groups. Two-way ANOVA analysis was used to compare differences between two independent groups.

Biochemical and Molecular Analysis

Estimation of total protein content.

Flower buds (0.5 g of fresh tissue) of 20-30 days are suitable to include prophase I stage, was

carried out. The tissues of the three studied groups and their control were powdered in liquid N2. Total soluble protein were extracted by 1 ml 80% ethyl alcohol, precipitated for 15 min at 4000 rpm at -5°C and dissolved in 1 ml phosphate buffer (pH 7.0). The protein extract were stained using Coommassie Brilliant Blue (G-250 Sigma) and the absorbency was recorded at 595 nm according to Bradford, (1976) method.

Estimation of Proline content

Flower buds (0.5 g of fresh tissue) were homogenized in 10ml extraction buffer then filtered. Equal volume of filtrate, ninhydrin and glacial acetic acid was activated at 100°C for 1 hour then cooled in an ice bath. The reaction mixture was extracted with 4ml toluene mixed, separated from aqueous phase and measured at 520 nm according to Bates *et al*. (1973).

ISSR for Genetic diversity:

PCR amplification was performed in a Perkin-Elmer/Gene Amp- PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 50ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mMdNTPs, 1 µM primer, 1 U Taq DNA polymerase and 30 ng template DNA.A set of ten ISSR primers with sequence (5´-3´) was used in the detection of polymorphism:

ISSR-3=$(AC)_8$YT; ISSR-4=$(Ac)_8$YG; ISSR-5=$(GT)_8$YG; ISSR-6=CGC$(GATA)_4$; ISSR-7=GAC$(GATA)_4$; ISSR-8=$(AGAC)_4$GC; ISSR-9=$(GATA)_4$GC; ISSR-10 =$(GACA)_4$AT; H-22=$(CA)_9$A; H-24=$(CA)_9$G.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized by UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

The banding patterns generated by ISSR-PCR marker were compared to determine the genetic relatedness of the first generation of *Vicia faba*. The genetic similarity coefficient (GS) between each two genotypes was estimated according to Dice coefficient (Sneath and Sokal, 1973).Dice formula measure the genetic similarity between individuals i and j: GSij = 2a/(2a+b+c). Where a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.

The cluster analysis was employed to organize the observed data into meaningful structures to develop taxonomies. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA).

3. Results:

Meiotic analysis:

Cytological analysis of meiotic division of *Vicia faba* L. flower buds treated with Biofertilizers powder and Ssp. of *Streptomyces chiabensis* revealed a high significant increase in total percentage of abnormalities induced after all treatments as compared to their respective control. The total percentage of abnormalities was decreased in the second division (Table 1).

The treated pollen mother cells (PMCs) of *Vicia faba* L. with Biogene induced a marked increase in the total percentage of abnormalities reaching (14.85**±1%) after X2 and was dose dependent (Table 1 and Fig 1). The increased percentage of abnormalities in the first meiotic division reached (14.85**±1) after X2 treatment as compared to its relative control, (Table 1 and Fig 1). The percentage of abnormalities declined in the second mitotic division reaching 8.49% after X2. Metaphase stage scored 24.76% abnormalities after treatment with X2. Ana-telophase stage had induced the highest percentage of abnormalities in the first division 31.66% after the use of X1 of Biogene, declined to 13.33% in second division (Table 2).

The pollen mother cells (PMCs) of *Viciafaba* L. treated with three concentrations of Potassiomag induced a significant increase in the total percentage of abnormalities and scored 10.5**±6.00% compared to 5.39± 4.0 to its respective control. The increased percentage of abnormalities was dose dependent (Table 1). The percentage of abnormalities in the first meiotic division reached its maximum after treatment with X2 (10.5**±6.00) as compared to its relative control, (Table 1, Fig 3). Ana-telophase stage had induced the highest percentage of abnormalities 34.88% in the first meiotic division after the use of X2 of potassiomag, while it declined to 9.88 % in second division (Table 1).

Table 1: Total percentage of abnormal PMCs and abnormal cells in 1st and 2nd divisions in *Vicia faba* L. plants after the three study groups:

1-Biogene

Treatment (Doses)	Total No of PMCs Cells	Total No of Abn. PMCS Cells	% of Abn. Cells±SD	1st Division						2nd Division					
				Abnormal Cells		Metaphase		Ana-telophase		Abnormal Cells		Metaphase		Ana-telophase	
				No of Abn. PMCS	% of Abn. ±SD	No of Cells	%	No of Cells	%	No of Abn PMCs	% of Abn.±SD	No of Cells	%	No of Cells	%
Control	3189	172	5.39±4.00	77	4.77±2	851	26.74	761	18.02	95	6.02±1	656	38.37	921	16.86
X	3552	334	9.40**±2.00	183	10.30*±3	893	24.25	882	30.53	151	8.49***±19	908	30.53	869	14.67
X1	3661	420	11.47**±1.00	237	12.68**±3	936	24.76	932	31.66	183	10.20***±7	937	30.23	856	13.33
X2	3610	515	14.26**±4.35	264	14.85**±1	821	23.49	956	27.76	251	13.69***±9	903	24.27	930	24.46

"Two way ANOVA": 1st vs. 2nd divisions: X difference: -32, ***highly significant at 0.001 level. X1 difference: -54, ***highly significant at 0.001 level. X2 difference: -13, ***highly significant at 0.001 level.

2-Potassiomage

Treatment (Doses)	Total No of PMCS Cells	Total No of Abn. PMCS Cells	% of Abn. Cells±SD	1st Division						2nd Division					
				Abnormal Cells		Metaphase		Ana-telophase		Abnormal Cells		Metaphase		Ana-telophase	
				No of Abn. PMCS	% of Abn. ±SD	No of Cells	%	No of Cells	%	No of Abn PMCs	% of Abn.±SD	No of cells	%	No of Cells	%
Control	3189	172	5.39±4.00	77	4.77±2	851	26.74	761	18.02	95	6.02±1	656	38.37	921	16.86
X	3421	200	5.84**±3.00	100	5.93**±10	886	28.00	799	22.00	100	5.76±3	874	36.00	862	14.00
X1	3379	285	8.43**±2.64	168	9.44**±1	896	29.82	883	29.12	117	7.31***±3	783	32.63	817	8.42
X2	3256	344	10.5**±6.00	193	10.41**±5	923	21.22	930	34.88	151	10.76***±8	717	34.01	686	9.88

"Two way ANOVA" for 1st vs. 2nd divisions: X difference: 0, non-significant. X1 difference: -51, ***highly significant at 0.001 level. X2 difference: -42, ***highly significant at 0.001 level.

3- Ssp. of *Streptomyces chiabensis*

Treatment (Doses)	Total No of PMCS Cells	Total No of Abn. PMCS Cells	% of Abn. Cells±SD	1st Division						2nd Division					
				Abnormal Cells		Metaphase		Ana-telophase		Abnormal Cells		Metaphase		Ana-telophase	
				No of Abn. PMCS	% of Abn. ±SD	No of Cells	%	No of Cells	%	No of Abn PMCs	% of Abn.±SD	No of Cells	%	No of Cells	%
"Control"	3429	275	8.01±3	173	9.75±7	901	36.36	873	26.54	102	6.16±9	774	25.81	881	11.27
X	3525	399	11.31**±1	226	12.40**±11	920	30.07	902	26.56	173	10.15***±4	799	27.31	904	16.04
X1	3563	509	14.28**±3	254	14.89***±8	845	26.52	860	23.37	255	13.72**±10	885	29.86	973	20.23
X2	3536	581	16.43**±0	258	15.06***±12	962	26.16	751	18.24	323	17.71**±2	919	23.92	904	31.66

"Two way ANOVA" for 1st vs. 2nd divisions: X difference: -53, ***highly significant. X1 difference: 1, ***highly significant at 0.001 level. X2 difference: 65, ***highly significant at 0.001 level.

The Ssp. induced a significant increase in the total percentage of abnormalities in PMCs of *Vicia faba* L. treated with three concentrations and scored 10.5**±6.00% compared to (8.01±3) its respective control. The increased percentage of abnormalities was dose dependent (Table 1). The percentage of abnormalities in the first meiotic division reached its maximum after treatment with X2 (16.43**±00) as compared to its relative control, (Table 1, Fig 5). Ana-telophase stage had induced the highest percentage of abnormalities 34.88% in the first meiotic division after the use of X2 of Potassiomag, while it declined to 16.04 % in second division after the dose X (Table 1).

Statistical analysis "F-test" revealed that the total percentage of abnormalities were highly significant after all treatments with the two biofertilizers as well as SSp of *Streptomyces chiabensis* on *Vicia faba*L. seeds.

Spindle disturbance was the most dominant abnormality observed in all phases at the first and second division except after the treatment of X2 of Biogene. The highest value 69.5% was recorded aftertreatment with X Potassiomag. Stickiness was the second dominant abnormality after all treatments in the different phases of the first and second division. The highest value was 54.75% after treatment with X2 Biogene. Multinucleate was recorded in low percentage after treatment with Biogene (6.99%) and SSp of *S. chiabensis*(1.89%) as dictated in Table 2 and pictured in Fig. 1.

Chiasma frequency:

The present study evaluates the chiasmata frequency in diplotene and metaphase I. The mean of chiasma frequency per bivalent in diplotene as well as metaphase I stage was found to be dose dependent The highest value of chiasma was recorded after the use of X1 Biogene reaching 3.83**±0.20/ bivalent and 23.00**±0.70/cell; as well as Potassiomag reaching 4.23**±0.25/bivalent and 25.38**±1.09/cell after treatment with X2 as compared to its respective control, (Table 3). The mean of chiasma frequency per bivalent as well as per cell pretreated with Ssp. did not declare any differences over its respective control. The stastistical analysis F-test clarified that all values of chiasma frequency affected by all doses of Biogene and Potassiomag were highly significant at 0.01 levels as compared to their respective control. However, all data had no significant values neither at 0.05 nor at 0.01 levels when F-test was applied to evaluate the given results of Ssp. of except after using 0.1gm in diplotene stage as can be seen in Table 3.

Mitotic analysis:

Mitosis of the first generation: Assessment of genotoxicity:

A comprehensive increase in mitotic indices of *Vicia faba* L. roots of the first generation seeds harvested from the pretreated seeds with the two biofertilizers Biogene, Potassiomag and SSp. of *S. chiabensis* was detected as compared to their respective control. The successive increase in mitotic division was dose dependent. The maximum values of mitotic indices were 10.15**±6.80, 11.06**±14.57 and 11.48**± 8.52 after pretreatment with the dose X2 of Biogene, Potassiomag and Ssp. respectively. The increase was highly significant at 0.01 level (Table 4). The accumulation in prophase stage was on the response of other stages in mitotic division of the three study groups.

Percentage of total abnormalities in the dividing cells of the first generation revealed a significant increase at 0.01 level as compared to its respective control, Table 4 and Fig 2. The highest percentage of abnormalities was at prophase stage recording 40.45, 45.32 and 42.51% in mitosis of the three study groups with Biogene, Potassiomag and Ssp. Percentage of abnormalities had decreased gradually during phases in mitosis of all study groups and recorded the minimum value 0.60% at telophase of the first generation pretreated with X1 Ssp. Fig.1. The induction of spindle disturbance and light stickiness were the common type of mitotic abnormalities. Micronucleus was rarely found in the mitotic division of the first generation, (Table 4 and Fig. 2).

Biochemical and molecular analysis:

Determination of total soluble protein:

An obvious increase in protein content after all treatments of the three study groups has been detected compared to their respective control in a dose dependent manner. The maximum value recorded was (32.00**±1.00), (33.60**±0.10) and (28.80**±1.20) mg/gm fresh weight after treatment with X2 of the biofertilizers Biogene, Potassiomag and Ssp. respectively. The statistical analysis indicates that all the results of protein occurred after all doses were highly significant at 0.01 levels as compared to their respective control.

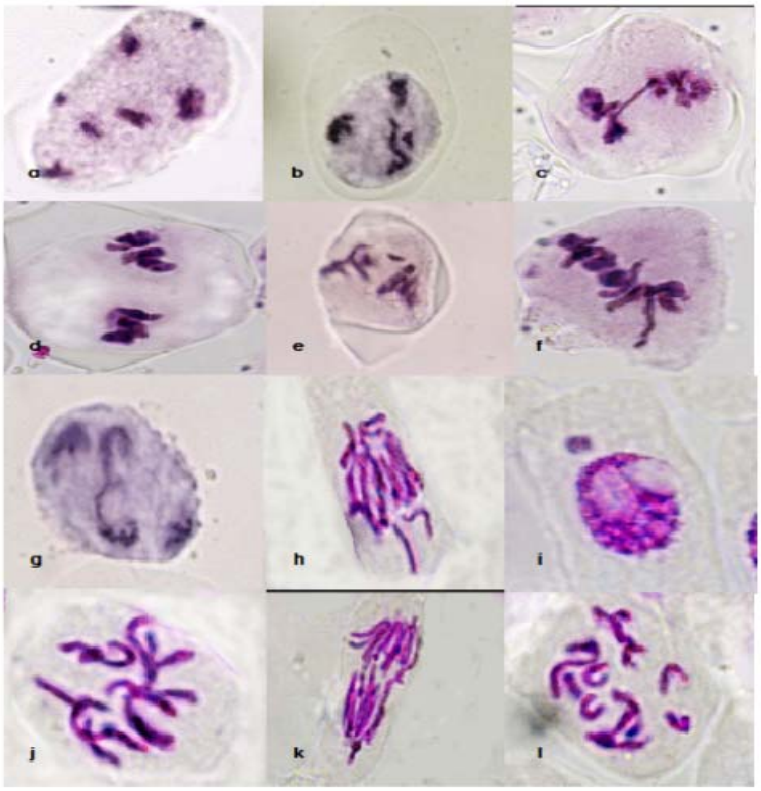
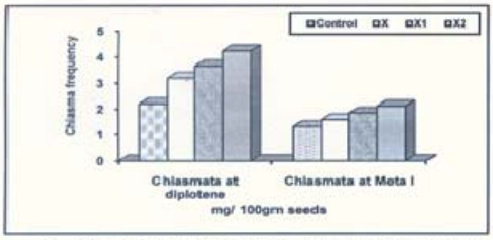


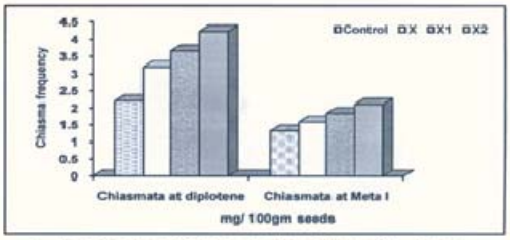
Fig.1: Types of abnormalities induced by the three used biofertilizers: (a - g: phases in meiotic division): a- Multinucleated cells after treatment with Biogene; b- Disturbed and sticky anaphase II after treatment with Potassiomage; c- Sticky anaphase I with single bridge; d- Sticky metaphase II with split spindle; e- Sticky metaphase II with fragment; f- Stiky metaphase II; g- Sticky anaphase II with bridge and break; (h - l phases in mitotic division): h: Disturbed anaphase; i- Micronucleus induced by Ssp.; j- Disturbed metaphase; k- sticky anaphase with bridge; l- C- metaphase.

Table 2: Types and percentage of abnormalities in PMCs after treatment with the three biofertilizers.

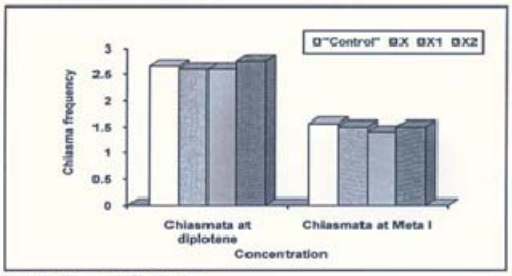
TREATMENT (CONC)	TOTAL NO OF PMCS	NO OF ABN. PMCS	%OF ABN. PMCS	Stickiness		Disturbance		Bridge		Laggards		Fragment		Multinucleate	
				No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells
1. Biogene.															
X	3552	334	9.40	135	40.41	159	47.60	16	4.79	19	5.68	1	0.29	4	1.19
X1	3661	420	11.47	191	45.47	211	50.23	13	3.09	5	1.19	0	0	0	0
X2	3610	515	14.26	282	54.75	147	28.54	19	3.68	31	6.01	0	0	36	6.99
2. Potassiomag.															
X	3421	200	5.84	61	30.50	139	69.50	0	0	0	0	-	-	-	-
X1	3379	285	8.43	96	33.68	168	58.94	14	4.91	7	2.45	-	-	-	-
X2	3256	344	10.50	131	38.08	184	53.48	20	5.81	10	2.90	-	-	-	-
3. Ssp. *Streptomyces chiabensis.*															
X	3525	399	11.31	159	39.84	215	53.88	21	5.26	4	1.00	-	-	0	0
X1	3563	509	14.28	146	28.68	315	61.88	48	9.43	0	0	-	-	0	0
X2	3536	581	16.43	221	38.03	306	52.66	25	4.30	18	3.09	-	-	11	1.89
Doses: Biogene: X: 0.83gm; X1: 1.66gm; X2: 3.32gm; Potassiomag: X: 0.4gm; X1: 0.8gm; X2: 1.6gm; Ssp.: X: 0.025g; X1:0.05gm; X2: 0.1gm															



a- Biogene: X= 0.83gm; X1= 1.66gm; X2= 3.32gm



b- Potassiomag: X= 0.4gm; X1= 0.8gm; X2= 1.6gm



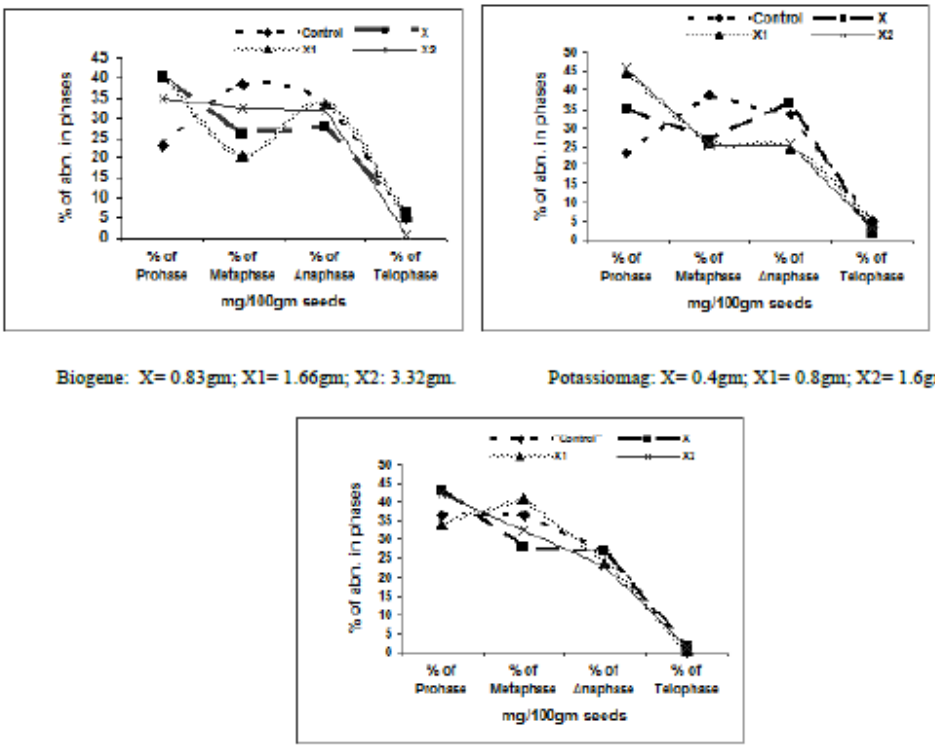
c-Ssp.: X= 0.025g; X1= 0.05gm; X2= 0.1gm.

Figure 2: Bar chart of Chiasma frequency at diplotene and metaphase I per bivalent in *Vicia faba* PMCs after the three study groups. ((F-test).*Significant compared to control at 0.05 level; **Highly significant at 0.01 level).

Table 3: Mitotic index and percentage of mitotic abnormalities in *Vicia faba*L. root tip cells of the first generation pretreated with the three biofertilizers

Treatment	Biogene				Potassiomag				Ssp. *Streptomyces chiabensis*			
	Total No. of cells	Mean of dividing cells	Mitotic index ± SD	% of Total abnormalities	Total No. of cells	Mean of dividing cells	Mitotic index ± SD	% of Total abnormalities	Total No. of cells	Mean of dividing cells	Mitotic index ± SD	% of Total abnormalities
Control	5050	66.6	6.59 ±9.58	11.71 ±1.92	5050	66.6	6.59 ±9.58	11.71 ±1.92	--	--	--	--
"Control"	--	--	--	--	--	--	--	--	5175	89.8	8.67 ±10.50	11.58 ±3.57
X	5040	88.8	8.80* ±6.64	25.22** ±3.50	5000	88	8.80* ±7.51	27.27** ±3.74	5025	91.8	9.13 ±8.46	29.60** ±5.63
X1	5040	96.4	9.56** ±12.60	27.17** ±3.63	5100	100.8	9.88** ±11.65	32.14** ±6.46	5040	103.2	10.23 ±14.86	32.17** ±5.26
X2	5090	103.4	10.15** ±6.80	25.53** ±2.60	5000	110.6	11.06** ±14.57	36.52** ±10.06	5000	114.8	11.48** ±8.52	28.74** ±1.14

*Significant from control at 0.05 level (F-test). **Highly significant from control at 0.01 level (F-test).
Doses of: Biogene: X= 0.83gm; X1= 1.66gm; X2: 3.32gm; Potassiomag: X= 0.4gm; X1= 0.8gm; X2= 1.6gm;
SSp. of *S. chiabensis.*: X= 0.025g; X1= 0.05gm; X2= 0.1gm.



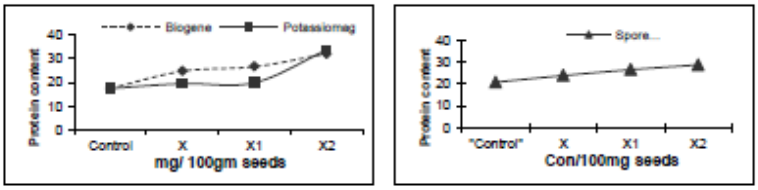
Biogene: X= 0.83gm; X1= 1.66gm; X2: 3.32gm. Potassiomag: X= 0.4gm; X1= 0.8gm; X2= 1.6gm.

Ssp.: X= 0.025g; X1= 0.05gm; X2= 0.1gm.

Fig. 3: Frequency and percentage of abnormalities of mitotic phases in *Vicia faba* L. root tips of the first generation of the three study groups.

Table 4: Percentage and types of abnormalities in *Vicia faba* L. root tips of the First generation pretreated with the three study groups:

TREATMENT (CONC)	TOTAL NO OF PMCS	NO OF ABN. PMCS	%OF ABN. PMCS	% OF DIFFERENT TYPES OF ABNORMALITIES											
				Stickiness		Disturbance		Bridge		Laggards		Fragment		Multinucleate	
				No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells	No of abn. cells	% of abn. cells
1. Biogene. X= 0.83gm; X1= 1.66gm; X2: 3.32gm															
X	3552	334	9.40	135	40.41	159	47.60	16	4.79	19	5.68	1	0.29	4	1.19
X1	3661	420	11.47	191	45.47	211	50.23	13	3.09	5	1.19	0	0	0	0
X2	3610	515	14.26	282	54.75	147	28.54	19	3.68	31	6.01	0	0	36	6.99
2. Potassiomag. X= 0.4gm; X1= 0.8gm; X2= 1.6gm															
X	3421	200	5.84	61	30.50	139	69.50	0	0	0	0	-	-	-	-
X1	3379	285	8.43	96	33.68	168	58.94	14	4.91	7	2.45	-	-	-	-
X2	3256	344	10.50	131	38.08	184	53.48	20	5.81	10	2.90	-	-	-	-
3. Ssp. *Streptomyces chiabensis.* X= 0.025g; X1= 0.05gm; X2= 0.1gm															
X	3525	399	11.31	159	39.84	215	53.88	21	5.26	4	1.00	-	-	0	0
X1	3563	509	14.28	146	28.68	315	61.88	48	9.43	0	0	-	-	0	0
X2	3536	581	16.43	221	38.03	306	52.66	25	4.30	18	3.09	-	-	11	1.89



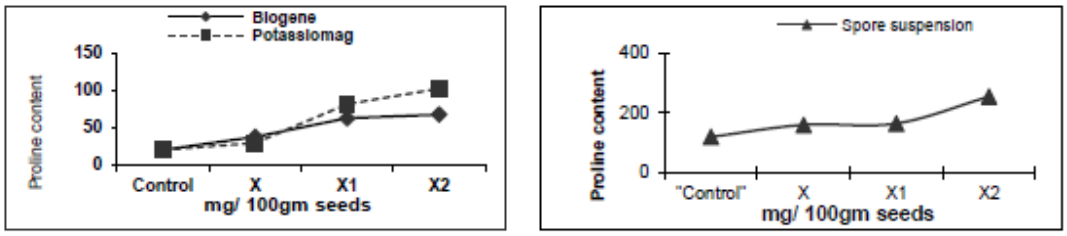
**Highly significant compared to control at 0.01 level (F-test).

Doses of: Biogene: X= 0.83gm; X1= 1.66gm; X2=**3**.32gm.; Potassiomag: X= 0.4gm; X1= 0.8gm; X2= 1.6gm.; Ssp.: X= 0.025g; X1= 0.05gm; X2= 0.1gm.; "Control"- saline solution= 0.85%

Fig. 4: Protein content in flower buds of *Vicia faba* L. µg/g fresh weight after treatments with Biogene, Potassiomag and Ssp. of *Streptomyces chiabensis.*

Determination of proline contents: A successive increase in proline content after all treatments of the three study groups has been detected compared to their respective control in a dose dependent manner. The maximum value recorded was 67.78**±0.2, 102.68**±2.68 and 254.36**±0.64 mg/gm fresh weight after treatment with X2 of the biofertilizers Biogene and Potassiomag and Ssp. respectively (Fig. 4).

The statistical analysis indicates that all the results of protein occurred after all doses were highly significant at 0.01 levels as compared to their respective control.



**Highly significant compared to control at 0.01 level (F-test).
Doses of: Biogene: X= 0.83gm; X1= 1.66gm; X2= 3.32gm; Potassiomag: X=0.4gm; X1= 0.8gm; X2= 1.6gm.; Ssp.: X= 0.025g; X1= 0.05gm; X2= 0.1gm. "Control" - saline solution (0.85%).

Figure 5: Proline content in flower buds of *Viciafaba* L. µg/g fresh weight treated with Biogene, Potassiomag and Ssp. of *Streptomyces chiabensis.*

Genetic diversity of the First generation seeds: ISSR analysis.

The diversity of the first generation of the three study groups was studied using ten ISSR primers to the germinated seeds (Table 5). Out of 279 fragments generated, 219 were found to be polymorphic (78.5% polymorphism). A total of 50 amplified fragments were considered as unique (specific) bands (17.92%). The primer ISSR-9 generates the highest number of fragments 42, while primer H-24generates the lowest number of fragments 10. The primer H-24 induce 4 specific bands (40%), while ISSR-3 exerts the minimum number of unique bands (3.7%).

The cluster analysis was carried out to represent the genetic similarity in a graphic dendogram of the three representatives of the first generation compared to their respective control, Fig. 6 . The UPGMA analysis based on ISSR character difference was carried out to the first generation of the three experimental groups and the control. The seeds of the first generation pretreated with Biogene and Ssp. splits in the same group (at 0.5 num); while the generation from untreated group splits off at 5num. The generation pretreated with Potassiomag splits at 25 num.

Table 5: Selected primers and genetic variations among the First generation of the pretreated *Vicia faba* seeds with X2 biofertilizers: Biogene, Potassiomage and Ssp. of *Streptomyces chiabensis* in the ISSR analysis.

Primer	Sequence (5'-3')	Size of amplification products bp.	Total number of bands	Unique (specific) bands	Polymorphic bands	Species Specific percentage for primers
ISSR-3	$(AC)_8YT$	1200 -300	27	1	22	3.70
ISSR-4	$(AC)_8YG$	1200 - 220	32	6	27	18.75
ISSR-5	$(GT)_8YG$	650- 160	26	2	21	7.69
ISSR-6	$(GATA)_4CGC$	1600 - 210	39	9	28	23.٠٨
ISSR-7	$(ATA)_4GAC$	1600 - 250	22	4	21	18.18
ISSR-8	$(AGAC)_4GC$	950 - 170	32	8	29	25.00
ISSR-9	$(GATA)_4GC$	2000 - 300	42	8	39	19.٠٥
ISSR-10	$(GACA)_4AT$	1100 - 200	26	4	23	15.38
H-22	$(CA)_9A$	800 - 200	23	4	21	17.39
H-24	$(CA)_9G$	520 - 200	10	4	9	40.00
Total number of bands		279	50	219	50	٢١٩
			27.9			
	Mean	21.95		٢١.9		
	Percentage		17.92	78.5	17.92	

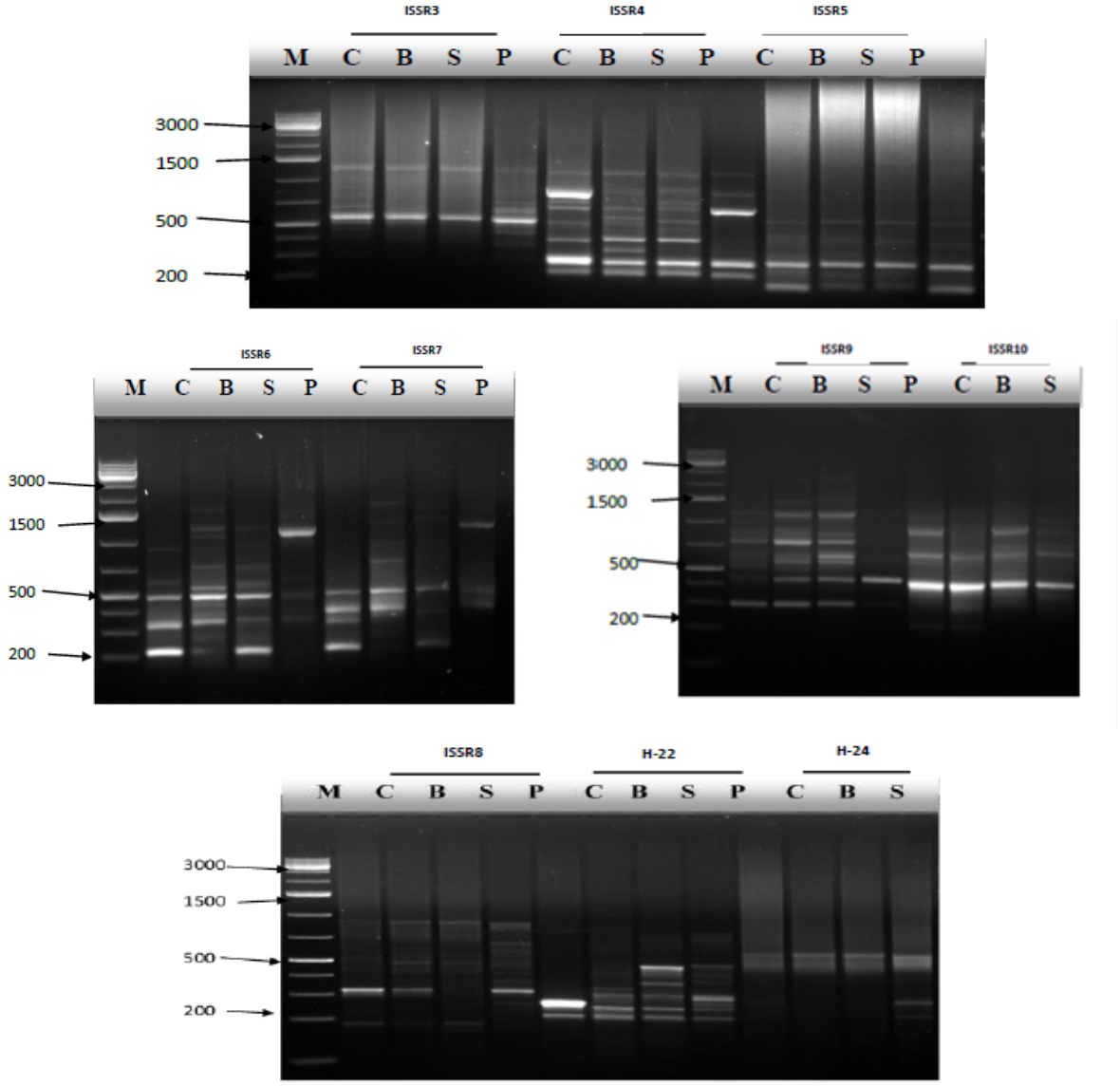


Fig.6: ISSR banding pattern produced by each of the ten primers: Lane M: DNA ladder (300bp); Lane C: control; Lane B: Biogene; Lane S: Ssp. of *S. chiabensis*; Lane P: Potassiomag.

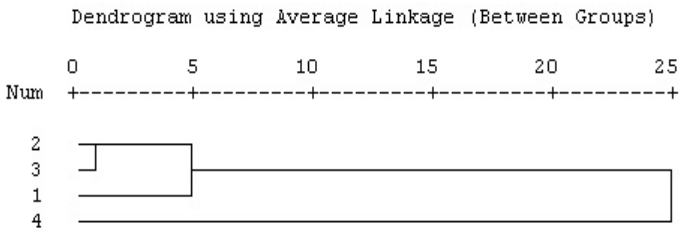


Fig. 7: UPGMA- Dendogram based on 219 ISSR characters illustrating genetic similarity between the studied generations (1: control; 2: Biogene; 3: Ssp.: 4: Potassiomage).

4. Discussion:

The used biofertilizers Biogene and Potassiomag as well as Ssp. of *S. chiabensis* have a potential to stimulate all biological activities of *Vicia faba* L. during the process of cell division. This stimulation was correlated by generating an uninterrupted sequence of increase in mitotic index of the first generation after the increase in total soluble protein, proline and increasing rate of homologous recombination calculated as chiasmata frequency during meiotic division.

Concerning the effect of biofertilizers after the three study groups on PMCs of *Vicia faba* L. flower buds, they induced a highly significant percentage of abnormalities in a dose dependent manner. The percentage of chromosomal aberrations in the second division declined compared to those at the first division after most of all doses. Moreover, the abnormalities in telophase stage in the second meiotic division had the least percentage as compared to their counterpart in the first division, indicating that cells recovered and that has been a temporary effect. These data were supported by those previously reported by Shehab *et al.* (1983), Rashad (1990), and Shehab *et al.* (2004).

The mean of chiasma frequency per PMCs at diplotene and metaphase I by bivalent chromosomes of *Vicia faba* L. plants treated with various doses of biofertilizers and Ssp. was found to be increased significantly in a dose dependent manner. The mutations in condensins - proteins involved in chromosome compaction - increases the axis length and thus the number of loops, and this concomitantly increased the number of DSBs (Mets and Meyer, 2009). However, the study of chiasma frequency is a valuable criterion to measure and determine the degree of both variability in chromosome behavior and pollen fertility (Kumar and Singhal, 2013). Moreover, the individual bivalents of *Vicia faba* chromosomes show autonomous behavior with regard to chiasma formation as earlier studied by Friebe (1979).The exchange of genetic material by homologous recombination during meiosis is essential for generating genetic diversity in living organisms and are considered as cytological manifestation to insure proper chromosome segregation (Shyam and Sergey, 2007). Chiasma frequency is widely studied as it is considered among the vital steps of chromosomal behavior during meiosis. Pairing homologous chromosomes is very essential for completion of this important event in sexual reproduction, as chromosomes may fail to pair either due to asynapsis or desynapsis resulting in reduction in pollen fertility (Gulfishan *et al*., 2010; Avijeet *et al*., 2011 and Ranjbar *et al*., 2012).

The progressive increase in mitotic indices of the first generation was highly significant and was dose dependent. The stimulatory effect on cell division was reported earlier after using gibberellic acid as a direct effect of increasing rate of biosynthesis and functional protein (El Keredy *et al.,* 1975). Stimulating activity of mitosis was previously reported after the use of biofertilizers by stimulating the plant defense response (Montesinos, 2002). Soil microorganisms such as *Agrobacterium tumefaciens* or *Bacillus subtilis* can stimulate nodulation, through the production of bioactive molecules. This is supported by the observations that plant growth regulators like gibberellin and other plant hormones are produced by bacteria and actionomycetes (Ranjeet *et al.* 2002). Furthermore, this stimulation of plant growth by actinomycetes referred to the production of pteridic acids A and B considering them as plant growth promoters with auxin-like activity (Hasegawa *et al*., 2006).

The high significant increase in mitotic indices was concomitant with the large amount of cells present in prophase stage recording significant values. These increased number of prophase cells were then infiltrated by the "metaphase-checkpoint "and or anaphase promoting complex, leading to the low percentage of cells present in metaphase and normal percentage of cells in telophase compared to their respective control. This adaptation in distribution of dividing phases followed by stimulation in mitotic index were previously noted by Hu *et al*., 2000 and Howell *et al*., 2007.

However, the increase in cell division was found to be related to the length of telomeres, the nucleoprotein structure protecting the end of chromosomes, and its activity promote malty entry into cell cycle division (Haussmann and Marchetto, 2010). On the other hand, progression in cell cycle is regulated at two points, the G1/S and G2 /M. Cytokinins was found to activate cell division through induction of D-type of cyclins mediators of internal and environmental stimuli to drive cell division (Miyazawa *et al*. 2003).

The close relationship between telomere clustering and chromosome synapsis has led to the suggestion that the bouquet (in which the ends of the chromosomes cluster together on the inner surface of the nuclear envelope) may help to facilitate pairing and synapsis (Lee *et al*., 2012), so the clustering may serve to confine homologous sequences to a small volume of the nucleus so as to promote synapsis which is typically initiated near the telomeres (Amie *et al.*, 1999 and Cho *et al*., 2014).

The results of the three study groups revealed the dominancy of abnormalities in the type of disturbance followed by stickiness in PMCs and in

mitotic cells. However, different types of abnormalities was recorded in a low percentage as: bridges, laggards and fragments as well as micro and macronucleus and multinucleate cells and were occasionally found in interphase. Micronuclei might originate from a lagging chromosome or fragment at ana-telophase as described earlier bySchlegel and MacGregor, 1982. Micronuclei derived from a whole chromosome have higher probability to survive and undergo condensation in synchrony with the main nuclei other than micronuclei derived from a chromosome fragment (Gusta vino *et al.*, 1987). Micronuclei have true mutagenic effect, which may lead to loss of the genetic material and have been regarded as an indication of the mutagenecity of their inducer (Ruan*et al.*, 1992 and Burroughs *et al.*, 1996). Genotoxicity was studied as the dose-response relationship for aneugens and clastogens (Zeiger *et al*., 2009; Liu *et al*. 2010 and Elhajouji *et al.* 2011). The induction of mutagenesis was reported as a direct effect of four water extracts of fruit and leaves on *Vicia faba* L. root tips as initial screening to validate their cytotoxicity by inducing micronuclei (Khalifa *et al*., 2014).

The quantitative estimation of total protein revealed an increase in their percentages. However, the acceleration rate of cell division in *V. faba* root cells treated with biofertilizers might be due to the increased rate of biosynthesis of biochemical contents required for cell division and may have the potentiality to alter the transition process or the transcriptional one via modulating the activity of some enzymes responsible for enhancement protein synthesizing machinery (Shehab *et al.,* 2004).

The quantitative estimation of the free amino acid proline which represent one of the macromolecular contents revealed a significant increase in a dose dependent manner. Proline is a dominant organic molecule that accumulates in many organisms upon exposure to altered environment and plays multiple roles in plant adaptation to stress; moreover, it is a wildly distributed osmolyte accumulated under alteration in environment of the different studied organisms and also in eubacteria (Kishoor *et al*., 2005; Yamada *et al*., 2005 and Barka*et al*., 2006). The possible explanation of stimulation all division process in the present study even during the growth of the first generation of parent treated with biofertilizer include: the production of hormones IAA; the production of enzymes ACC deaminase which reduce the level of ethylene in roots during development; nitrogen fixation; production of siderophores; solubilizing and mineralization of nutrients and improvement of abiotic stress resistance (Hayat *et.al*., 2010).

The presence of ISSR polymorphic bands in the first generation as well as the increase in chiasma frequency to 1.74 times more than their respective control indicates the induction of genetic polymorphism in these genotypes as well as genetic variability. The study of ISSR and analysis of degree of polymorphism for different progeny as well as UPGMA analysis was extensively used to evaluate the genetic variation among offspring (Wei *et al*., 2008; Noormohammadi*et al*., 2013). The study of ISSR revealed its importance in planning hybridization in essential crops. The UPGMA would be more efficient when different characters were included for the same taxa.

Considering all data gained in the present study, it might be reported that biofertilizers Biogene and Potassiomag calibrated by Ssp. of *Streptomyces chiabensis* have a regular pronounced stimulating effect on cell division.

On the other hand, high frequency of chiasmata reflected the stimulation of recombination process and repair the damage occurred in the double strand breaks during meiotic recombination cause significant gene diversity and variability in the offspring. The data revealed by ISSR indicated the production of genetic variability in the progeny. However, and regarding to the previous data, these biological control agents are safe and economic fertilizers although the minimum level of nuclear abnormalities induced during mitotic and meiotic cells development.

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