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Genotoxic Effects of Heavy Metals on Mitotic Chromosomes of *Trigonella Foenum-Graecum* L.

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ABSTRACT

Lead, cadmium, and copper are common environmental pollutants in most industrialized countries. Soil with heavy metal pollution has raised concern in recent years due to its possible destructive effects on plants system. In the present investigation, we studied the genotoxic effect of lead, cadmium, and copper on mitotic chromosomes *of Trigonella foenum-graecum* L. for the first time. The root tips of *T. foenum-graecum* were treated with four graded concentrations (viz. 50, 100, 150, and 200 ppm) of lead, cadmium, and copper. After hydrolyzing the root end in hydrochloric acid solution, they were stained with acetocarmine. After squashing the root end, they were studied under a microscope. To this end, the mitotic index (MI) and the total percentage of abnormality (TAP%) were analyzed. Studying of the root end of *T. foenum-graecum* showed that this plant is diploid and its chromosomal base number is 2x=2n=16. Cytological monitoring revealed that Pd, Cd, and Cu exhibit mitodepressive behaviors at higher concentrations. Moreover, the mitotic index decreases, but the incidence of different anomalies, such as sticky chromosome, c-mitosis, micronucleus, laggard chromosome, bridge, and precocious movement, increase through increasing heavy metal concentration. Among the mitotic abnormalities observed in all treatments, the highest percentage was related to the sticky chromosome. The highest genotoxic potential was observed in the lead, followed by cadmium and copper. The results showed that heavy metal pollution led to a significant MI reduction and TAP% increase in root tip meristems of *T. foenum-graecum*. This should be considered as a risk warning of the environmental pollution on plants, especially medicinal plants.

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Introduction

Heavy metal toxicity, found in industrial wastes, is a widespread environmental problem globally and has toxic impacts on plants, animals, and even humans. Since heavy metals do not break down and remain in the environment for a long time, they are categorized as genotoxic agents. Chen *et al*. (2017) have also observed that plants are the main metabolic pathway for transferring toxic elements from the soil to humans. The hyperaccumulation of toxic metal in plants depends on physiological function, such as uptake rate, translocation efficiency, and deposition rate, particularly within growing tissue (Lasat *et al.,* 2005). Heavy metal accumulation causes damage and alters genetic material during the cell cycle (Gebhart, 1984; El-Shahaby *et al.,* 2003; Hajmoradi and Taleb beydokhti, 2019a, 2019b).

Metals can affect many physiological and biochemical mechanisms within plants and also the extent of toxicity. The genotoxic potential of lead, cadmium, and copper has made them particularly challenging pollutants (George, 2000; Kumar and Tripathi, 2007; Yucel *et al.,* 2008; Pandey and Upadhyay, 2010; Abubacker and Sathya, 2017; Ackova, 2018; Stoyanov *et*

al., 2018; Aprile and De Bellis, 2020). Cadmium proved to have extremely toxic effects on all living organisms. Cadmium (Cd) is one of the very mobile elements in soil, which can be easily transported and distributed to all plant organs (Gruenhage and Jager, 1985; Kabata-Pendias and Pendias, 1992).

Lead (Pb), as a non-essential element, is proved to have adverse phytotoxic effects on morphology, seeds germination, growth of the seedling, photosynthesis, content of water, nutrition of mineral and activity of enzymes in plants (An, 2006; Chen *et al*. 2017; Kumar and Bhardwaj, 2017; Ackova, 2018).

A small amount of copper (Cu) is required by plants; however, it becomes toxic if it exceeded a particular concentration. Although copper is vital for the health of all living things, an excessive amount of it in the body has several effects on health by causing different sicknesses or even death (Ezeh *et al.,* 2018).

Root tips of seedlings are frequently used for chromosome or DNA damage assessment in mitotic cells of higher plants. Fenugreek (*T. foenum-graecum,* Fabaceae) is a plant around 60-90 cm tall. It has green leaves consisting of three small obovate to oblong leaflets, small white flowers, and pods that contain small, golden-brown seeds. Fenugreek is an herb long used in alternative medicine. This herb may have numerous health benefits. Fenugreek is used as a spice (seeds), herb (dried or fresh leaves), and vegetable. Since the water and soil in agricultural areas may be polluted with heavy metals, the plants cultivated in the polluted area will certainly affect the plant kingdom.

The present study aimed to evaluate the susceptibility of mitotic chromosomes of *Trigonella foenum-graecum* to the genotoxic effect of lead nitrate (Pb $(NO_3)_2$), Cadmium ($CdCl_2$), and Copper ($CuCl_2$).

Materials and Methods

Plant material

The seeds of Fenugreek, from the family Fabaceae, were used for the experiments. For the root tip germination, the seeds' surface was first completely sterilized, rinsed, and then placed in a Petri dish containing a filter paper. Next, the Petri dish was covered and incubated for 2-3 days at 22-25 °C.

Lead, cadmium, and copper treatment

Solutions with different lead, cadmium, and copper concentrations (50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L) were prepared from Pb $(NO_3)_2$, $CdCl_2$, and $CuCl_2$ using the dilution method. After the germination stage, the seeds were treated with different heavy metals concentrations for 3 hrs. To control them, some root meristems were kept in distilled water. Then the root tips were completely rinsed with distilled water and fixed in ethanol: acetic acid (1:3) solution for 24 hrs. The roots were then removed to a container having 70% ethanol for future use (Kumar and Srivastava, 2015).

Mitotic slide preparation

In this stage, to soften the root tip tissues, they were hydrolyzed in 1 N HCl for one minute. Then, they were stained with 4% acetocarmine under a heating condition. The slides were finally prepared through the squash technique, and their genotoxic effect was investigated. The light microscope under oil immersion was used to measure the mitotic index and chromosomal abnormalities in a different phase of mitosis. A minimum of 400 cells was scored from each slide (Ranjbar *et al.*, 2012). The mitotic index was calculated by dividing the number of cells x 100 / total number of cells, as suggested by Love (Love, 1949). Total Abnormality Percentage (TAP) was calculated by dividing the number of abnormal cells x 100 / total number of cells (Kumar and Bhardwaj, 2017).

Statistical analysis

Data analysis was done using SPSS 16.0. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). P-values $\leq$ of 0.05 were considered to be significant. Data were normalized using square root normalization in SPSS software version 9.0.

Results

Mitotic index

Observing mitotic cells is a rapid way to evaluate and observe chromosomes. The degree of cytological aberrations in mitosis is a

dependable criterion for measuring the mutagen sensitivity of the species and the effect of mutagens (Ignacimuthu and Babu, 1989). All studied mitotic cells of *T. foenum-graecum*, were diploid and possessed a chromosome number of 2n = 2x = 16. Mitosis was normal with relatively minor chromosomal anomalies in the control set. However, a concentration-dependent increase in mitotic abnormalities was observed in the meristematic root cells of *T. foenum-graecum* in all Pb $(No_3)_2$, $CdCl_2$, and $CuCl_2$ treatments (Fig. 1, Table 1).

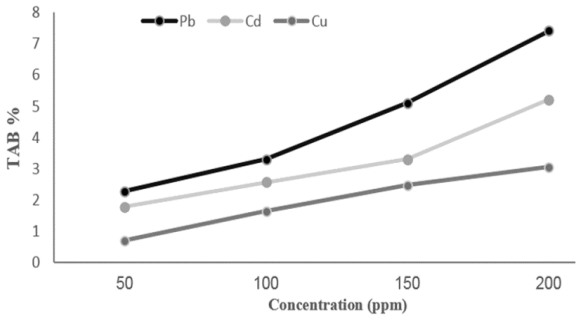

Fig. 1. Relationship between total abnormality (TAB%) and heavy metal concentration.

Table 1. Different types of heavy metal-induced chromosomal abnormalities at various stages of mitosis, MI% and TAB% in root tip cells of *Trigonella foenum-graecum*

Treatment			**Cd**		**Control**
Concentration (ppm)	**50**	**100**	**150**	**200**	**-**
Sticky chromosome	1.11±0.16	1.32±0.18	1.51±0.27	2.73±0.09	-
C- mitosis	0.21±0.17	0.32±0.27	0.53±0.09	0.79±0.05	-
Micronucleus	-	0.11±0.11	0.21±0.17	0.32±0.19	-
Laggard	-	0.24±0.14	0.37±0.18	0.49±0.16	-
Bridge	0.12±0.03	0.19±0.08	0.25±0.19	0.33±0.15	-
Precious movement	0.28±0.15	0.33±0.16	0.41±0.06	0.51±0.08	-
MI%	12.89±0.63^{b}	10.96±0.19^{a}	9.87±0.25^{c}	8.21±0.08^{c}	-
TAB%	1.78±0.35^{a}	2.57±0.20^{d}	3.31±0.56^{b}	5.22±0.64c	14.57±0.16^{a}
Treatment			**Cu**		**Control**
Concentration (ppm)	**50**	**100**	**150**	**200**	**-**
Sticky chromosome	0.64±0.13	0.93±0.18	1.19±0.05	1.37±0.03	-
C- mitosis	-	0.19±0.08	0.36±0.12	0.54±0.09	-
Micronucleus	-	-	0.17±0.09	0.24±0.14	-
Laggard	-	0.19±0.08	0.28±0.15	0.35±0.21	-
Bridge	-	0.15±0.15	0.18±0.17	0.21±0.05	-
Precious movement	-	0.14±0.17	0.25±0.19	0.31±0.16	-
MI%	13.74±0.55^{b}	12.38±0.45^{c}	11.24±0.72^{c}	9.37±0.61^{d}	-
TAB%	0.70±0.20^{b}	1.65±0.33^{a}	2.48±0.20^{a}	3.06±0.61^{b}	14.57±0.16^{a}
Treatment			**Pb**		**Control**
Concentration (ppm)	**50**	**100**	**150**	**200**	**-**
Sticky chromosome	1.40±0.14	1.56±0.22	2.65±0.09	4.31±0.13	-
C- mitosis	0.32±0.27	0.52±0.16	0.77±0.14	0.96±0.09	-
Micronucleus	0.19±0.08	0.24±0.14	0.36±0.12	0.44±0.06	-
Laggard	-	0.41±0.15	0.54±0.09	0.69±0.16	-
Bridge	0.21±0.17	0.36±0.08	0.47±0.07	0.58±0.09	-
Precious movement	0.13±0.13	0.19±0.08	0.28±0.15	0.39±0.21	-
MI%	11.51±0.25^{a}	10.65±0.18^{c}	9.41±0.92^{b}	7.68±0.42^{b}	-
TAB%	2.28±0.20^{a}	3.31±0.61^{c}	5.11±0.64^{b}	7.41±0.21^{a}	14.57±0.16^{a}

Abbreviations: MI: Mitotic Index; TAP%: Total Abnormality Percentage; (Mean numbers ± standard error followed by lowercase letters are statistically significant at $p < 0.05$).

The MI was used for the assessment of the cyto-genotoxicity of metals. The MI was decreased from 11.51±0.25 to 7.41±0.21, 12.89±0.61 to 8.21±0.08, and 13.74 to 9.37±0.61 with increasing the lead, cadmium, and copper concentration, respectively. The mitotic index decreases with increasing heavy metal concentrations (Fig. 2, Table 1). The highest mitotic index reduction was observed during the lead treatment, followed by the cadmium and copper treatments.

Chromosome abnormalities

The mitotic cells analysis in the Pb, Cd and Cu treatments indicated many chromosome-related abnormalities, such as stickiness, laggards, C mitosis, micronucleus, chromosome bridge, and precocious movement of the chromosome.

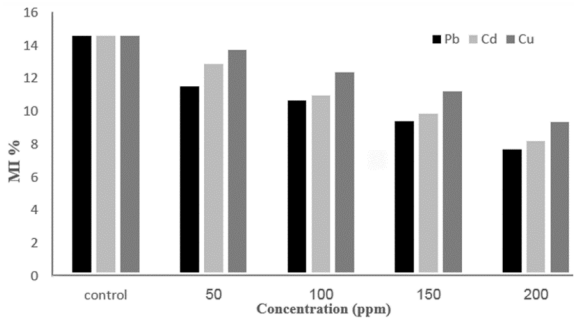

Fig. 2. Correlation between MI and heavy metal concentration.

The total abnormality percentage (TAP%) in treatment with lead increased from 2.28±0.20 to 7.41±0.21, increasing doses from 50 ppm to 200 ppm. In cadmium treatment, the TAP% increase from 1.78±0.35 to 5.22±0.64 with increasing the CdCl2 doses. In copper treatment, it increases from 0.70±0.20 to 3.06±0.61 at the same lead and cadmium concentrations. Stickiness and C-mitosis were more frequent in all treatment sets. The frequency of other abnormalities in lead, cadmium, and cooper treatments is as follows:
Laggards > bridge > micronucleus > precious movement.
Precious movement > Laggards > bridge > micronucleous.
Laggard > precious movement > micronucleous > bridge.

Discussion

Toxic and mutagenic effects of heavy metals on plant systems have been reported by earlier studies (Siddiqui, 2012; Hajmoradi and Taleb beydokhti, 2019a, b; Sarac *et al*., 2019; Aprile and De Bellis, 2020.). The present study showed an increase of chromosomal aberrations in mitotic cells of the Fenugreek treated with heavy metals. The present study further confirms that heavy metals pollutions can have genotoxic effects.
Chromosome stickiness indicates highly toxicity of mutagen, which may lead to death (Turkoglu, 2009). Non-separated stickiness chromosomes led to an increased incidence of sticky laggards and bridges. The sticky chromosome was the significant abnormality in early metaphase I of all treatments. Its frequency increased with increasing heavy metals (Table 1). Stickiness is a mitotic disruption that is unlikely to cause structural damage to the chromosome (Badr, 1983). Some heavy metal-induced mutations of genes may incorrectly code some non-histone proteins involved in organizing chromosomes, which, in turn, leads to stickiness. The metal itself may react with the histone proteins and make them sticky (Ritambhara and Kumar, 2010). The number of cells including chromosome stickiness varied in all treatments. Chromosome stickiness is an indicator of an abnormal chromosome with a sticky surface, leading to the death of the cell (Singh, 2015). This abnormality can be attributed to genetic and environmental factors (Pagliarini, 2000; Ranjbar *et al.,* 2012). The chromosomal stickiness is highly frequent because of the disturbance in the cell's nucleic acid metabolism (Chidambara *et al.,* 2006).
Root tips, treated with Pb, Cd, and Cu, induced C- metaphase in all treatments except for 50 ppm Cu. In such abnormalities, the chromosomes are thick and short in metaphase and show no equator orientation. C-mitosis is a consequence of the mitotic spindle inactivation caused by the loss of microtubules of the spindle fibers connected with the centromere's delayed division (Kumar and Srivastava, 2015). Partial or incomplete C-mitosis leads to multipolar spindles, aneuploid nuclei, and micronuclei and makes cells exhibit normal mitoses. C-mitosis might cause polyploidy, resulting in the formed cells' degeneration without more division (Deysson, 1968; Grant, 1978; Abubacker and Sathya, 2017; Stoyanov *et al*., 2018; Aprile and De Bellis, 2020). The C-metaphase incidence in *T. foenum-graecum* root tips depends on the dose.
The micronuclei formation reflects a neugenecity, resulting in the loss of genetic material (Kumar and Bhardwaj, 2017; Stopper and Muller, 1997). Gilli (1941) and Levan (1951) suggested that the acentric chromosome formation from lagging chromosomes or fragments during anaphase may resemble a micronucleus at telophase if the involved chromatin material is large enough. It was proved that cells with micronucleus might have degenerated in the F2 or divide (Arora *et al.,* 1969; Das, 1962). A positive correlation was observed between the micronuclei induction frequency in root tips and the mitotic index (Degrassi and Rizzoni, 1982). As a result, the

micronucleus can be considered as a signal that can detect pollutions that lead to mutations. This type of abnormality was not based on only 50 and 100 ppm Cu and 50 ppm Cd (Fig. 3, Table 1).

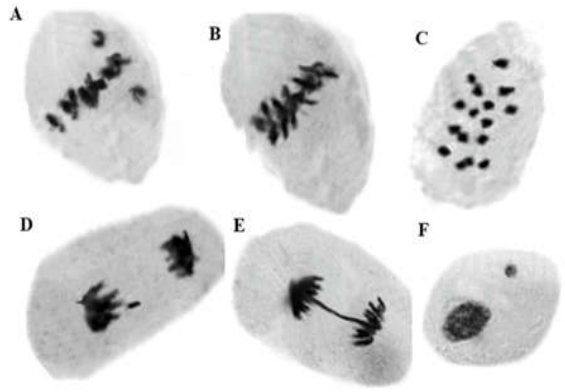

Fig. 3. Different chromosomal abnormalities in root tip cells of heavy metal-induced *T. foenum-graecum*; (A) precious movement of chromosome; (B) sticky chromosome; (C) C-mitosis; (D) laggard; (E) bridge; (F) micronucleus.

Another phenomenon was lagging chromosomes (Fig. 3) in various percentages during anaphase in all treatments, except for 50 ppm Pb, Cu, and Cd. Laggards were observed at higher mutagen doses, particularly under lead treatments. The chromosome lagging occurs because it may either poorly attach to spindle fiber or not attach at all. Sticky chromosomes and altered homology of the paired chromosome are two other causes of the laggard chromosome (Magoon and Cooper, 1958). Das and Roy (1989) attributed the spindle fibers' failure to carry the chromosomes to Polar regions, and the effect of mutagens, in turn, led to the mitosis at anaphase. Chromosome bridges indicate a full chromosome structure breakdown, which leads to the formation of long and thin chromatin threads. Chromosome bridges are probably because of sticky chromosomes and subsequent free anaphase separation failure; besides, an unequal chromosome segment translocation may cause chromosome bridge formation (Gomurgen, 2000). Swanson (1965) attributed this phenomenon to paracentric inversion. The highest degrees of bridges were observed in lead nitrate and the lowest was seen in copper chloride treatments. At higher doses of treatment, bridges were also more frequent at anaphases. More than one bridge and lagging chromosomes were observed in some mitotic cells. According to Shaikh and Godward (1972), the number of chromosomes involved in the exchange determines the frequency of bridges. The single bridge is induced from the sister chromatid fusion at a common breakage point. On the other hand, non-homologous chromosome translocation, producing unlike arms, leads to the double bridge formation (Yagyu and Morris, 1957). The thickness of the bridge and the number of chromosomes involved depend on the treatments. Chromosome stickiness in different plants can be attributed to genetic and environmental factors (Nirmala and Rao, 1996). This abnormality with different thicknesses was observed in all treatments except for 50 ppm Cu (Fig. 3, Table 1).

The scattering spindle dysfunction from the loss of spindle fiber microtubules, early chromosome criminalization, and chemical breaking of the nucleoprotein backbone's protein moiety resulted in the precocious movement of chromosomes (Kumar and Srivastava, 2015; Patnaik *et al.,* 1984). Univalent chromosomes generated in the late prophase stage because of early completion of chiasma in early metaphase I may produce micronuclei which, in turn, results in micro-pollen formation and pollen grains with unbalanced numbers of chromosomes, such as aneuploids (Defani-Scoarize *et al.,* 1995; Mansuelli *et al.,* 1995). All lead, cadmium, and cooper doses exert a toxic effect on cell division, leading to precocious movement. This abnormality was not observed only in mitotic cell concentration < 50 ppm of cooper treatment (Fig. 3, Table 1).

Conclusion

The results showed that Pb, Cd, and Cu might cause abnormal division of mitotic cells in *T. foenum-graecum*. Moreover, it was observed that they have toxic effects on chromosomal morphology including chromosome stickiness, C-mitosis, laggard, anaphase bridge, micronucleus, and precious movement. According to the results, the toxic level of Pb was higher than that of two other heavy metals. The concentration of heavy metals has a considerable role in inhibiting the cell division of

root tip cells, leading to the decreased mitotic index. The result indicates a great danger from heavy metal pollutants to the environment and plants.

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Conflict of interests

The authors declare that they have no conflict of interests.

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بررسی اثرات ژنوتوکسیک فلزات سنگین بر کروموزوم‌های میتوزی گیاه *Trigonella foenum-graecum* L.

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چکیده

سرب، کادمیوم و مس آلاینده‌های محیطی معمول در اغلب کشورهای صنعتی هستند. با توجه به اثر مخرب خاک آلوده به فلزات سنگین در گیاهان، بررسی این فلزات در سال‌های اخیر مورد توجه قرار گرفته است. در این مطالعه ما اثر ژنوتوکسیک سرب، کادمیوم و مس را بر کروموزوم‌های میتوزی گیاه *Trigonella foenum-graecum* L. برای اولین بار بررسی کردیم. انتهای ریشه گیاه *T. foenum-graecum* توسط غلظت‌های افزایشی سرب، کادمیوم و مس (۵۰، ۱۰۰، ۱۵۰ و ۲۰۰ پی‌پی‌ام) تیمار شدند. انتهای ریشه‌ها بعد از هیدرولیز در محلول اسید هیدروکلریک، توسط استوکارمن رنگ آمیزی شدند. بعد از له کردن انتهای ریشه‌ها، اسلایدها در زیر میکروسکوپ مورد مطالعه قرار گرفتند. در انتها، ایندکس میتوزی (MI) و درصد کلی ناهنجاری‌ها (TAP٪) محاسبه شدند. مطالعه‌ی انتهای ریشه نشان داد که گیاه *T. foenum-graecum* دیپلوئید است و عدد پایه‌ی کروموزومی آن ۲x=۲n=۱۶ است. بررسی سیتولوژیکی نشان داد سرب، کادمیوم و مس در غلظت‌های بالا به عنوان مختل کننده‌ی میتوز رفتار می‌کنند. علاوه بر این، با افزایش غلظت فلزات سنگین، ایندکس میتوزی کاهش می‌یابد ولی ناهنجاری‌های مختلف مانند کروموزوم چسبناک، C–میتوز، میکرونوکلئوس، کروموزوم سرگردان، پل و حرکت زودهنگام کروموزوم افزایش می‌یابد. در بین ناهنجاری‌های دیده شده در تمام تیمارها، بالاترین درصد مربوط به چسبندگی کروموزوم است. بالاترین پتانسیل ژنوتوکسیک در سرب بعد کادمیوم و مس دیده شد. نتایج نشان داد که آلودگی فلزات سنگین منجر به کاهش قابل ملاحظه MI و افزایش درصد TAB در انتهای مریستمی ریشه‌ی *T. foenum-graecum* می‌شود. این مطالعه باید به عنوان زنگ خطر اثرات آلودگی محیطی در گیاهان خصوصاً گیاهان دارویی در نظر گرفته شود.

واژگان کلیدی: سیتوتوکسیک؛ شنبلیله؛ فلزات سنگین؛ ناهنجاری‌های کروموزومی

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