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Abstract

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Stachys inflata Benth. belonging to Lamiaceae growing in the vicinity of zinc and lead mine around Zeh abad village, Qazvin province, Iran. The aim of this study is find out how *S. inflata* is affected by a long-term exposure to zinc and lead pollutants focusing on the meiotic behavior of pollen mother cells (PMCs) and pollen fertility. The chemical analysis of soil and water samples from the studied area by the ICP-MS indicates presence of lead and zinc in a concentration much higher than the standard. The results revealed that polluted soil and water with heavy metals had a significant impeding effect on the division of PMCs in *S. inflata* as noticed by the increase in meiosis abnormality percentage, decrease in meiotic index (MI) and pollen fertility compared to the control. The present data indicate that this species is not meiotically stable. In general, in pollen mother cells of specimen grown in polluted soil exhibited an increased incidence of chromosome stickiness, B-chromosomes, chromosome bridges, laggard chromosomes, micronuclei, desynapsis, cytomixis and formation of tripolar and pentapolar cells.

INTRODUCTION

The genetic factors and environmental condition effect growth and development of plants. Any change in these factors result in variation in plants. The environment is life sustaining system consisting both biotic and abiotic components, which interact with each other and influence on their surroundings. The environmental pollution caused by man's activities degrades these entities.

In recent years industrialization is growing rapidly due to which the pollution load in water, air and soil is increasing day by day. Heavy metal pollution of the soil and water has raised concern due to its possible impact not only on human health but also on the plant system (Ravindran 1978; Kawada and Suzuki 1998; Hassan & Ahmad, 2000; Singh 2001; Bhat et al. 2007; Siddiqui, 2007; Tripathi and Girjesh, 2010; Abdul Raoof and Siddiqui, 2013, Bifeng et al. 2017, Bhuyan S. and Islam S., 2017, Wu W. 2018). Like other developing countries, in Iran the ecosystems are also exposed to various types of pollutants that are disturbing the natural biodiversity in many ways.

Uptake of heavy metal can cause destructive changes in plants such as inhibition of root growth, as the morphological effects of metal toxicity (Samardakiewicz et al., 2005). Metal toxicity in plants has been reported by various authors (Bollard and Butler 1966; Brown and Jones 1975; Foy *et al.* 1978; Chidambarm *et al.* 2009). Pb, Zn and Other metal ions, such as Ni, Cu, Al, Cr, Hg and Co causes disarray in the pattern of the mitotic and meiosis cycle and produces chromosome aberrations, such as the development of micronuclei, chromosome bridges, chromosome stickiness, etc (Ravindran 1978;Seregin et al., 2001; Yucel et al. 2008, Zohari et. al. 2012; Kumar and Bhardwaj, 2017; Abubacker1 and C. Sathya, 2017).

Various kinds of developmental disturbances in anthers and ovules of plants, resulting in a high percentage of aborted ovules and pollen grains (Ostrolucka, 1989; Mičieta and Murin, 1996; Izmaiłow, 2000; Kościńska-

Pająk, 2000; Biskup and Izmaiłow, 2004; Kłosowska et al., 2009). The various pollen viability tests are based on staining techniques that determine pollen enzymatic activity, membrane integrity, and the stainability of the cytoplasm and nucleus (Khatun and Flowers, 1995; Nepi and Franchi, 2000; Vižintin and Bohanec, 2004; Slomka et al., 2010).

Among heavy metals, lead (Pb) and zinc (Zn) are the most widespread. A certain level of zinc in the plant is necessary for natunal growth of plants. But its higher concentration is highly toxic. Lead is one of the hazardous anthropogenic heavy metal. Lead taken up from soil through the roots, and 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 limited data is available on its influence of lead and zinc on plant genetic material.

Most of the studies on the effects of pollution on plants has been conducted on cultivated plants particularly the crop plants with little work focused on the effects of pollution on plants in the natural ecosystem. Therefore the aim of this study is to find out the possible genotoxic effects of pollutants in *Stachys inflata* Benth. belonging to Lamiaceae as the wild plant species. The present paper describes the meiotic abnormalities and pollen viability in *S. inflata* induced by polluted soil and water.

Material and methods

Site description

Specimens of the *S. inflata* were collected from the national plant population at locality exposed to mining pollution in Zeh abad village, Quzvin provinence. The lead and zinc mine is located at NW of Qazvin, Iran (Latitude=36° 28, Longitude=49° 25). The specimens of same species were collected from 10 km distance of the mine with no apparent source of pollution as control. The studied site and sample area are shown in figure 1.

Soil and water sampling

In this research, soil and water sampling from the natural growth area of *S. inflata* was 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 in a solution of 10% nitric acid in an ultrasonic bath for 15 minutes, followed by repeated rinsing with bidistilate water and finally rinsing with ultrapure water. Until collection containers were kept in sealed polythylene bags. Water samples were stabilized with ultrapure nitric acid (0.5% HNO3). For determination of concentrations of pb and zn metals in water, A samples was sent to the laboratory. All the determinations were carried out by inductively coupled plasma quadrupole mass spectrometry (ICP-Q-MS).

Approximately 5-6 kg of soil samples were taken from a depth of about 80 cm and a 20 mesh was passed to separate the rubble and grains. After transferring the specimens to the laboratory, 80 mesh were screened and sent to the laboratory for analysis by ICP-MS device.

Plant material

The specimens of *S. inflata* were collected from polluted site. The species identified using available references by first author (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 until used.

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 from young anthers squash technique and stained with 2% acetocarmine. All slides were made permanent using Venetian turpentine (Wilson, 1945). 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 200 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, as suggested by Löve (1949). Photographs of chromosomes were taken on an Olympus BX-41 photomicroscope at initial magnification of _ 1000. 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. For this purpose pollen grains were first obtained from flowers of herbarium specimen and then stained with acetocarmin/glycerin (1:1). Slides were stored at room temperature for 24-48 hours. 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 were well stained and considered infertile when were empty or unstained. Percent pollen fertility was calculated by the following formula:

Pollen fertility (%)= $\times$ 100

RESULT

Amount of lead and zinc in samples of water and soil

The concentrations of lead and zinc that were determined in soil and water samples taken from Zeh Abad mining area along with their standards limits are presented in Table 2 and 3.

Lead and zinc are the most abundant heavy metals in nature. They are an essential nutrient but could be also toxic for humans and plants. Maximum permissible limit for lead and zinc in soil is 0.01 mg/l and . . . . . . , respectively[14]. The pb and zn values obtained in the analyzed soil are . . . .. and . . . .. respectively that are more much than the standard values (table 1). The concentrations of Zinc and lead in water are also analyzed. The amount of pb and zn in sampled water are . . . . and . . . .., respectively. The obtained values are greater than their standard (table 2). The standard value of lead and zinc in water are . . . . . . and. . . .., respectively.

Due to the high concentration of lead and zinc in water and soil, the probability of the effect of these two elements on the growth of plants in the area is very high. Lead and zinc are absorbed through contaminated soils and water and enter the plant.

Cytological results in *Stachys inflata*

All the studied samples from both populations (polluted and unpolluted) of *S. inflata* were diploid and possessed 2n = 2x = 32 chromosome number (Figure 1A).

A total of 967 diakinesis/metaphase I (D/MI), 795 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. inflate* (table 1). 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 cells and pentapolar which have been discussed below (Figure 1). Data with regard to the meiotic stage as well as abnormalities that observed in each stage is presented in table 3.

Sticky chromosomes were observed in both populations. However, high percent of this phenomenon was found in polluted population of *S. inflate* (table 3). Sticky chromosomes were dominant at diakinesis and early metaphase I (39.96%) and metaphase II (18.81%) in polluted population but in the control population this abnormality was only found in prophase (7.8%) (Figure 1B).

B-chromosomes or accessory chromosomes, which occur in addition to the standard or A-chromosomes in some of the plants, are smaller than other chromosomes and do not form any association with them. 11.93 % of pollen mother cells of polluted population displayed B chromosome, while the control population did not show this phenomenon. This abnormality was found only in metaphase I cells of polluted plant (Figure 1C).

In polluted population, the number of cells with univalents presenting precocious migration to the poles were high, while this abnormality was not observed in control population. Precocious migration to the poles were found during metaphase I/II (17.9% and 13.41% respectively) and causing desynapsis.

In polluted population chromosome bridges resulting from stickiness were observed in 17.03% and 10.89% of anaphase I and anaphase II respectively (Figure 1D), while in control population 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 cells of polluted population and 3.58% of telophase I cells of control population (Figure 1E).

PMCs with Laggard chromosomes were found in 20% of anaphase I and 7.97% of anaphase II in polluted population (Figure 1F). Only 2.56% of control population cells at anaphase I were displayed this abnormality.

Micronucleus is another abnormality that was only found in polluted population in telophase II (13.81%, Figure 1G).

While in polluted population of *S. inflata*, tripolar and pentapolar cells were observed in 14.98% and 7.97% respectively (Figure 1H and 1I), the control population displayed tripolar cells in only 3.11% of telophase II cells.

About 300 tetrads from per population were examined for calculating the meiotic index. The meiotic index was about 69% in test population and 98% in control population (Table 1). The most frequent abnormalities observed at the tetrad stage were irregular triads, pentads and irregular tetrads with micronucleus.

Pollen fertility

Pollutants and different environmental stresses have played a major role in influencing the pollen viability and pollen size of different plant species (Gottardini et al. 2004, Higashitani 2013, Rezanejad 2013, Paupière et al. 2014; Rani and Kumar, 2016.).

The viability of pollen grains of *S. inflata* was determined by staining method. In the present study, an attempt was made to assess the effect of Zn and Pb on pollen of *S. inflata* growing at polluted and unpolluted sites. Results for pollen viability are presented in tables 1. The lowest number of viability was observed at the polluted population whereas the highest number was observed at unpolluted population (table 1).

The percentage of pollen viability for *S. inflata* in polluted population and control were 52% and 95%, respectively. Pollen viability was found to be proportional to the present heavy metals in given sites. Figure 2 shows viable and non-viable pollen grains of *S. inflate.*

Base on heavy metals emissions, remarkable effects on pollen fertility of species under study were observed. Viable and fertile pollen grains were found to be darkly stained and round in shape while non-viable pollen grains were found to be transparent and have shriveled structure. Pollen viability of plant species studied was found to be correlated with the heavy metals of sites where these plants were growing.

Pollen viability consider as a good markers of the course of microsporogenesis. Normal meiosis produces regular pollen grains in size with highly viable, but disturbed meiosis reduces pollen viability and causes variability of pollen grain 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 lead and zinc in surface waters and soils of the region. So that the amount of lead and zinc heavy metals in the samples of water and soil is exceeded from standard.

Cytological study of *S. inflata* clearly show that the percentage of PMC with meiotic abnormalities in the plants growing in polluted areas is distinctly higher as compared to the control specimens. Present investigation on *S. inflata.* confirms that heavy metal lead and zinc acts as a strong mutagenic agent in plants. The percentage of abnormalities increased from 3.22% in the control to 60.73% in the case of plants in polluted area. Higher percentages of meiotic abnormalities were observed in meiosis I as compared to meiosis II stage and most of the abnormalities have been observed at metaphase-I, II and anaphase-I, II stages of meiosis.

A comparison of total meiotic abnormalities in test and control specimens show that the maximum meiotic abnormalities in plants exposed to pollutants is 39.96% while the maximum of abnormality in control plants is 7.8% (table 1). Among these abnormality sticky chromosome was the most frequently observed chromosomal aberration in the polluted population of *S. inflata.* furthermore, the frequency of remaining abnormalies was in the following order:

sticky chromosome > cytomixis > Laggards > desynapsis > bridge > tripolar > micronucleous > B chromosome > pentapolar.

Chromosome stickiness and laggards that found in polluted population with high percent, may be caused by genetic and environmental factors (**Pagliarini, 2000**). Laggards may produce micronuclei, if they fail to reach the poles in time to be included in the main telophase nucleus (Koduru and Rao, 1981; Utsunomiya et al., 2002), 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).

B-chromosomes only found in polluted population. These accessory chromosomes when present in high numbers affect negatively the growth and vigor of the plants (Jones and Houben, 2003).

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. Desynapsis occurs either due to the action of recessive disorganization genes in a homozygous situation or early chiasma terminalization which 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 producing aneuploid gametes causing reduction in pollen fertility of plants (Veilleux 1985; Sheidai et al. 2007, Ranjbar et. al. 2012)

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 (Gomurge, 2000). The thickness of bridges and the number of chromosomes involved in their formation varied among different meiocytes of test population. 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 fibers or late chiasma terminalisation or may even result from low chiasma frequency or from the presence of asynaptic or desynaptic genes (Nicklas and Ward 1994, Pagliarini 1990). 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).

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 cells or multipolar 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 localities has also showed higher percentages of pollen sterility. The high percentage of stained pollen grains (95%) was recorded for control population of *S. inflata*. This result is predictable based on meiotic behaviour data and of the low percentages of irregularities in this population (table 3). So in population that chromatin exchange in their PMCs does not occur or occurs at a very low frequency and other meiotic abnormalities are almost negligible, most of the pollen grains are fertile. In contrast, a low percentage of pollen viability (52%) in test population of *S. inflata* can be explained by having high percent 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 affects the meiotic course considerably and results in reduced pollen viability. Previous studies also showed that there is a direct relationship between occurrence of cytomixis and other abnormality with reduced pollen viability (Lattoo et al. 2006; Singhal & Kumar 2008; Ranjbar et. al. 2011; Zoair et. al. 2012).

In conclusion, the present study showed that the lead and zinc amount in soil and water are so much more than standard and have considerable effects on increases chromosomal aberrations in pollen mother cells of *S. inflata*; so perhaps it can be concluded that these heavy metals may exhibit genotoxic effect on plant. In addition to this, 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 cell division of pollen mother cells of *S. inflate*.