Assessment of the genotoxicity of wastewater samples on *Vicia faba* L.

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Abstract: Genotoxicity impact of wastewater samples from sewage and mixed with industrial effluent from Kafer El-Sheikh, Egypt, on cultivated crops was assessed using the *Vicia faba* root-tip cytogenetic bioassay. The results showed that the irrigation with wastewater decreased the mitotic index (MI), caused significant increases of micronucleus (MCN) frequencies and anaphase aberration (AA) as stickness,, lagging, and bridges. The results also showed that continuous irrigation by wastewater several times may pose a potential genotoxic risk to cultivated plants. The results of the present study suggest that the *V. faba* cytogenetic bioassay is efficient, simple in genotoxicity studies of wastewater, and that there is a correlation between the genotoxicity and the irrigation with wastewater.

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1. Introduction

Pollution is a crucial threat to our environment that the increasing discharge of hazardous chemicals into the environment has affected the balance of natural ecosystems. This consequently attracts the attention of several researchers and governmental agencies to the health of living organisms (Leme and Marin-Morales, 2009). Environmental pollution constitutes a great health hazard to human, animals, and plants with local, regional and global implications. Pollution has adverse effects on land, water and its biotic and a biotic component (Al-Dulaimi *et al.*, 2012). Among the damages caused by chemical agents, exposed organisms are under genotoxic and mutagenic effects. These effects have shown to be worrying, due to its capacity to induce genetic damage that can lead to several health problems and also affect future generations, since these alterations can be inheritable (Ribeiro, 2003). Agriculture expansions in Egypt depend mainly on irrigation but water supply from irrigation canals is not sufficient enough. Therefore, farmers in many parts of the Nile delta urgently use drainage of water in irrigation their fields, because of these waters are consider the only source for irrigation purposes (Khalifa *et al.*, 2003). Estimation indicates that more than fifty countries of the world with an area of twenty million hectares are treated with polluted or partially treated polluted water (Mahmood, 2006).Plants grown in contaminated soils when consumed by peoples can result in health problems (Wahid *et al.*, 2004) like diarrhea, mental retardation, liver and kidney damage (Matsuno *et al*., 2004 and Uzair *et al*., 2009).

Vicia faba considered one of the most important legume crops in Egypt, being used for human food and animal feed, due to its high nutritive value (Bond, 1966). In our study we used it as a bioindicator for pollution that *V. faba* is one of the most commonly used plant materials for cytological, radiobiological, and physiological studies. In addition to its many favorable properties as a test material (Kihlman, 1971), *V. faba*, offer a wide range of possibilities of cytogenetic analyses, because the chromosomes of this species are large and few in number (2n = 12) and thus easy to study (Villalobos-Pietrini *et al*., 1978). Besides, *V. faba* has its own metabolic activation system (Takehisa *et al*., 1982), the treatments may be applied to the roots directly and the complete experiment is rather inexpensive. This plant has been commonly used to study the effects produced by physical and chemical mutagens, having the frequency of aberrations as an efficient indicator of mutagenic response (Villalobos-Pietrini *et al*., 1994). *Vicia faba* has been recommended by the International Program on Chemical Safety (IPCS) to determine the root tip meristem chromosomal aberration assay for screening of chemicals for clastogenicity (Kanaya *et al*., 1994). Also the effectiveness of *V. faba* chromosomal aberration assay has been used in assessing water quality conditions *in situ* (Grant *et al*., 1992).The objectives of this study were to (i) use *V. faba* as a bioindicator to evaluate the effect of water pollution and (ii) evaluate the cytotoxicity effect of the irrigation by polluted water.

2. Materials and methods

Water sources

The wastewater was collected from different sites in Kafr El-Sheikh city, Kafr El-Sheikh governorate, Egypt. The sources of water were tap water, agricultural water from Meet yazeed branch canal (Lat. = 30˚ 57 40″ E, Long. = 31˚ 5′ 54″ N) in addition to agricultural

polluted water from two different sites [Bitaytah darin which contained agriculture drainage water (Lat. = 31˚ 2′ 33″ E, Long. = 31˚ 8′24″ N) and Kitchener drain which contained agricultural water mixed with sewage and industrial wastes (Lat. = 31˚ 3′ 8″ E, Long. = 31˚ 8′ 30″ N)] (Figure 1). Moreover, both distilled water used as a negative control and 300mM aqueous hydrogen peroxide as a positive control mutagen.

Seeds germination

Seeds were soaked in water for 12 hours for each water source and then transferred to pots half-filled with sand. Three pots were used for each water source and three seeds were sown in each pot. The irrigation was continued till primary and secondary roots were appeared.

Mitosis division

Roots of 1-2 cm long for each examined plant were cut and fixed in a freshly prepared carnoy's fixative (3:1 v/v) (absolute ethyl alcohol: glacial acetic acid) for 24 hours. Fixed roots were kept in 70% ethyl alcohol in a refrigerator until use. Mitosis was carried out using the feulgen squash technique. Treated roots were washed with distilled water, hydrolyzed in 1 N HCl at 60 ºC for 10 minutes, washed by distilled water, stained in basic fuchsin stain for at least two hour at 37º C in the dark. 1-2 mm from the terminal of deeply stained root tips was squashed on a clean dry slide by using a drop of 45% acetic acid. At least ten fields in ten well spread slides for each treatment was examined carefully under the binuclear light microscope (Olympus Japan). Mitotic indexes (MI), percentage of mitotic abnormalities were calculated. Types of and percentage of abnormalities were also recorded and photographed using Digital camera (SONY).

Statistical analysis

The data of cytological characters were analyzed using Graph Pad prism version 5.01. Correlation was determined by applying Pearson's method. Statistical significance was defined as $P<0.05$. The coefficient of determination (R^2) was estimated using IPM SPSS statistics version 20 software (SPSS, Inc., Chicago, USA).

3. Results

Mitotic index (MI)

Mitotic index measurements under polluted water in addition to tap water and control were given in Table (1), Figure (2). All values of mitotic index recorded in all plants that irrigated with polluted water were low compared to control (13.2%). The lowest value was recorded in plants under irrigation by water collected from Bitaytah drain (7.7 %), whereas the highest value of MI was recorded in plants irrigated with water from Meet yazeed canal (11.9). Plants watered by H_2O_2, tap water and water from Kitchener drain recorded values of 8.1%, 10.5% and 10.9 % respectively.

Phase frequency

Data of phase frequency was given in Table (1). Prophase frequency in all plants that irrigated with polluted water recorded low values compared to control (47.8%) except for in case of plants irrigated by water from Kitchener drain (48.2%). The reduction varies overall all irrigated plants. The lowest value was recorded in plants which irrigated by water collected from Bitaytah drain (28.3%). While the other irrigated plants gave relatively high values. It was observed that all values of metaphase frequency in plants under the polluted water were higher than that of control (10.5%) except for plants irrigated by water from Meet yazeed canal which had an equal value to the control. The highest value was recorded in the plants that irrigated by water from Bitaytah drain (16.5 %). Values of Tap water, water from Kitchener drain and under the effect of aqueous of H_2O_2 show no significance difference.

The highest value for anaphase frequency (18.4%) was recorded in the plants that irrigated by water from Bitaytah drain compared to control (11.2%), whereas the lowest value was recorded in the plants that irrigated by water from Kitchener drain (9.2%). Values recorded in the plants that irrigated water from Meet yazeed canal were similar to that recorded for the control (11.2%). On the other hand, a slight increase in anaphase frequency was recorded in the plants that irrigated with Tap water (13%).

Telophase frequency values that recorded in the plants that irrigated by water from Bitaytah drain, Kitchener drain and plants watered by H_2O_2 were (36.8%, 36.9% and 37.6 %) respectively. These results exhibited a significant increase compared with control (30.5 %). On the contrary, the values in case of the plants that irrigated by Tap water and that irrigated by water from Kitchener drain were (27.1% and 29.8% respectively) which gave a negative effect compared to the control.

Abnormality percentage

The percentage of abnormal cells recorded at various stages is given in Table (1). For prophase abnormalities, it was noticed that, there was no abnormalities in the plants of control while the highest percentage of abnormalities was recorded in the plants watered by H_2O_2 (4.8%), whereas the lowest value was recorded in the plants that irrigated by water from Kitchener drain (1.6%). For metaphase, the percentage of abnormal cells was very high in the plants watered by H_2O_2 (22.8%) followed by (17.8%) in case of plants irrigated by water from Bitaytah drain compared with the control (5.95%).The other obtained values from plants that irrigated by other different polluted water were slightly high than control.

For anaphase, it was observed that the highest percentage of abnormalities was recorded in the plants that irrigated by water from Bitaytah drain (69.2%) compared to control (29.9%). The lowest value was recorded in plants under the irrigation by water from Kitchener drain (40.9%). Generally, all irrigated plants showed higher values of abnormalities than control.

For telophase, it was observed that all values of abnormalities in all plants under study were increased

compared to control except for plants that irrigated with Tap water which showed the same value as in control (0.9%). The highest value was recorded in plants that irrigated by water from Bitaytah drain (11.4%). The rest values imposed a moderate effect compared to the plants that irrigated by water from Bitaytah drain.

The percentage of total abnormalities is given in Table (2) and represented graphically in Figure (3). This parameter was increased in all irrigated plants compared to control (3.3%). The highest value was scored in the plants that irrigated by water from Bitaytah drain (21.7%) where the lowest one was recorded in the plants that irrigated by water from Meet yazeed canal (8.2 %). Values recorded in plants that watered by H_2O_2, tap water and by water from Kitchener drain were (12.7%, 8.7% and 9.4%) respectively.

Types and percentages of mitotic abnormalities were listed in Table (2) and represented in Figure (4). In the present study all polluted water induced different types of abnormalities such as stickiness, breaks, bridges, lagging chromosomes, micronuclei, unequal distribution, multipolar cell, and C- Metaphase.

In comparison to the control (0.3%), the highest value of stickiness, were recorded in plants which irrigated by water from Bitaytah drain (3%), Figure (4) (A & B). On the other hand, the lowest value was recorded in plants watered by tap water (1.6%) and in the plants that irrigated by water from Meet yazeed canal (1.9%). The values in case of H_2O_2 and at the irrigation by water from Kitchener drain were closed (2.4 and 2.5%) respectively.

Chromosomal morphology

By microscopic examination, the chromosomal alterations in anaphase cells as fragments, bridges, isochromosomes and chromosomes with inactivated centromeres were scored. In order to compare the frequencies obtained by each treatment with those scored in their own control. Chromosome breaks recorded in all plants under study as in Figure (4) (C & D). Compared to control (1.5 %), the highest value of this phenomenon was recorded in the plants that irrigated by water from Bitaytah drain (7.3%). The lowest value was recorded for irrigation with Tap water (2.6 %). Some types of chromosome, chromatin and multibridges were recorded in all plants under the study Figure (4) (E & F). In comparison to the control (1.5 %) the highest values were recorded in the plants that irrigated by water from Bitaytah drain (8 %). The lowest value was recorded in the plants that irrigated by water from Meet yazeed canal 2.5%). Values of other plants showed significant increase in this phenomenon compared to control.

Type of lagging chromosomes (Figure 4, G) was also observed all plants of the experiment. The highest value was recorded in plants which irrigated by both tap water and water from Meet yazeed canal (1%).), whereas the lowest value was recorded in case of irrigation with H_2O_2 (0.4%).

Micronuclei type, Figure (4, H and I) were recorded in the plants which watered with H_2O_2 and at the irrigation by water from Bitaytah drain with values of (1.4% and 2.3 %) respectively. Unequal distribution of chromosomes, Figure (4, J), was also recorded in plants that watered by H_2O_2 and in others irrigated by tap water with values of (0.2% and 3%), respectively.

Multipolar cells, Figure (4, K) were recorded only in plants which watered irrigated by water from Bitaytah drain (0.2%). Types of C- Metaphase Figure (4, L) were also recorded only during the irrigation with H_2O_2 with value of (0.2%).

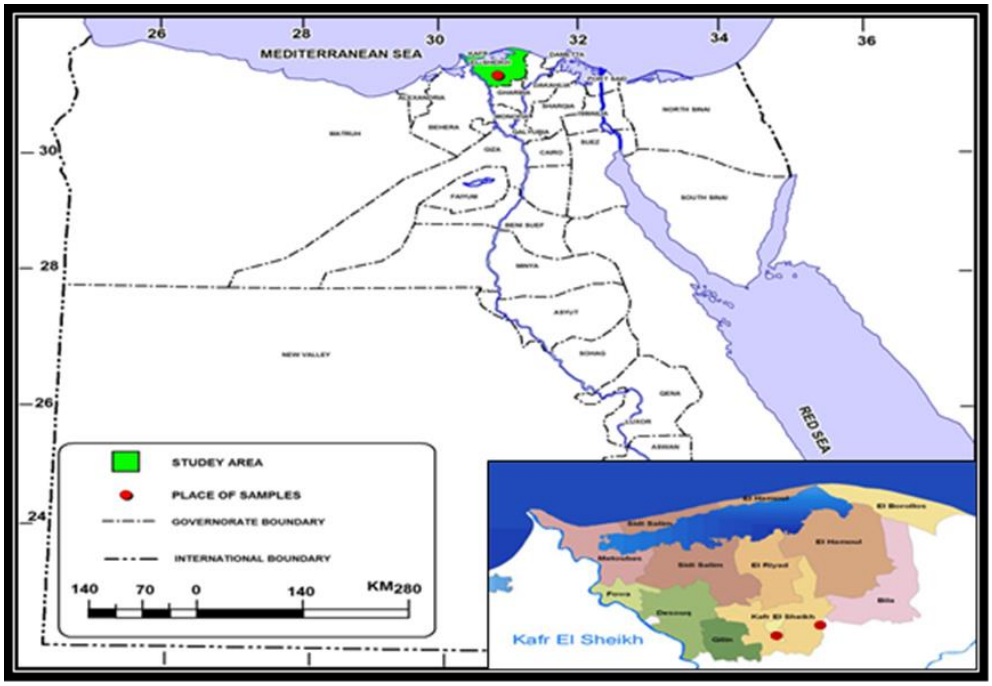

Figure 1: Map of Egypt which shows the study area in Kafr El-Sheikh governorate.

Table 1: Percentage of mitotic index, mitotic stage frequencies and abnormalities in *V. faba* root tip cells which irrigated by polluted water, tap water, distilled water (negative control) and H_2O_2 (positive control). Ns: non significant, * =$P \leq 0.05$, *** = $P \leq 0.001$

Water source	Mitotic index ±SD	Percentage of mitotic stage frequencies				Percentage of mitotic stage abnormalities			
		Prophase %	Metaphase %	Anaphase %	Telophase %	% of prophase	% of metaphase	% of anaphase	% of telophase
Distilled water as control negative	13.2 ± 0.6	47.8	10.5	11.2	30.5	0	5.95	29.9	0.98
H_2O_2 as control positive	8.1 ± 0.8	39.4	12.4	10.7	37.6	4.8	22.8	62.9	3.9
Tap water	10.5 ± 0.1	47.1	12.9	13	27.1	2.5	8.1	41.3	0.9
Agricultural drainage water	7.7 ± 0.09	28.3	16.5	18.4	36.8	2.3	17.8	69.2	11.4
Surface water	11.9 ± 0.8	41.4	10.5	11.2	36.9	2.7	7.3	50.95	5.4
Mixed water	10.9 ± 0.7	48.2	12.9	9.2	29.8	1.6	8.8	40.98	3.6
F – value	45.9***	Ns	2.771*	4.922***	Ns	Ns	2.514*	6.036***	6.271***

Table 2: Mitotic index, total abnormal cells and types of mitotic abnormalities induced by polluted water, tap water, distilled water (negative control) and H_2O_2 (positive control) in *V. faba* root meristems. Ns: non significant**, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$**

Water source	Total no. of abno. cell (%)	Types and percentage of mitotic abnormalities							
		Stickiness	Break	Bridge	Lagging chromosome	Micronucei	un equal distribution	Multi-polar cell	C – Metaphase
Distilled water	3.3 ± 0.8	0.3	1.5	1.5	0	0	0	0	0
H_20_2	12.7± 1.6	2.4	3.4	4.4	0.4	1.4	0.2	0	0.2
Tap water	8.7± 0.5	1.6	2.6	3.2	1	0	0.3	0	0
Bitaytah drain	21.7 ± 4.6	3	7.3	8	0.7	2.3	0	0.2	0
Meet yaseed canal	8.2 ± 2	1.9	2.8	2.5	1	0	0	0	0
Kitchener drain	9.4 ± 1.3	2.5	2.7	3.7	0.5	0	0	0	0
F – value	34.56***	4.257**	4.857**	4.915**	2.634*	7.575***	ns	ns	ns

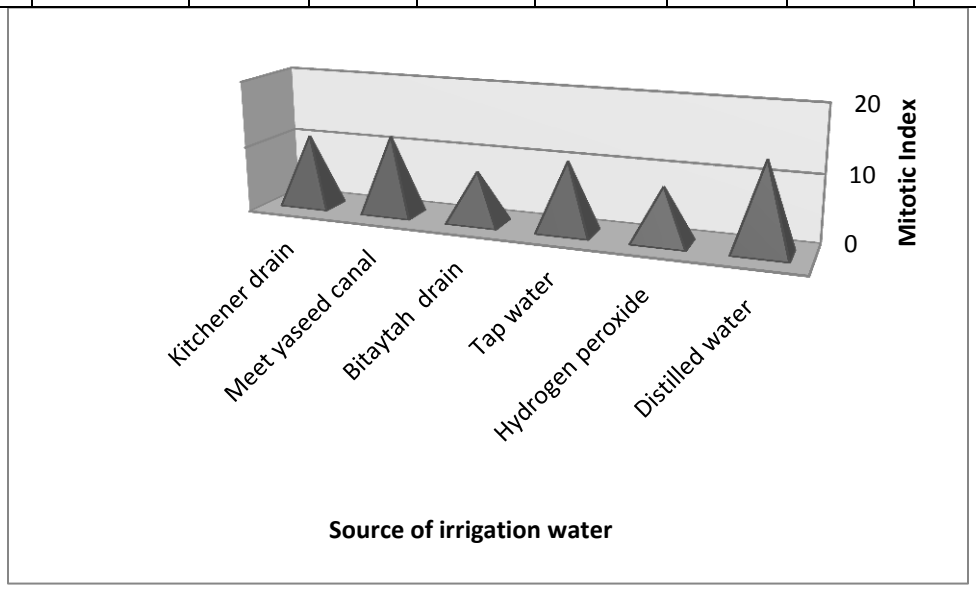

Figure 2: Effects of different water sources on mitotic activity of *V. faba* root tip cells.

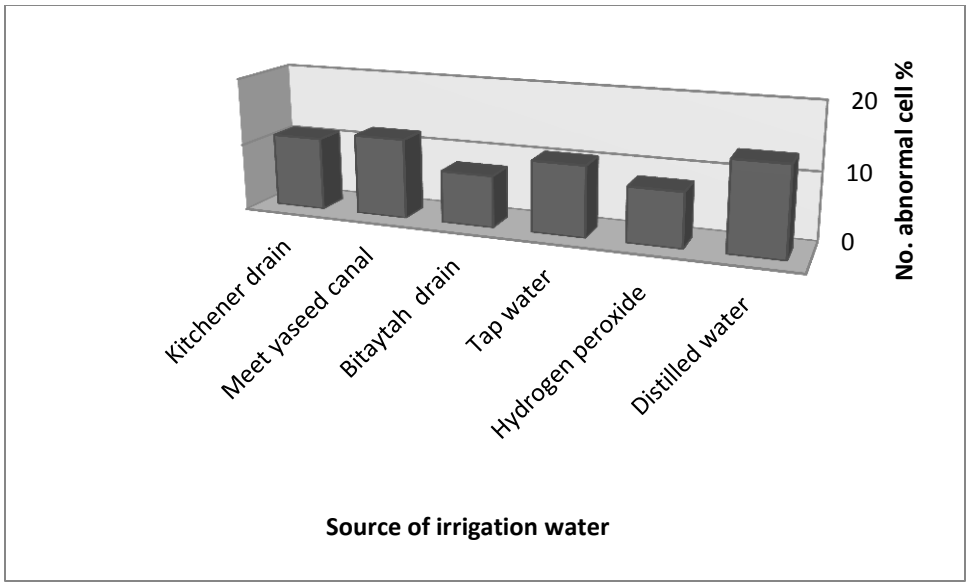

Figure 3: Percentage of mitotic abnormalities in *V. faba* root tip cells under different water sources.

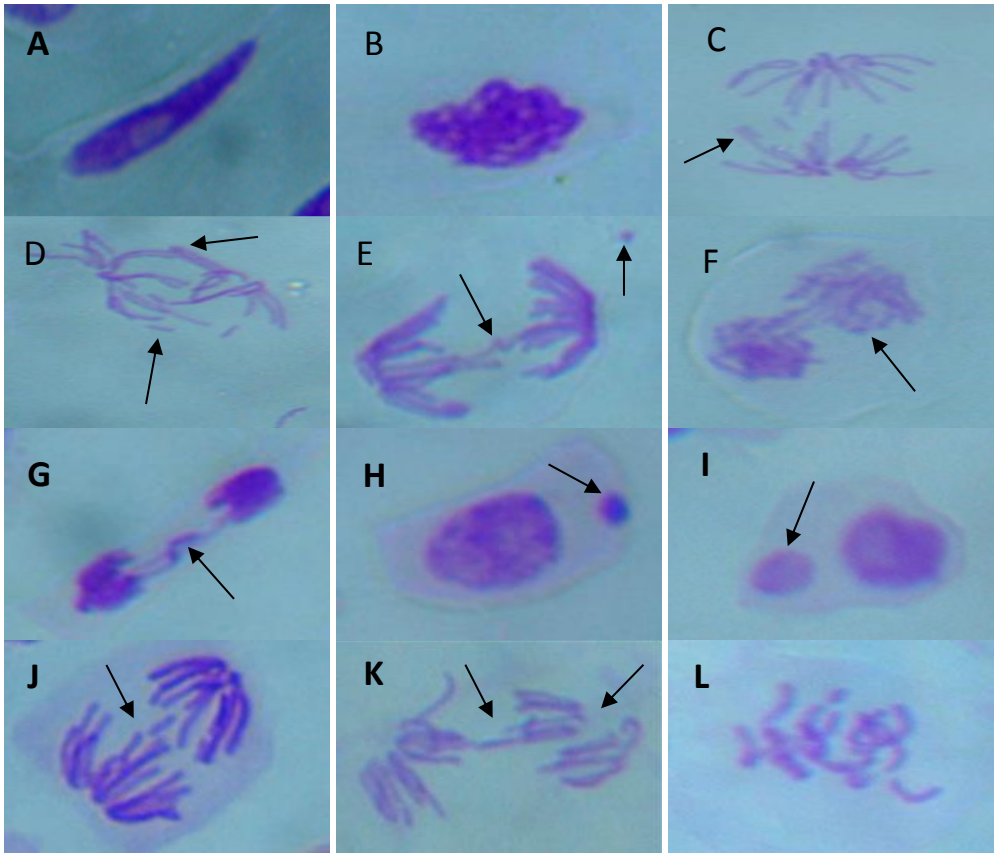

Figure 4: Types of abnormalities induced in different stages of mitosis in *V.faba*root tip cells irrigated with different sources of water: (A) Stickiness in interphase, (B) Stickiness in prophase, (C) Chromosome breaks in anaphase, (D) Chromosome breaks and chromosome bridge in anaphase, (E) Chromosome bridge and micronuclei in anaphase, (F) Chromatin bridge in telophase, (G) Lagging chromosome and chromosome bridge in anaphase, (H) Micronuclei (1/10 from the size of the main nucleus) in interphase, (I) Micronuclei (1/3 from the size of the main nucleus) in interphase, (J) Unequal distribution in anaphase, (K) multipolar cell and chromosome bridge in anaphase, (L) C – metaphase.

4. Discussion

Not surprisingly that the irrigation of the cultivated crops with polluted water induced mitotic changes in root tips of *V. faba* compared to control. These changes vary from the reduction of mitotic index, changes in phase index and induction of percentage and types of chromosomal aberrations.

Cytotoxicity defined as a decrease in the mitotic index and it considered as an acceptable measure of cytotoxicity for all living organism (Smaka-Kinel *et al.*, 1996). Mitotic index is considered a parameter that allows estimating the frequency of cellular division (Leme and Marin-Morales, 2009 and Marcano *et al*., 2004). In present study, the mitotic index values showed a significant reduction values. This trend of reduction was previously observed by Sik and Aki (2009) in Allium cepa under effects of industrial wastewater. The reduction in mitotic index by cytotoxic substances and polluted water in the present study may be due to the effect on microtubule configuration (Armbruster *et al*., 1991), or the blocking of the mitotic cycle during interphase stage (Mohandes and Grant 1972), or the inhibition of DNA synthesis (Beu *et al*., 1976; Chand, and Roy 1981), which could be due to blocking of G1, there by suppressing DNA synthesis (Schnelderman *et al*., 1971). Blocking in G2 prevents the cell from entering mitosis (Van't Hoff, 1968) and inhibits nuclear protein synthesis in the cell cycle (Kim and Bendixen 1987), which leads to inhibit the formation of various metabolic events necessary for mitosis (Rost and Morrison 1984).

In this study, there was slight significant increase in the frequency of both metaphase and anaphase cells in some treatments, while in some others, there was a significant decrease in the frequency of anaphase cells. These results agreed with Egito *et al*. (2007) in study of cytotoxic and genotoxic potential of surface water on onion (*A. cepa*). Furthermore, the mitotic abnormalities were detected similar to those observed in *A. cepa* of wastewater (Nielsen and Rank 1994 and Amin 2002). Moreover, there was a negative correlation between mitotic index and mitotic abnormalities as previously recorded in Allium cepa (Kovalchuk *et al*., 1998 and Bushra et al., 2002). The highest values of mitotic abnormalities at anaphase were agreed with that obtained in Vicia faba under the effects of some chromium salts (Gómez-Arroyo and Villalobos-Pietrini 1983), insecticides heptachlor (Gómez-Arroyo, 1985) and in situ detection of mutagens in an aquatic environment (Grant *et al*., 1992).

The scoring of chromosomal alterations in anaphase cells supply adequate data to assess damage at the genetic level that is produced by environmental pollutants and the highest values of mitotic abnormalities was recorded in metaphase and telophase cells (Grant *et al*., 1992). Our results showed mitotic abnormalities in metaphase and telophase cells of plants treated by wastewater. These results agreed with that noticed by El- Shahaby *et al*. (2003), who indicated the genotoxic effect of industrial wastewater using the *A. cepa* chromosome aberration.

Moreover, the present study showed that many abnormalities such as stickiness, breaks, bridges, lagging chromosomes, micronuclei, unequal distribution and multipolar cell. In addition to the highest number of sticky chromosomes was recorded under the effect of industrial effluent compared to negative and positive control. These results were in agreement with that obtained in onion bulbs (*A. cepa*) (Olorunfemi *et al*., (2011), they observed the sticky chromosomes at metaphase and anaphase stages were abundant in the Allium test which indicated that these effluents contain substances that are very toxic.

Stickiness may be due to defective functioning of one or two types of specific non-histone proteins involving chromosome organization (Gaulden 1987 and Turkoglu 2007). It may be occur through immediate reactions with DNA during its inhibition periods, causing DNA-DNA or DNA-protein cross linking (Turkoglu 2007). Sticky chromosomes indicate a highly toxic, irreversible effect probably leading to cell death (Fiskesjo 1985, 1988). Darlington and Mcleish (1951), also they suggested that stickiness might be due to degradation or depolymerization of chromosomal DNA.

C-mitosis type of abnormalities in forms of C-metaphase was recorded in this study only in positive control. Besides, the lagging chromosomes were recorded in some other treatments. This may be due to irregular orientation of chromosomes, which might be attributed to the failure of the spindle apparatus to organize and function in a normal way (Patil and Bhat 1992).The malfunction of the spindle mechanism could be attributed to the reactivity of metal ions with the tubulin SH group (Dash et al., 1988), or may be due to the direct results of breaks and fragmentation, which lead to the loss of centromeres and the stopping of their movement (Gari *et al*., 1998).

A high number of breaks and bridges were recorded in all treatments compared to negative and positive control. The formation of bridges could be attributed to chromosomal breakage and reunion (Halıem, 1990). Furthermore, attributed bridges and fragments to clastogenic effects which resulting from chromosomal and chromatin breaks were detected in *A. cepa* (Kovalchuk *et al*., 1998). Chromosome breaks cannot be repaired and are indicative of permanent genetic damage (Halıem, 1990 and Bickham *et al*.,

2000). Likewise, the present study recorded many types of micronuclei (MN). A similar result was reported by Smaka-Kinel *et al*. (1996) in *A. cepa* and Shugart *et al*. (2003) in animal. This type of aberration may occur through chromosome breaks or fragments or spindle poisoning, which is an anomalous disjunction of chromosomes during anaphase (Fiskesjö 1997).

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