

NICOSULFURON GENOTOXICITY ON GERMINATED MAIZE (*ZEA MAYS* L.) GRAINS

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ABSTRACT

Nicosulfuron (NS) is a chemical substance extensively used on maize (*Zea mays* L.) production as weed-killer (herbicide). It is very effective and destructive on weeds, but genotoxic effects of this herbicide on *Z. mays* has not been investigated. It is aimed with this study to investigate the genotoxic effects of NS herbicide on roots of germinated *Z. mays* grains. The NS was performed different doses (50, 125, 250, 500 and 750 ppm) during 24, 48 and 72 hours. DNA isolation from root samples was carried out using the CTAB method. DNA fragments from *Z. mays* genome were amplified by PCR technique using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) primers. In addition, root terminal cells of *Z. mays* were stained using 2% orcein and morphologies of cell nucleuses were investigated. Analyzed electrophoresis profiles of RAPD and ISSR-PCR have clearly indicated some changes such as the appearance of new bands or disappearance of bands as compared to the control. RAPD and ISSR-PCR analyses were clearly revealed that NS causes serious DNA sequence changes on *Z. mays* genome. Additively, some nuclear alterations were observed like micronuclei, irregular nucleus, nuclear buds, etc. depending on the increase of herbicide dose and exposure times.

KEYWORDS:

Nicosulfuron, *Zea mays* L., Genotoxicity, ISSR-PCR, RAPD-PCR, Orcein cell stain.

INTRODUCTION

Nicosulfuron (2-(4,6-dimethoxy-pyrimidin-2-ylcarbonyl sulfamoyl) - *N*, *N*-dimethylnicotinamide) is an herbicide often used in maize farming [1]. It is a selective herbicide used against harmful plants in maize fields as annual or perennial grassy weeds and some broad-leaf weeds plants like *Amaranthus retroflexus* L., *Chenopodium album* L., *Digitalis sanguinalis* (L.) Scop., *Echinochloa crus-galli* (L.) P. Beauv., *E. colonum* (L.) Link, *Portulaca oleracea* L., *Setaria viridis* (L.) P. Beauv., *Solanum*

nigrum L., *Sorghum halepense* (L.) Pers. and *Xanthium strumarium* L. [2-4]. Nicosulfuron (NS) rapidly penetrates to weeds via leaf after its application. Then, development of weeds halts and leafs transform to red-purple color. Weeds turn yellow and dry among 15-20 days [3, 4]. The indiscriminate use of various pesticides or herbicides in agriculture has caused environmental contamination and changes in genetic structure of organisms in the environment [5-8]. Regular application of herbicides in agricultural practices is a potential threat to genetic constitution of crop plants and animals [7]. Therefore, investigating the potential genotoxic effects of herbicides both on use for or against the plants have an importance. In this manner, PCR performed RAPD and ISSR primers for detecting the genotoxicity of herbicide treated plant systems are gave reliable, evaluable and significant outcomes.

To date, there is not any study about genotoxic effects of NS herbicide on *Z. mays*. In this study, it is aimed to investigate the potential genotoxic effects of the herbicide on *Z. mays*, using RAPD and ISSR-PCR techniques supported by morphologically investigations on germinated maize grains root tip cell nucleuses.

MATERIALS AND METHODS

Preparation of Plant and Herbicide Material. It was used commercially obtained maize grains (Tuğba Ltd. Company, Aydın, Turkey) preferred in maize industry as plant material in Turkey. Maize grains were germinated in the sterile petri dishes using filter paper wetted with sterile water at room temperature. The germinated maize had about 1 cm root length were used by herbicide treatments. Nicosulfuron herbicide (Baturam, Pileryum Ltd. Company, Turkey) was diluted with sterile distilled water at different concentrations in 50 ppm, 125 ppm, 250 ppm, 500 ppm and 750 ppm. Each concentration was performed for 24 h, 48 h and 72 h. The control test was performed with only sterile water. Maize roots were stored at -20°C for DNA isolation until use. For cell staining, maize root tips were stored in fixative solution (3:1 ethanol, glacial acetic acid) at +4°C.

TABLE 1
RAPD primers tested for amplification of maize genomic DNA.

Tested Primers	Primer Sequence (5'-3')	T _m (°C)	Appositeness
P-5	CTGCGACGGT	34	-
P-9	GGGAAGAGAG	32	-
P-11	GGCCGATGAT	32	-
P-13	ACCGCCTTGT	32	-
P-14	CAGCACTGAC	32	-
P-16	TGGTGGCCTT	32	+
P-17	GTAGCACTCC	32	-
P-21	ACGGTGCCTG	34	-
P-23	CGCCCAAGCC	36	+
P-24	CGCCCTGGTC	36	+

TABLE 2
ISSR primers tested for amplification of maize genomic DNA.

Tested Primers	Primer Sequence (5'-3')	T _m (°C)	Appositeness
ISSR-01	AGAGAGAGAGAGAGAGG	52.8	+
ISSR-02	GAGAGAGAGAGAGAGAT	50.4	-
ISSR-06	GAGAGAGAGAGAGAGAC	52.8	-
ISSR-10	GGGTGGGTTGGGGTG	58.8	-
ISSR-27	GTGCGTGCGTGCGTGC	59.4	-
ISSR-829	TCTCTCTCTCTCTCG	52.8	-
ISSR-847	CACACACACACACARC	53.7	-
ISSR-861	ACCACCACCACCACCACC	60.5	+
ISSR-862	AGCAGCAGCAGCAGCAGC	60.5	-
ISSR-866	CTCCTCTCTCTCTCTCTC	60.5	-

DNA Isolation. Maize roots stored at -20°C were ground to fine powder and transferred to eppendorf tubes. Total genomic DNA was extracted from root tips by a modified hexadecyltrimethyl ammonium bromide (CTAB) method [9]. The quantity and quality of DNA were determined by using a Nanodrop Spectrophotometer (Shimadzu, Kyoto, Japan). DNA samples were diluted with sterile distilled water to 2 ng/μL.

RAPD-PCR Analysis. RAPD analysis was performed according to the method of Williams et al. [10]. A set of 10 random 10-mer primers was purchased from Thermo Inc. (Burlington, MA, USA). For selection of suitable primers, an initial primer screening was carried out with these primers after screening. The three primers (Table 1) produced clear, reproducible, unique polymorphic banding patterns and generated RAPD profile variations that displayed the band intensity, disappearance of bands and appearance of new bands of amplified DNA in the NS-treated genomic DNA samples.

PCR amplifications were performed in 25 μL of reaction mixture containing 10 ng of template DNA, 1×Taq polymerase buffer and 1 U of Taq polymerase (FIREPol, Solis BioDyne, Estonia), 1.5 mM MgCl₂, 1 mM dNTP, and 1 μM of primer. Amplifications were carried out in Thermo Arktik Thermal Cycler (Thermo Scientific, USA) that was programmed for the initial denaturation step 85°C for 15 sec, 95°C for 5 sec and at 92°C for 1 min 55 sec, followed by 45 cycles of 95°C for 5 sec, 92°C for 55 sec, 32-36°C for 1 min, 72°C for 2 min, and a final elongation at

72°C for 7 min. PCR reactions were repeated twice to ensure reproducibility of amplified products. The PCR products were separated on a 1.3% agarose gel containing ethidium bromide (0.5 μg/mL) and photographed with the Gel Logic 212Pro imaging system (Carestream, USA). Molecular weights of PCR products were estimated using a 100 bp DNA Ladder (Fermentas).

ISSR-PCR Analysis. ISSR analysis was performed according to the method of Wongsawad and Peerapompisal [11]. ISSR-PCR analysis was performed according to the sequences obtained from the University of British Columbia (Canada). For selection of suitable primers, an initial primer screening was carried out with 10 primers after screening. Two primers amplified clear, reproducible and unique polymorphic banding patterns and they were used for further analysis (Table 2).

Amplification of ISSR fragments from *Z. mays* genomic DNA was performed in a total reaction volume of 25 μL containing 8 ng of template DNA, 1× Taq polymerase reaction buffer, 1.5 mM MgCl₂, 0.1 mM each of dNTPs (dATP, dCTP, dGTP, and dTTP), 0.2 mM primer and 1 U of Taq DNA polymerase (FIREPol, Solis BioDyne). Amplifications were performed in Thermo Arktik Thermal Cycler (Thermo Scientific): 4 min denaturation at 94°C and 45 cycles of 45 sec each denaturation at 94°C, 50 sec annealing at 47-60.5°C (primer depended) for ISSR amplification, and a 1.5 min extension at 72°C, followed by a final extension at 72°C for 7 min. PCR-amplified fragments were separated on 1.2% agarose

gel containing ethidium bromide (0.5 µg/mL). Gels were visualized under UV light and digitally photographed with Gel Logic 212Pro imaging system (Carestream). Molecular weights of ISSR-PCR products were estimated using a 100 bp DNA Ladder (Fermentas).

Cell Staining. The fixed maize root tips were stained with 2% orcein dye for 10 min and slightly squashed to accomplish a better spreading of the material over the slide. The material was analyzed in light microscope and digitally photographed (Olympus BX51 binocular light microscope, with 1000 magnification). The mitotic index (MI) was calculated by number of cells which undergone mitotic divisions divided by total cells in a microscopic field into 100 [12].

RESULTS AND DISCUSSION

Due to the usage of NS herbicide on maize farming, commercial maize (*Z. mays*) was preferred as model organism to detection of genotoxic effects

of the herbicide. Three RAPD primers (p16, p23 and p24) produced unique polymorphic banding patterns among 10 primers. Analysis of electrophoresis profiles of these three primers in the NS-treated genomic DNA samples indicated apparent changes such as the appearance of new bands or disappearance of bands as compared to the control (Figure 1). Also, the variation observed in RAPD profiles, time and concentration-dependent. Depend on RAPD analysis results it was detected that NS caused DNA damages in genome of maize root cells. Higher plants are recognized as excellent genetic models to detect effects of all environmental mutagens or pesticides and are frequently used in monitoring studies [6, 7, 13-15]. RAPD-PCR technique has been used successfully to detect various types of DNA damage and mutations in organisms induced by various types of contaminants [16-18]. Furthermore, many studies have reported that the RAPD assay is more sensitive than classic tests such as the comet and micronucleus assay, as RAPD analysis is capable of detecting temporary DNA changes at lower concentrations of pollutants [19-21]. RAPD also shows to be a powerful means of detecting a broad range of DNA damage due to environmental contaminants [22].

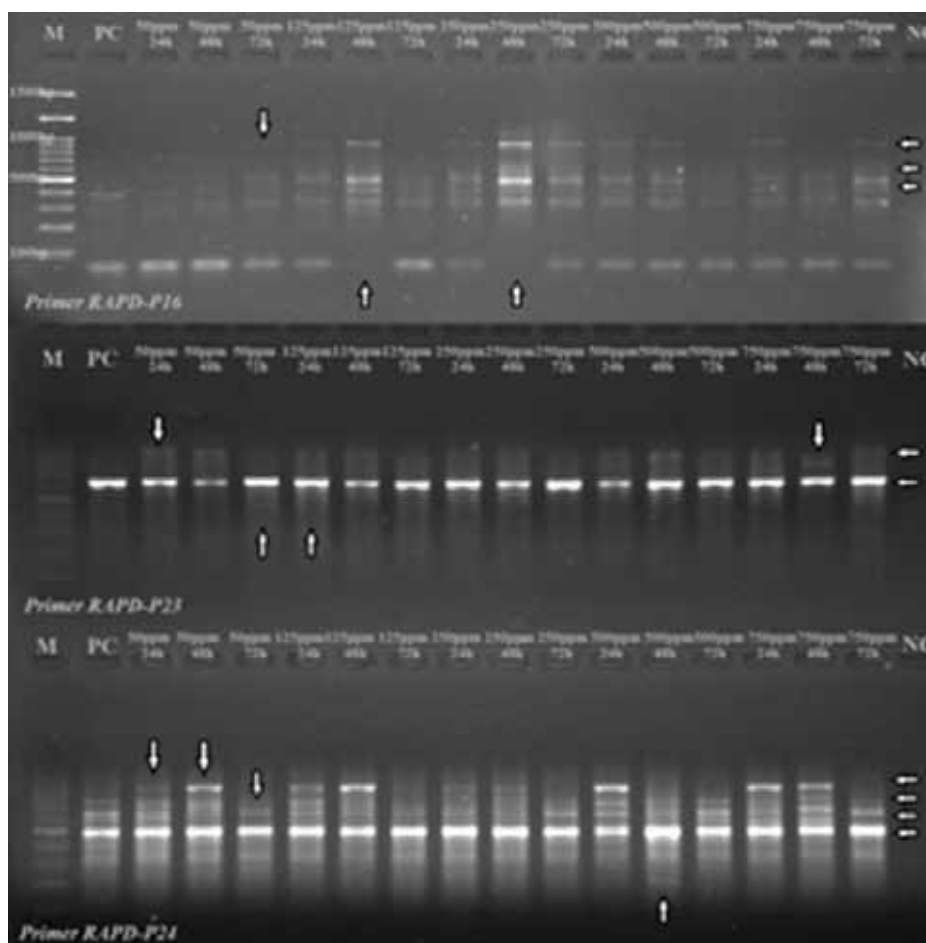


FIGURE 1

The RAPD banding patterns amplified with p16, p23 and p24 primers from maize genomic DNA.

M: 100bp DNA ladder (Fermentas), PC: positive control, NC: negative control. The appearance of new bands or disappearance of bands as compared to the positive control were showed with white arrows.

There are plenty of RAPD-PCR-based studies about detection of genotoxic effects of pollution agents on plants or animals. Aslam et al. [23] shown genotoxic effects of heavy metal cadmium on *Cap-sicum annuum* L. plant (an important spice crop of India) using RAPD-PCR method. Ghosh et al. [24] preferred analyses of RAPD band profiles for detection of genotoxicity of multi-walled carbon nanotube on *Allium cepa* root cells. Salem et al. [22] declared that use of RAPD-PCR technique is an advantage and first evidence in detection of fish genotoxicity induced by heavy metals from landfill leachates. Mentioned studies are supportive for the present study and the preference of RAPD primers to detect-ing an herbicide genotoxicity is an advisable ap-proach.

ISSR-01 and ISSR-861 primers produced unique polymorphic banding patterns among 10 primers. The amplified ISSR banding profiles of NS-treated maize genomic DNA showed a consistent in-crease in appearance and disappearance of bands with increasing concentration of the herbicide (Fig-ure 2). There are some changes in the ISSR banding patterns between control and NS-treated groups as changes in the number, the position and the intensity of the bands. The banding profile alterations had an increase according to the exposure time of herbicide. Accompanied by these alterations support that NS causes DNA damages in the genome of maize root cells. The ISSR-PCR evaluations are supported by RAPD-PCR results. Recent studies show that ISSR

technique is very popular in genotoxicity analyses. PCR-based molecular markers are widely used as tools to detect and characterize soma-clonal varia-tions and induced mutations at the genome level [25]. Especially in the mutation breeding, ISSR-PCR technique provides a reliable and rapid detection means for the screening of mutants [26]. ISSR-PCR technique is a popular analysis method recently used in detection of DNA damage and alterations in or-ganism genome exposed to hazardous materials. Therefore, we performed this technique for support-ing and confirmation of our RAPD-PCR results. ISSR-PCR technique is more sensitive and reproducible to study polymorphism induced by environmen-tal stress [27, 28]. Bajpai et al. [28] used ISSR-PCR technique for investigation of chromium (+VI) geno-toxicity in *Pyxine cocoes*. Genotoxic effects of glyphosate herbicide on *Bulinus truncates* were in-vestigated by Bakry et al. [29]. It was declared that the changes in the number, position and intensity of DNA bands induced by glyphosate herbicide may be attributed to the fact that the herbicide can induce genotoxicity through DNA damage. Similarly, the changes in the ISSR banding pattern between control and NS-treated groups were obvious, including the changes in the number, the position and the intensity of the bands. Therefore, we propose that NS causes DNA damages in genome of maize root cells. This result was supported with results of the other molec-ular technique (RAPD-PCR analyses) in the present study.

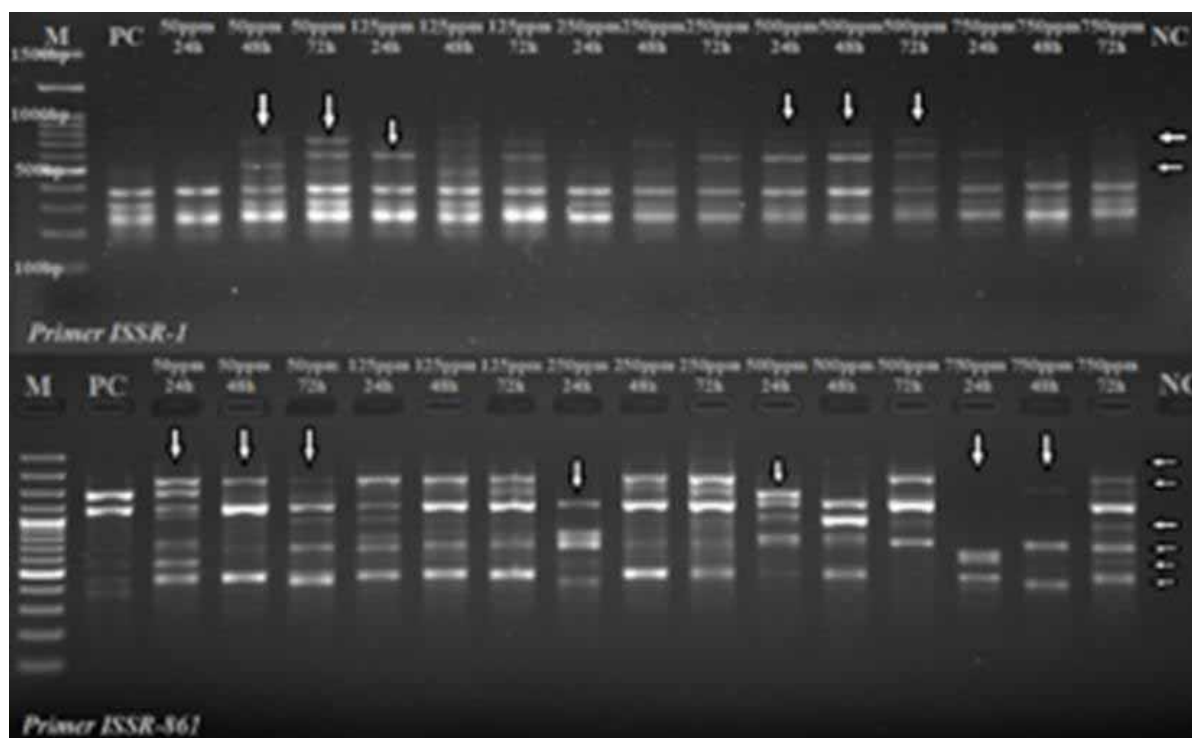


FIGURE 2

The ISSR banding patterns amplified with ISSR-01 and ISSR-861 primers from maize genomic DNA. M: 100bp DNA ladder (Fermentas), PC: positive control, NC: negative control. The appearance of new bands or disappearance of bands as compared to the positive control were showed with white arrows.

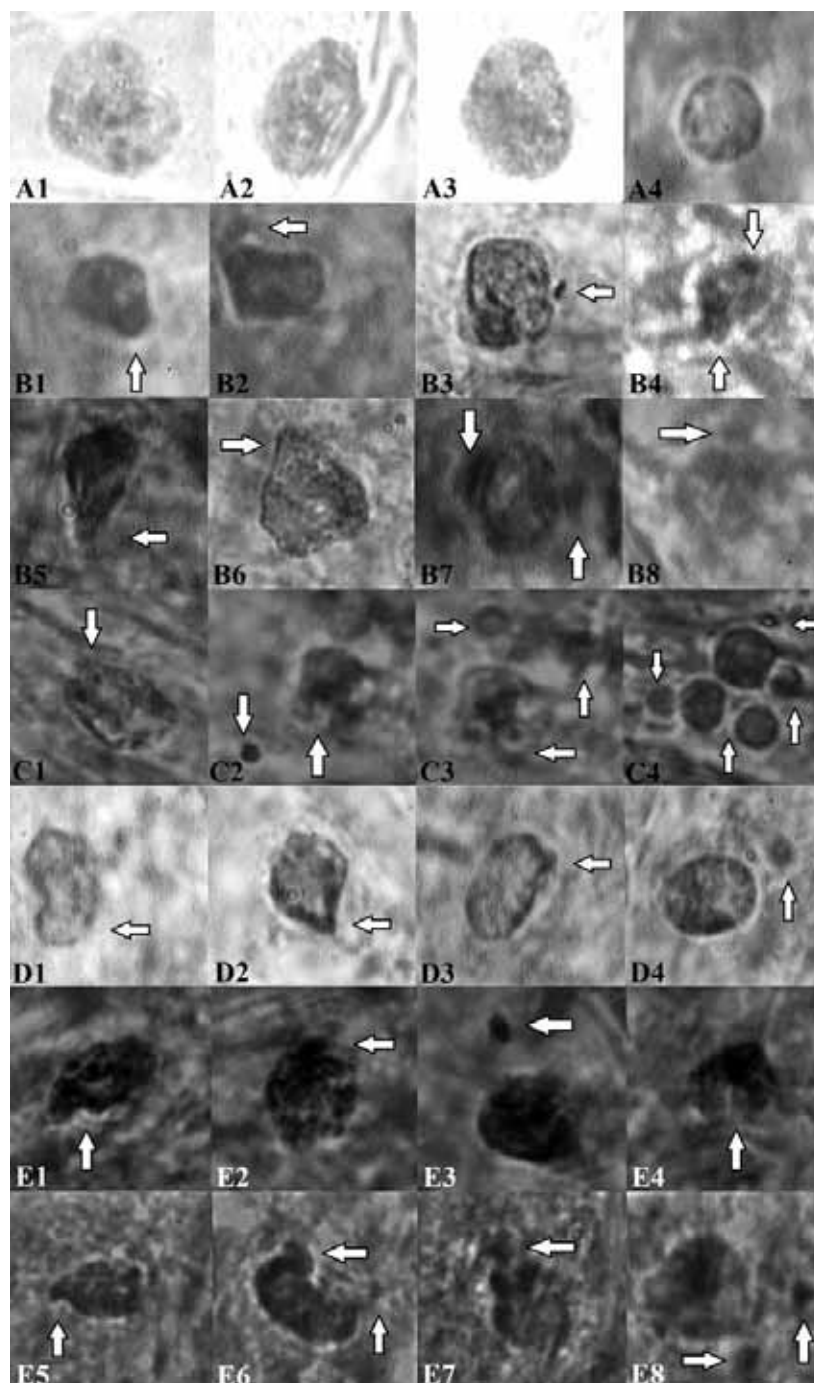


FIGURE 3

Nuclear alterations induced by nicosulfuron herbicide.

A: control group, B: 125 ppm test group B1: irregular nucleus (24h), B2-B4: nuclear bud formed by excess material (48h), B5-B6: irregular nucleus, B8: nuclear bud (72h), C: 250 ppm test group, C1: irregular bud (24h), C2-C3: micronucleus with nuclear bud (48h), C4: abnormal mitosis, micronucleus and mini cell formations (72h), D: 500 ppm test group, D1: irregular nucleus like kidney and nuclear bud (24h), D2-D3: nuclear buds (48h), D4: micronucleus formation (72h), E: 750 ppm test group, E1-E2: irregular nucleus and nuclear bud (24h), E3-E4: micronucleus formation and nuclear bud (48h), E5: irregular nucleus, E6: nuclear bud like kidney, E7: micronucleus formation, E8: micronucleus (72h).

The nuclear morphological alterations characterized by the presence of irregular nuclei, nuclear buds and micronuclei were observed (Figure 3). Irregular nuclei and nuclear buds were observed in herbicide concentrations between 125-750 ppm, and

all exposure times of these concentrations. The irregular nuclei were different formations as horn (Figure 3: B4, B6, D2, D3, E5), kidney (Figure 3: D1, E6) and horseshoe (lobated nucleus) (Figure 3: E3, E4, E7). It is determined a significant augmentation in

the frequency of nuclear alterations, according to increase in concentration and time. Micronucleus formation had observed since 250 ppm concentration. Also, it was observed that frequency and number of micronucleus in root cells increased according to as the gradual increase in concentration and exposure time to NS herbicide (Figure 3). The evaluation of nuclear abnormalities, along with the chromosome aberrations, has shown to be a sensitive analysis, for making the investigation of test agent actions even more accurate in relation to their effects on the DNA of exposed organisms. The presence of lobulated nuclei and polynuclear cells can indicate a cell death process [13]. Also the alterations such as micronuclei can be and their induction is commonly used to detect genetic damages derived from exposure to a mutagenic agent [5]. Micronuclei tests are considered one of the most promising tests for the evaluation of environmental mutagenicity, since it is efficient, simple and fast [5, 30, 31]. It is known that the micronuclei analysis is a sensitive method to detect environmental mutagens [30, 31, 32].

It observed that the nuclear morphological alterations characterized by the presence of irregular nuclei, nuclear buds, and micronuclei as shown in Figure 3. Irregular nuclei and nuclear buds were observed in herbicide concentrations between 125-750 ppm, and all exposure times of these concentrations. The irregular nuclei were different formations as horn, kidney and horseshoe (lobated nucleus). Similar formations were showed by Fernandes et al. [5, 6]. It is determined with present study a significant augmentation in frequency of nuclear alterations, according to increase in concentration and time. Micronuclei formation had observed since 250 ppm concentration. Also it was observed that frequency and number of micronucleus in root cells increased according as the gradual increase in concentration and

exposure time to NS herbicide (Figure 3). Fernandes et al. [6] observed a concentration-dependent increase in frequency of micronucleus and nucleus alterations. Micronuclei analysis has been considered by many authors as the most effective and simplest endpoint to analyze the mutagenic effect promoted by chemicals [30-32]. According to Leme et al. [13], this is due to the fact that micronuclei result from damages, not or wrongly repaired, in the parental cells, being easily observed in daughter cells as a similar structure to the main nucleus, but in a reduced size. Thus, micronuclei arise from the development of some chromosome aberrations, for instance, chromosome breaks and losses.

After the MI results were analyzed, a concentration-dependent MI inhibition was observed. This is indicated the cytotoxic potential of NS herbicide in maize. MI was the lowest (8.25) at the 750 ppm concentration and 72 h. With increasing concentration and exposure time of the NS herbicide, concentration and time dependent decrease in the mitotic index was noticed. The effect of NS herbicide concentration and exposure time on mitotic index was significantly different for 50, 125, 250, 500 and 750 ppm as compared to the control (Figure 4). The decrease in mitotic activity probably indicates mito-depressive effect of NS herbicide.

Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA [32]. The interactions between herbicides and DNA molecule may cause various nuclear anomalies in plant cells. It is clearly showed that NS herbicide causes some nuclear anomalies in maize root tip cells.

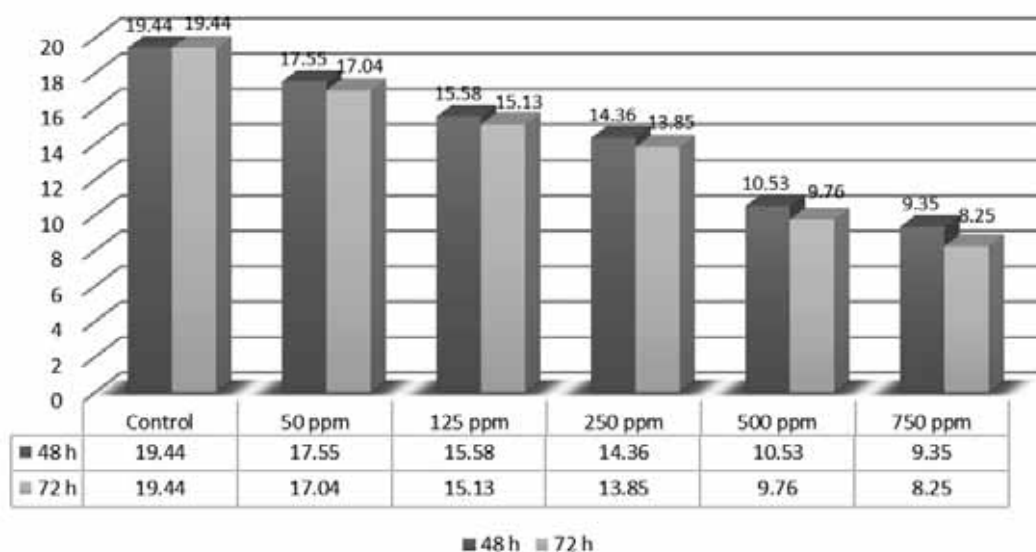


FIGURE 4

Mitotic index decreasing of *Allium cepa* root tips control and nicosulfuron herbicide treated groups (for 48 and 72 hours with 50, 125, 250, 500 and 750 ppm).

CONCLUSIONS

Herbicides are used extensively to improve crop yields in conventional farming. Effective chemicals against different plant pathogens are extensively preferred. Use of high dose herbicide causes contamination and accumulation in the environment and may lead acute or chronic toxic effects in organisms. It is known that widespread use of herbicides has caused serious health problems and fatalities in the world. The induction of DNA damage with herbicides can potentially cause to adverse reproductive outcomes in humans such as cancer and many other acute or chronic diseases. Genetically damaged crops may potentially have a negative effect on both natural ecosystems and human health. It is not known genotoxic effects of most synthetic herbicides on agricultural crops. The NS is a synthetic herbicide and uses against weeds in maize farming. It is detected that NS herbicide causes many alterations and aberrations on maize genomic DNA. This determination was clearly supported by experimental results using molecular and microscopy techniques. Dramatically, genotoxic effect of NS was observed in recommended using doses. Incorrect use of this herbicide by farmers may increase the risk even more. The seriously increase in genotoxic effect level of highest dose (750 ppm) used in this study verify the risk. Exposure time to herbicide is also an important criteria caused increasing the DNA damage. Harmful effects of DNA damage occurred on exposed organisms may continue even after stopping the exposure. Dangerous effects of this herbicide on genetic material may also threaten the other animals feed with maize. It is suggested that NS use in low concentration (<100 ppm) and control possible dose accumulation through herbicide treatment. According to MI analysis results, the NS herbicide exhibited cytotoxicity by decreasing the mitotic index in a dose and time dependent manner. This proves that the NS herbicide may have mito-depressive effect on maize.

ACKNOWLEDGEMENTS

This study was supported by Anadolu University Scientific Investigation Projects Commission (Project No: 1404S117).

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Received: 23.04.2018
Accepted: 31.08.2018

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