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Effect of heavy metals on meiosis cell division in *Stachys inflata* Benth.

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ABSTRACT

Stachys inflata Benth. belonging to Lamiaceae, grows in the vicinity of zinc and lead mine around Zehabad village, Qazvin Province, Iran. The aim of this study is to determine how *S. inflata* is affected by a long-term exposure to zinc and lead pollutants. The focus, here, is on how the meiotic behaviour of pollen mother cells as well as pollen fertility are affected by heavy metal pollutants. ICP-MS analyses of soil and water samples indicated the presence of lead and zinc in a concentration much higher than the recommended standard. The results revealed that soil and water polluted by heavy metals had a significant impeding effect on the division of PMCs in *S. inflata*. An elevated meiosis abnormality rate, as well as decreases in meiotic index and in pollen fertility were observed compared to the control. The data indicate that this species is not meiotically stable. In general, the pollen mother cells of specimen grown in polluted soil exhibited an increased incidence of chromosome stickiness, B-chromosomes, chromosome bridges, laggard chromosomes, micronucleus, desynapsis, cytomixis and formation of tripolar and pentapolar cells.

Keywords: Heavy metal, Meiosis abnormality, Pollution, *Stacys inflate.*

INTRODUCTION

The genetic factors and environmental conditions affect growth and development of plants. Any alterations in these factors may result in plant variation. The environment surrounding plants is a life sustaining system consisting of both biotic and abiotic components. These components interact with each other and influence their surroundings. The environmental pollution caused by human activities is one such factor that may degrade the sustenance of the surrounding entities. In recent years, a rapid growth in industrialization has led to an increasing rate of pollution in water, air, and soil. There are different sources of metal contamination such as agricultural chemicals, industries, mining and fuel. Mining further more produced effluents including different types of pollutants which are becoming potential soil contaminants. Heavy metal contamination in soil and water has raised concerns due to its possible impacts, not only on human health but also on the plant system (Ravindran 1978; Kawada & Suzuki 1998; Singh 2001; Heidary *et al*. 2016; Bifeng *et al*. 2017; Wu *et al*. 2018). Studies conducted in various plant species showed that various chemical compound such as diethylsulphate and sodium azid (Bhat *et al*. 2007; Siddiqui *et al*. 2007), ethylene imine, diethyl sulphonate, ethyl methylsulphonate (Hassan & Ahmad 2000), elicit different chromosomal aberration in meiosis and mitosis, leading to reduced pollen fertility.

Tripathi & Girjesh (2010) reported genetic loss through lead and zinc induced by chromosomal stickiness in grass pea. Like other countries, the ecosystems of Iran are continually exposed to various types of pollutants that are disturbing the natural biodiversity in various ways. Uptake of heavy metals may cause destructive changes in plants such as inhibition of root growth and morphological defects (Samardakiewicz *et al*. 2005).

Metal toxicity in plants has been reported by various studies (Bollard & Butler 1966; Brown & Jones 1975; Foy *et al*. 1978; Chidambarm *et al*. 2009; Ackova 2018). Lead (Pb), zinc (Zn) and Other metal ions, such as nickel (Ni), copper (Cu), Aluminium (Al), chromium (Cr), mercury (Hg) and cobalt (Co) have been reported to cause

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disarray in the pattern of the mitotic and meiosis cycles and to produce chromosome aberrations, such as the development of micronuclei, chromosome bridges, chromosome stickiness, among others (Ravindran 1978; Seregin *et al*. 2001; Yucel *et al*. 2008; Zohari *et al*. 2012; Kumar and Bhardwaj 2017). Various kinds of developmental disturbances in anthers and ovules of plants have resulted a high percentage of aborted ovules and pollen grains (Micieta & Murin 1996; Izmaiłow 2000; Koscinska-Pająk 2000; Biskup & Izmaiłow 2004; Kłosowska *et al*. 2009; Ismael *et al*. 2018). Various pollen viability tests are based on staining techniques that determine pollen enzymatic activity, membrane integrity along with the stainability of the cytoplasm and nucleus (Khatun & Flowers 1995; Nepi and Franchi 2000; Vizintin & Bohanec 2004; Slomka *et al*. 2010).

Among heavy metals, Pb and Zn are the most widespread. While certain level of zinc in plants is necessary for their natural growth, higher concentrations are highly toxic. Lead is one of the hazardous anthropogenic heavy metal. It is, usually, taken up from soil through the roots, then translocated to the aerial parts at a rate of several percent (Yucel *et al*. 2008). Large quantities of Pb and Zn compounds in liquid, solid and gaseous wastes in the environment can ultimately have significant adverse biological and ecological effects. However, only a few studies have, so far, investigated the influence of lead and zinc pollutions on the genetic material of the plants.

Most of the studies reported the effects of pollution on plants have focused on cultivated plants, in particular, the crop plants. However, little has been conducted to focus on the effects of pollution on plants in the natural ecosystem. Therefore, the aim of this study is to investigate the possible genotoxic effects of heavy metal pollutants in *Stachys inflata* as a wild species and also to describe the meiotic abnormalities and pollen viability in *S. inflata* induced by lead and zinc polluted soil and water.

MATERIALS AND METHODS

Site description

The lead and zinc mine of Zeh Abad is located at NW of Qazvin Province, Iran. The national plant population of polluted specimens of the *S. inflata* were collected from a locality exposed to mining pollution (Latitude = 36° 28' 13", Longitude = 49° 25' 02", elevation: 1094 m a.s.l.). Control population of the same species were collected from a distance of 4 km away from the mine with no apparent source of pollution (Latitude = 36° 29' 15", Longitude = 49° 27' 01", elevation: 1060 m a.s.l.). The studied site area is shown in Fig. 1.

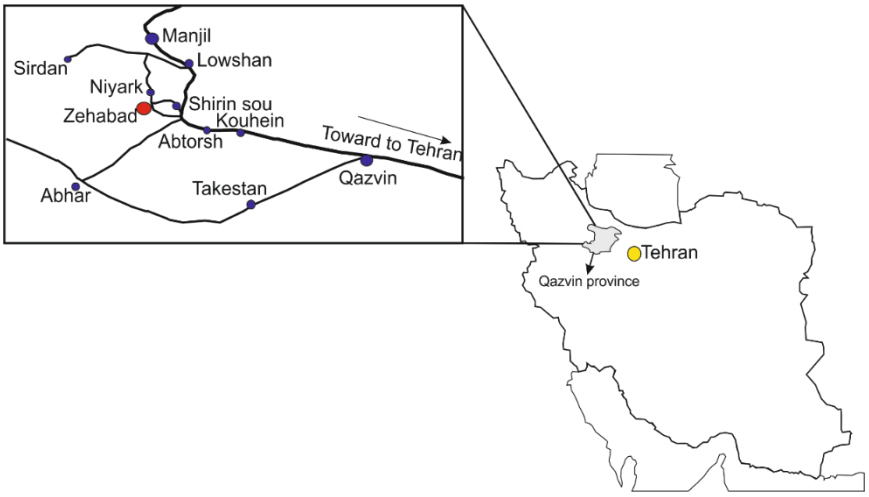

Fig. 1. Map of Zeh Abad mine, showing study area location.

Soil and water samplings

In this study, soil and water samplings from the natural growth area of *S. inflata* were carried out to investigate the effects of Zeh Abad mine pollutions on the division of meiosis in this plant.

Water samples were collected in high density polyethylene containers previously washed by a 10% nitric acid solution in an ultrasonic bath for 15 min, followed by repeated rinsing with bidistilate water and finally by ultrapure water. Until the collection time, containers were kept in sealed polyethylene bags. Water samples were stabilized using ultrapure nitric acid (0.5% HNO_3 solution). Water samples were transferred to a laboratory to determine Pb and Zn concentrations.

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All of the determinations were carried out by an inductively coupled plasma quadrupole mass spectrometry (ICP-Q-MS).
Approximately, 5-6 kg of soil samples were taken from a depth of around 80 cm and was passed through a 20 mesh to separate the rubble and grains. After screening the soil samples with 80 mesh, soil specimens were transferred to the laboratory for analysis by ICP-MS device.

Plant material
The specimens of *S. inflata* were collected from their natural habitat in the polluted site near the source of pollutions. The species were identified using available references (Rechinger, 1982). For cytogenetic study, 15 flower buds from at least five plants at an appropriate stage of development were fixed in Carnoy's solution (96% ethanol, chloroform and propionic acid, 6:3:2) right after being taken out of the plants for 24 h at room temperature and then stored in 70% alcohol at 4°C for further procedures.

Cytogenetic analysis
Cytogenetic analysis was performed on *S. inflata* flower buds fixed in Carnoy's solution as described before. For cytological studies slides were prepared using young anthers squash technique and stained with 2% acetocarmine. All slides were made permanent using Venetian turpentine. Assessment of meiotic behaviour was performed by evaluating all possible pollen mother cells (PMCs) on each slide. Tetrads were also analyzed in test and control populations. Approximately, 300 cells at the tetrad stage were observed in both populations.
On the basis of these counts, the meiotic index (MI) was calculated by dividing the number of normal tetrads by the total number of tetrads observed, this value multiplied by 100 (Tedesco *et al*. 2002). Photographs of chromosomes were taken by an Olympus BX-41 photomicroscope at initial magnification of 1000X. Chromosome counts were made from well-spread metaphases in intact cells, by direct observation and from photomicrographs.

Pollen fertility
Pollen fertility was estimated by the pollen ability to stain. So that, pollen grains were first obtained from flowers of herbarium specimen of *S. inflata* and then stained with acetocarmine/glycerin (1:1). Slides were stored at room temperature for 24-48 h. The stainability was determined using samples of 1000 pollen grains per flower. Slides were examined and documented with an Olympus BX-51 photomicroscope. The pollen grains were considered fertile when they were well stained and considered infertile when they were empty or unstained. Pollen fertility rate was calculated by the following formula:

$$\text{Pollen fertility (\%)} = \frac{Total\ number\ of\ fertile\ pollen\ grains}{Total\ number\ of\ pollen\ grains} \times 100$$

RESULTS

Lead and zinc concentrations in the water and soil samples
The lead and zinc concentrations determined in soil and water samples of Zeh Abad mining area along with their standard limits are presented in Table 1 (Standards for quality of soil resources and its guides, 2014) and Table 2 (Sewage outlet standard, 2014).
According to the standards, maximum permissible limits for Pb and Zn in soil are 50 and 200 mg kg^{-1}, respectively, while their levels in the analysed soils were 4730 and 3065 mg kg^{-1}, severally, that are 94.6 and 15.325 times higher than their respective standard values as illustrated in Table 1.
The Pb and Zn concentrations in water samples were 59 and 21 mg L^{-1}, respectively, while the standard values for these two elements in water are 0.1 and 2 mg L^{-1}, respectively, 590 and 10.5 times lower than their respective sampled levels (Table 2).
Due to the high Pb and Zn concentrations in water and soil, the probability of their effects on the plants growth in the area seems to be very high. The elements may be absorbed through contaminated soils and water, then enter the plant.

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Table 1. Heavy metals concentrations in soil.

Heavy metal	**Pb** (mg kg^{-1})	**Zn** (mg kg^{-1})
Metals concentrations	4730	3065
Standard	50	200

Table 2. Heavy metals concentrations in water.

Heavy metal	**Pb** mg l^{-1}	**Zn** mg l^{-1}
Metals concentrations	59	21
Standard	0.1	2

Cytological effect of Pb and Zn on PMC cells of *S. inflata*

A total of 667 diakinesis/metaphase I (D/MI), 894 anaphase I/telophase I (AI/TI), 774 methaphase II (MII) and 985 anaphase II/telophase II (AII/TII) cells were analysed in the test and control populations of *S. inflata*. The meiotic irregularities observed in the studied populations included: chromosome stickiness, B-chromosomes, desynapsis, cytomixis, chromosome bridges resulting from stickiness, the occurrence of laggard chromosomes, formation of micronuclei in tetrad cells, formation of tripolar and pentapolar cells which have been discussed below (Fig. 2). The data regarding the meiotic stages as well as abnormalities observed in each stage are presented in Table 3.

Sticky chromosomes were observed in both populations. However, higher rates (%) of this phenomenon were found in polluted population of *S. inflata* (Table 3). Sticky chromosomes were dominant during diakinesis and early metaphase I (39.96%) and metaphase II (18.81%) in polluted population, while this abnormality in the control was only found diuring prophase (7.8%) (Fig. 2B). B or accessory chromosomes, which occur in addition to the standard or A-chromosomes in some plants, are smaller than others with no relation to them. In the present study, 11.93% of pollen mother cells of polluted population displayed B chromosome, while the control did not exhibit this phenomenon. This abnormality was found only in cells of polluted plants during metaphase I (Fig. 2C).

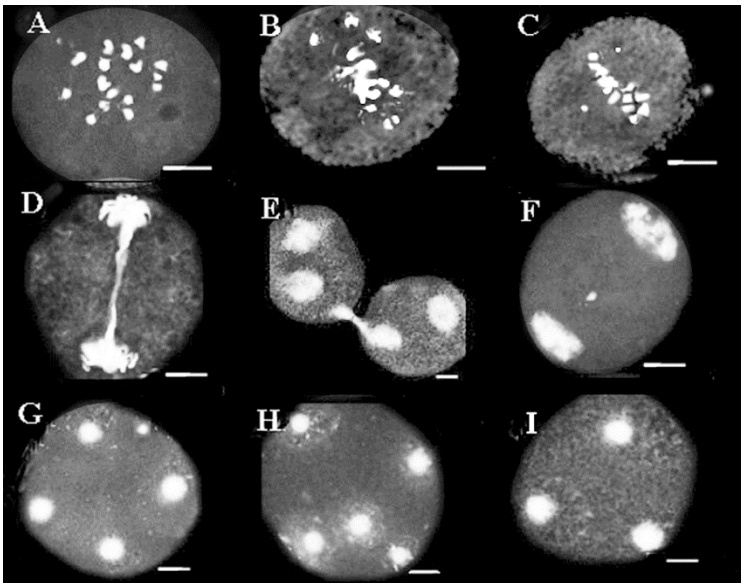

Fig. 2. Meiotic cells with different abnormality in polluted population of *Stachys inflata.* A: Diakinesis showing 2n=2x=32 chromosome; B: Sticky & laggard; C: B chromosome; D: Bridge in anaphase I; E: Cytomixis between two cells in telophase I; F: Laggard in telophase I; G: Micronucleous in telophase II; H: Pentapolar and I: Tripolar cells. Scale bar: 3 µm.

In polluted population, the number of cells with univalents presenting desynapsis was high. The control did not exhibit this abnormality. Precocious migration to the poles were found during metaphase I/II (17.9% and 13.41%, respectively) causing desynapsis. In polluted population, chromosome bridges resulting from stickiness were

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observed in 17.03% and 10.89% of anaphase I and anaphase II respectively (Fig. 2D). In control, only 2.05% of PMC at anaphase I displayed this abnormality. The phenomenon of cytomixis consists in the migration of chromosome between meiocytes through cytoplasmic connection. This phenomenon occurred in 29.13% of anaphase I/telophase I and 17.74% of metaphase II in polluted cells (Fig. 2E). In control, only 3.58% of PMCs in telophase I exhibited cytomixis. PMCs with laggard chromosomes were found in 20% of anaphase I and 7.97% of anaphase II in polluted population (Fig. 2F). Only 2.56% of control cells at anaphase I displayed this abnormality. Micronucleus as another abnormality was found in 13.81% polluted cells during telophase II (Fig. 2G). Tripolar and pentapolar phenomenon were observed in 14.98% and 7.97% of cells in the polluted population, respectively (Figs. 2H and 2I). The control displayed tripolar cells in only 3.11% cells during telophase II.

Table 3. Number of pollen mother cells (PMCs) analysed, percentage of PMCs meiotic behaviour, meiosis index and pollen fertility in polluted and unpolluted populations of *S. inflata.*

Character/Species	test	Standard division	control	Standard division
Total cell number	1844	-	1677	-
D/MI	553	21.15656	114	14.60137
% D/MI	29.98	-	24.68	-
% Sticky & laggard	39.96	7.386474	7.8	1.16619
% B chromosome	11.93	12.46435	-	-
% Desynapsis	17.90	4.534314	-	-
AI/TI	405	11.9499	390	14.72956
% AI/TI	21.96	-	23.25	-
% Cytomixis	29.13	6.572671	3.58	2.607681
% Bridge	17.03	7.082372	2.05	1.095445
% Laggard	20	7.375636	2.56	2.683282
MII	372	13.67333	402	12.0499
% MII	20.17	-	23.97	-
% Desynapsis	13.41	3.969887	-	-
% Cytomixis	17.74	3.577709	-	-
% Sticky&laggard	18.81	7.615773	-	-
AII/TII	514	19.01999	471	22.25848
% AII/TII	27.87	-	28.08	-
% Laggard	7.97	5.403702	-	-
% Bridge	10.89	5.366563	-	-
% Micronucleous	13.81	9.528903	-	-
% Tripolar	14.98	12.08967	3.11	6.053098
% Pentapolar	7.97	5.564171	-	-
% Meiotic Index	58	12.76821	97	14.48726
% Pollen fertility	52	8.239516	95	6.87125
n	16	-	16	-

Abbreviations: D/MI = diakinesis/metaphase I; AI/TI = anaphase I/telophase I; MII = metaphase II; AII/TII = anaphase II/telophase II.

About 300 tetrads per population were examined for calculating the meiotic index. The meiotic index was about 58% in test population and 97% in control (Table 3). The most frequent abnormalities observed at the tetrad stage included irregular triads, pentads and irregular tetrads with micronucleus.

Pollen fertility

Pollutants and other environmental stresses have reported to play a major role in influencing the pollen fertility and pollen size in different plant species (Gottardini *et al*. 2004; Higashitani 2013; Rezanejad 2013; Paupiere *et al*. 2014; Rani & Kumar 2016). The fertility of pollen grains of *S. inflata* was determined by staining method (Fig. 3). In the present study, an attempt was made to assess the effect of Zn and Pb on the *S. inflata* pollen growing at polluted site in comparison with the control. The results obtained from pollen fertility study, are presented in Table 3. The pollen grains of polluted population exhibited less fertility compared to the unpolluted one (Table 3). The pollen viability rate (%) for *S. inflata* in polluted population and control were 52% and 95%, respectively. Pollen viability was found to be proportional to the presence of heavy metals in given sites. Fig. 3 illustrates viable and non-viable pollen grains of *S. inflata.* Based on heavy metal emissions, remarkable effects on the studied pollen fertility were observed. Viable or fertile pollen grains were found to be darkly stained and round in shape, while non-fertile ones were transparent and had shrivelled structure. Pollen fertility of plant species studied was found to be correlated with the heavy metals of sites, where these plants were growing.

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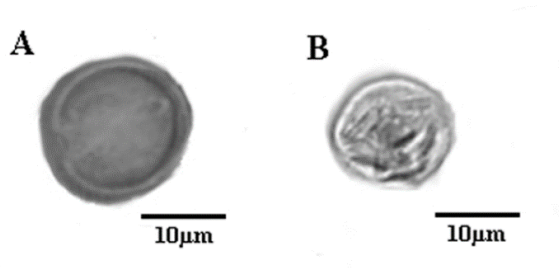

Fig. 3. Viable (A) and non-viable (B) pollen grains of polluted population of *Stachys inflata*.

Pollen fertility is considered to be a good marker of the course of microsporogenesis. Normal meiosis produces regular pollen grains in size with highly fertile, but disturbed meiosis reduces pollen fertility and causes variability in pollen size (giant pollen and very small). These variety can result from inbreeding depression, autopolyploidy, segmental allopolyploidy, hybridization, mutations, and also environmental effects (Stace 1991).

DISCUSSION

The results obtained from the study of heavy metals in soil and water indicate that the pollution arising from mine activity with the release of waste deposits in inappropriate places has caused the accumulation of heavy metals such as Pb and Zn in surface waters and soils of the region. This was supported by the result of the soil and water sample analyses using ICP-MS device, exhibiting that the amount of Pb and Zn in these samples are exceeded from standard (Tables 1-2).

Cytological study of *S. inflata* clearly showed that the PMC rate (%) with meiotic abnormalities in the plants growing in polluted areas was higher than in the control specimens (Table 3). The present study on *S. inflata* exhibited that heavy metals such as Pb and Zn act as a strong mutagenic agent in plants. The rate (%) of abnormalities in studied taxon increased from 3.22% in the control to 60.73% in polluted area. Higher rates (%) of meiotic abnormalities were observed in meiosis stage I compared to meiosis II, such that most of the abnormalities were found at metaphase-I, II and anaphase-I, II of meiosis.

A comparison between the test and control specimens exhibited that the maximum meiotic abnormality in those exposed to pollutants was 39.96%, while in control was 7.8% (Table 3). Among these abnormalities, sticky chromosome was the most frequently observed chromosomal aberration in the polluted population of *S. inflata* (Fig. 4). The frequency of abnormities were in the following order: Sticky chromosome > cytomixis > laggards > desynapsis > bridge > tripolar > micronucleous > B chromosome > pentapolar.

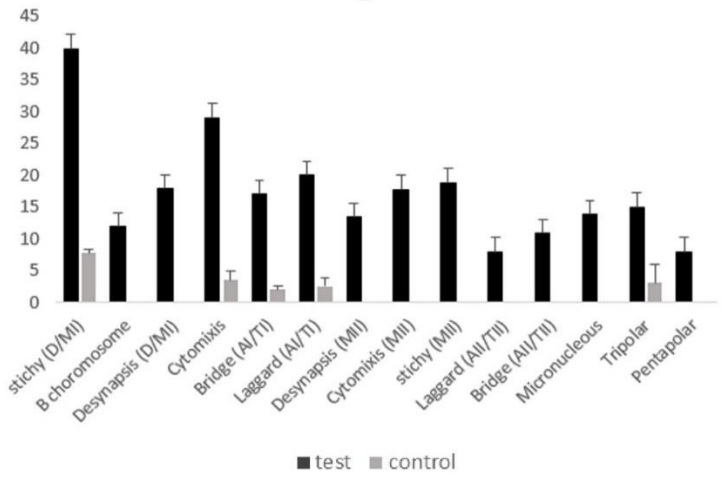

Fig. 4. Comparison of meiotic abnormalities between polluted and control populations of *Stachys inflata.*

Chromosome stickiness and laggards observed in polluted population with high rate (%), may be due to genetic and environmental factors (Pagliarini 2000). B-chromosomes were only found in polluted population. These accessory chromosomes once present in high numbers, affect negatively the growth and vigour of the plants (Jones & Houben 2003).

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Desynapsis is considered as the precocious separation of bivalents in metaphase of meiosis I leading to the formation of varied degrees of univalent in polluted population. It occurs due to the action of recessive disorganization genes in a homozygous situation. Early chiasma terminalisation is considered as another reason for desynapsis. This abnormality may lead to the formation of meiocytes with double normal chromosome number. In several cases such univalent may have difficulty during anaphase I movement and become lagged. Therefore such defective cells may produce aneuploid gametes and leads to reduction in pollen fertility of plants (Veilleux 1985; Sheidai *et al*. 2007; Ranjbar *et al*. 2012). This abnormality has been reported by Siddiqui (2012) in *Vigna mungo* under lead treatment. Also, Zohair *et al.* (2012) have observed precocious chromosomes in examined species under polluted conditions.

Chromosome bridges may be due to chromosomal stickiness and subsequent failure of free anaphase separation or may be attributed to an unequal translocation of chromosome segment (Gomurgen 2000). The thickness of bridges and the number of chromosomes involved in their formation varied among different meiocytes of test population. This abnormality have been reported by Yucel *et al*. (2008) and Kumar & Srivastava (2015) under polluted condition. Genetic as well as environmental factors have been considered as the reason for chromosome stickiness in different plant species (Nirmala & Rao 1996).

The chromatin transmigration between PMCs through cytoplasmic channels was the common feature in polluted population. Since cytomixis creates variation in the chromosome number of the gametes, it could be considered a mechanism of evolutionary significance (Ghaffari 2006). Chromosomes that produced micronuclei during meiosis, were eliminated from microspores as microcytes. The micronucleus reached the microspore wall and formed a kind of bud, separated from the microspore (Baptists-Giacomoelli *et al*. 2000). The eliminated microcytes in the polluted population gave origin to small and sterile pollen grains. Laggards may be related to impaired attachment of kinetochores to the spindle fibres and late chiasma terminalisation. Laggard chromosomes may even result from low chiasma frequency. The presence of asynaptic or desynaptic genes are another reason for observing laggard chromosomes (Pagliarini 1990; Nicklas & Ward 1994).

Ascending chromosomes generally consist of univalent chromosomes formed during late prophase stages by precocious chiasma terminalization in early metaphase I. Laggards and non-oriented chromosomes may produce micronuclei leading to the formation of micro-pollen, and probably to gametes with unbalanced chromosome numbers (Mansuelli *et al*. 1995), such as aneuploids (Defani-Scoarize *et al*. 1995). Heavy metals such as Cd also caused sticky chromosomes in *Pisum sativum* (Fusconi *et al*. 2006, 2007; Siddiqui *et al*. 2009). In line with our finding, Prus-Gowacki *et al*. (2006) and Yucel *et al*. (2008) reported laggards induced by heavy metals. Failure of chromosome movement occurred in one of the poles of cells in anaphase. Any distortion or breakage in the spindle may result in formation of tripolar or multipolar cells due to random sub-grouping of the chromosome. Such abnormal tetrads lead to the decrease in meiotic index of polluted population (58%) and causing formation of infertile pollen grains.

Besides the abnormalities in various stages of meiosis stages, the population from polluted locality has also exhibited higher rates (%) of pollen sterility. The high rate of stained pollen grains (95%) was recorded for control population of *S. inflata*. This result was predictable based on their meiotic behaviour data and low rates (%) of irregularities in this population (Table 3). In the population that chromatin exchange in their PMCs does not occur or occurs at a very low frequency, while other meiotic abnormalities are almost negligible, most of the pollen grains are fertile.

In contrast, a low rate of pollen viability (52%) in polluted population of *S. inflata* can be explained by having high rates of cytomixis, micronucleus, laggard, chromosome stickiness and other abnormalities. In this population a relatively high frequency of chromatin transfers in different stages of meiosis and consequently, low pollen viability was observed. So, it can be concluded that meiotic abnormalities affect the meiotic course considerably, resulting in reduced pollen viability. Previous studies also reported that there is a direct relationship between occurrence of cytomixis, laggard, chromosome stickiness, other abnormality and reduced pollen viability (Lattoo *et al*. 2006; Singhal & Kumar 2008; Ranjbar *et al*. 2011; Zoair *et al.* 2012).

CONCLUSION

In conclusion, this study exhibited that the lead and zinc concentrations in the soil and water of studies area were so much more than the standards and had considerable effects on increased chromosomal aberrations in pollen mother cells of *S. inflata*. It, therefore, may be concluded that these heavy metals may exhibit genotoxic effect on

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plant. Furthermore, polluted population with greater chromosome abnormality, displayed greater pollen sterility. This result indicates that irregularities observed at meiosis probably have a direct relation with pollen fertility. This could be due to toxic effect of Pb and Zinc on meiotic division of pollen mother cells of *S. inflata*.

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اثر فلزات سنگین بر تقسیم میوز در گیاه *Stachys inflata* Benth.

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چکیده

گیاه سنبله‌ای ارغوانی (*Stachys inflata* Benth.) متعلق به تیره نعناعیان به طور طبیعی در اطراف معدن سرب و روی اطراف روستای زه‌آباد در استان قزوین، رویش دارد. هدف از این مطالعه، بررسی اثر طولانی مدت آلاینده‌های سرب و روی بر روی گیاه *S. inflata* با تاکید بر رفتار میوزی سلول‌های مادر گرده و باروری دانه‌های گرده آنها بود. تجزیه شیمیایی نمونه‌های آب و خاک منطقه توسط دستگاه ICP-MS، بیانگر غلظت بالای عناصر سرب و روی بسیار بیش از حد استاندارد است. نتایج نشان داد آب و خاک آلوده به فلزات سنگین اثر قابل توجهی بر تقسیم سلول‌های مادر دانه گرده در گیاه *S. inflata* دارد، به طوری که باعث افزایش ناهنجاری‌های میوزی، کاهش شاخص میوزی و باروری دانه گرده در مقایسه با گیاه شاهد شده است. به طورکلی، سلول‌های مادر گرده در نمونه آلوده ناهنجاری‌هایی مانند افزایش چسبندگی کروموزومی، B کروموزوم، پل، لاگارد، میکرونوکلئوس، دسیناپس، سیتومیکسیس و تشکیل سلول‌های سه قطبی و پنج قطبی را نشان دادند.

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