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Effects of Spent Drilling Fluids from Natural Gas Fields on Seed Germination and Root Development of Maize (*Zea mays* L.)

Zhe Wang [1,2] and Mingde Hao [1,*]

[1] Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources, Yangling 712100, China; wangzhe4048@163.com
[2] University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: mdhao@ms.iswc.ac.cn; Tel./Fax: +86-29-8701-2210

Abstract: The use of drilling waste for land reclamation is a cost-effective way to improve soil fertility and to decrease landfills. However, the potential phytotoxic and cytotoxic effects of this waste on crops have not been investigated in detail. Here, we evaluated the toxicity of spent drilling fluids (SDFs) from a natural gas field using the non-target plant *Zea mays* L. (maize). Four different concentrations of SDFs (2%, 4%, 6%, and 8%, *w*/*w*) were used to test the toxic effects in two soils (aeolian and loessal). Different endpoints, including germination, root elongation, reactive oxygen species (ROS) accumulation, antioxidant activity, mitotic index, and chromosomal abnormalities, were used to test the effects of SDFs after four days of exposure. Higher levels (≥6%) of SDFs inhibited seed germination and root growth, and altered the oxygen status by increasing hydrogen peroxide (H_2O_2) and inhibiting superoxide ion (O_2^-) accumulation in the roots. SDFs-induced oxidative stress caused member damage, exacerbated cell injury, and reduced cell viability in the roots, compared with those untreated plants. The plants responded to high SDFs levels (≥6%) by upregulating antioxidants such as peroxidase, superoxide dismutase ascorbate peroxidase, and proline. A reduction in the mitotic index and induction of chromosomal abnormalities in root meristematic cells were indicators of the cytotoxicity of SDFs in maize seedlings. The upregulation of antioxidants due to the change of ROS and the induction of chromosomal abnormalities were more severe in roots grown in aeolian soil than in those grown in loessal soil. The present results provide insight into the mechanism underlying the phytotoxicity and cytotoxicity of SDFs and have implications for land reclamation to minimize deleterious effects on non-target crops.

Keywords: spent drilling fluids; land reclamation; seed germination; cytotoxicity; chromosomal abnormalities

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1. Introduction

Drilling for exploration and production in the oil and gas industry generates large volumes of waste, which constitutes the second-largest volume of waste behind produced water [1,2]. Different varieties of drilling fluids, such as water-, oil-, and synthetic-based, are used at the well sites. Upon completion of a gas and oil well, the spent drilling fluids (SDFs) is brough back to the ground and the disposal of SDFs becomes a major challenge especially when reconditioning, recycling and storage. Water-based drilling fluids are the most widely used system, and are considered less expensive than the other two types of fluids. The water-based drilling fluids typically consist of a base fluid, bentonite clay, organic material (lignite or lignosulphonates), weighting agents (e.g., barium sulphate), and various additives, to allow for good performance [3]. Some of the additives introduce potentially toxic compounds into the fluids, such as biocides, oil, completion or stimulation fluid components, corrosion inhibitors, and reservoir fluids, due to their different biodegradability. To meet the economic and environmental sustainability requirements

for industrial waste management, such as minimizing waste and decreasing disposal cost, various synthetic macromolecule polymers based on natural plants, such as cellulose and starch, are used in gas exploration to improve the properties of drilling fluids [4–6]. These natural materials are pollution-free and biodegradable, and are thus beneficial for alleviating the potential environmental risks [7–9]. This will greatly increase the possibility of SDFs as a soil amendment for land reclamation.

Alternatives for the disposal of drilling fluids, including chemical stabilization and solidification, use in construction, or transport to an approved landfill, were used in the oil and gas industry in China. Compared to other disposal options, land reclamation is a relatively effective and attractive strategy for managing the drilling fluids [10]. This process is used to treat and/or dilute the potentially harmful constituents (petroleum hydrocarbon, soluble salts, and/or plant-available trace metals) contained in SDFs. In addition, the use of SDFs for land reclamation is a practical and cost-effective way to improve soil fertility and decrease landfills. There are few studies addressing the impacts of SDFs on plant growth, which includes plant biomass, grain yield, nutrients, and trace elemental uptakes in plant tissue. Bauder et al. (1995) showed that SDFs applied at controlled rates were beneficial to the growth of sorghum and maize by increasing the Fe and Zn concentrations in plant tissues [11]. Bauder et al. (2005) showed that a single application of SDFs at rates up to 94 Mg ha^{-1} did not affect wheat yield compared to a negative control [12]. Zvomuya et al. (2011) demonstrated that soluble nutrient elements, such as N, P, and Mg in above-ground plant tissues increased with increasing loading rate in samples taken 45 days after SDFs application [13]. Yao et al. (2014, 2015) showed that twice-used SDFs significantly increased barley above-ground biomass, and all SDFs treatments increased the available potassium relative to that in the control [14,15]. Despite studies investigating the effects (negative and/or positive) of SDFs on various crops, comprehensive data on the underlying mechanism are limited, and the effects on the early growth of crops remain unclear. In agriculture, seed germination and early seedling growth are the two critical stages for the establishment of crops, and plants are highly sensitive to abiotic stressors, such as salinity, heavy metals, polycycle aromatic hydrocarbons [16–20]. The disruption of germination and/or seedling establishment indirectly affects plant yield, and a delay or reduction in plant emergence can affect final productivity. While land reclamation can be a relatively effective and promising option for the management of SDFs, the direct exposure of seeds to chemical agents in SDFs is a potential risk. Therefore, a comprehensive understanding of early seedling growth would establish a reference point and facilitate management decisions to optimize land applications of SDFs.

The objective of this study was to investigate the phytotoxic and cytotoxic effects of water-based SDFs, collected from a natural gas field, on maize (*Zea may* L.). The phytotoxicity was evaluated through germination, growth, and root elongation using a selected crop. The cytotoxicity was assessed by the mitotic and chromosomal aberrations index. This study provides meaningful information on the phytotoxicity of water-based drilling fluids in plants, which can be helpful for formulating future management strategies to minimize the deleterious effects of SDFs on non-target crop plants. Furthermore, the parameters of cytotoxicity can be used as endpoints to evaluate the ecotoxicological effects of water-based SDFs in land reclamation on the agroecosystem.

2. Materials and Methods

2.1. Sample Collection and Experimental Design

Water-based SDFs samples were collected from an active gas well site in the first gas production field of the Changqing Oilfield, located in the Ordos Basin. The operating area is an arid and semi-arid monsoon climate, and the soil types mainly include aeolian and loessal soil. The maize (*Zea mays* L.) is an agricultural crop, widely grown in this operating area. A completely randomized design was used in the greenhouse with different soils and SDF concentrations. The fact that many wells are drilled on agricultural lands raises concerns with the public with respect to selecting disposal methods. Therefore,

two typical soils (aeolian and loessal) with physicochemical properties generally found in this operating area could be used for reclamation with SDFs. The SDFs and soil used for treatment were air-dried and obtained a homogenized solid state before the experiment. The loading rates of SDFs in this experiment referred to previous research [11]. Spent drilling fluids were applied to plastic pot equivalent to 20, 40, 60, and 80 dry g of drilling fluids per kg soil (2%, 4%, 6%, and 8%) for the next germination assays.

2.2. Physicochemical Analysis

The physicochemical analysis of SDFs and soil (air-dried samples) was measured according to the methods of the Soil Analysis Standard. Water-soluble cations, sodium (Na), calcium (Ca), magnesium (Mg), pH, and electrical conductivity (EC), were measured from saturated extracts, and the sodium adsorption ratio (SAR) was calculated from Na, Ca, and Mg concentrations. Trace metals in the SDFs restricted in the Regulation of the Ministry of Ecology and Environment (China/GB 15618-2018), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn), were determined by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy), and mercury (Hg) content by CV-AAS (Cold Vapor-Atomic Absorption Spectrometry), as described in a previous article [21]. The specific constituents of concern (COC) (e.g., Cl^-, SO_4^{2-}, NO_3^-) in the water extracts of the SDFs were measured using an ion chromatograph ICS-1100 (Thermo Fisher Company), working in an external water mode.

2.3. Germination Assays

Maize XianYu 335 (*Zea mays* L.) seeds were surface-sterilized in 2.5% sodium hypochlorite for 5 min and thoroughly rinsed with distilled water before sowing. Germination assays were performed in 12 cm Petri dishes (20 seeds per dish, five replicates), using a mix of SDF and soil for different treatments. Dishes were sealed and incubated in a germination chamber equipped with daylight fluorescent lamps at 28 °C. The first 24 h were carried out in darkness. The air moisture ranged from 55% to 60%. Seed germination was indicated by the emergence of the coleoptile exposed to the soil surface, and plants were scored daily for 9 days. Seed germination kinetics were determined using a theoretical model described as the following formula [22]:

$$y(t) = y_{\max}/[1 + \exp(\frac{4\mu}{y_{\max}}(\lambda - t) + 2)] \quad (1)$$

Three parameters of germination kinetics were obtained from this model: the length of the lag phase (λ), during which the seeds acquired the aptitude to germinate; after the latency, the probability of germination rates (μ) of germination per unit time was the same for all seeds and constant with time; the maximum germination ($y_{\max}$), the plateau reached by $y(t)$ measured the number of viable seeds. $y(t)$ is the number of germinated seeds at time t. The time for the germination of 50% of the viable seeds was determined by

$$T_{50\%} = \lambda + \ln(2)/\mu \quad (2)$$

2.4. Root Growth

Root elongation was recorded at four days after sowing. According to the growth status of shoots, six seedlings were randomly selected that could represent the average growth of the plants from each Petri dish. A total of 30 seedlings were prepared to determine the length of the primary roots per treatment, using a digital pachymeter. Simultaneously, the numbers and lengths of adventitious roots were also recorded, the adventitious root length was defined as the length from the root tip to the base of the root. The primary roots from all germinated seeds were excised for biochemical, histochemical, and mitotic index (MI) evaluation.

2.5. Distribution and Accumulation of Superoxide Anion in Roots

For in situ localization of superoxide anion ($O_2 \cdot^-$) formation by nitroblue tetrazolium (NBT) staining [23], the primary roots were excised (at approximately 1 cm from the apex) and immediately incubated in 6 mM NBT in 20 mM Tris-HCl buffer (pH 7.0) at room temperature for 15 min. The reaction was stopped by the transfer of the primary roots to distilled water, and $O_2 \cdot^-$ was visualized as deposits of dark blue insoluble formazan compounds [24]. Each treatment was repeated at least five times with similar results. The absorption value at 560nm (OD560) was used to represent the content of $O_2 \cdot^-$, as previously described, with modification [25]. In brief, the primary roots (fresh weight of 1 g) were cut into pieces and homogenized in 3 mL of 20 mM Tris-HCl buffer (pH 7.0) and then centrifuged at 12,000× *g* for 5 min at 4 °C. The supernatant (2 mL) was mixed with 0.4 mL NBT solution, and incubated at room temperature for 1 h. The content of H_2O_2 was measured, as described earlier, and the value was calculated using the extinction coefficient of 0.28 uM^{-1} cm^{-1}, and expressed as μM g^{-1} FW [26].

2.6. Determination of Antioxidants Activity

Fresh samples (0.5 g) of the primary roots were ground with the help of a mortar, using liquid nitrogen, and homogenized in 50 mM phosphate buffer (pH 7.4) under chilled conditions. The homogenized mixture was centrifuged at 12,000× *g* for 10 min at 4 °C. These samples were subjected to analysis of the activity of enzymatic antioxidation, like superoxide dismutase (SOD; 1.15.1.1), guaiacol peroxidase (POD; 1.11.1.7), ascorbate peroxidase (APX; 1.11.1.11), catalase (CAT; 1.11.1.6), and the activity assays were performed following the methods described previously [27]. Proline contents were measured following the methods described earlier [28].

2.7. Lipid Peroxidation, Membrane Leakage, and Cell Viability Analysis

Levels of lipid peroxidation in primary roots were estimated using the product of Verma and Dubey (2003) [29]. Membrane permeability was used to evaluate cells injured, and electrolyte leakage in fresh roots was measured using an electrical conductivity meter, following the method of Shakir et al. (2018) [30]. Trypan blue exclusion methods were used to detect cell death [31]. An amount of 10 to 20 roots grown for 4 d in all treatments were immersed in a 0.4% solution of trypan blue (SOLARBIO) and incubated in the dark overnight and examined under a light microscope.

2.8. Cytogenetic Analysis

Root apices from 4-day-old seedlings were excised and immediately placed in formalde hyde–acetic acid–ethanol (FAA) fixative for 24 h, and then preserved in 70% ethanol for cytological analysis. The fixed roots were first treated with 1% (*w*/*v*) cellulase (R-10) and 6% (*w*/*v*) pectolyase (Y-23) (one gram cellulase or pectolyase dissolved in 100 mL of distilled water) at 37 °C for 2 to 3 h, and then the mitotic index was calculated following the methods described previously [32]. Five slides were prepared per treatment, with six fields per slide, to evaluate the presence of chromosomal aberrations (CA). Cytotoxic effects were evaluated by calculating the mitotic index (MI), and genotoxic effects were assessed by counting several types of CA observed in the meristematic cells.

2.9. Statistical Analysis

Data are expressed as the mean ± standard deviation. Statistical analysis was performed using one-way ANONA and multivariate analysis with JMP software 10.0 (SAS Institute). Differences between treated samples and controls were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Spent Drilling Fluids and Soil Properties Prior to Application

The SDFs used in this study were water-based drilling fluids, and the main components were bentonite, potassium polyacrylamide, carboxymethyl cellulose, carboxymethyl starch, sodium hydroxide, potassium chloride, calcium carbonate, sawdust, and limestone. The density of the SDFs was 1.68 Mg m^{-3}, and the content of solids was 21.6%. The pH, EC, SAR, and the specific COC in the water extracts of the SDFs are listed in Table 1. The water extracts of the SDFs contained 950.55 mg kg^{-1} chloride (Cl^-), 1551.65 mg kg^{-1} sulfate (SO_4^{2-}), and 12.44 mg kg^{-1} nitrate (NO_3^-). The heavy metal contents of the SDFs are presented in Table 2. The heavy metals in the SDFs did not exceed the limits established for the total content of toxic heavy metals in a soil environment.

Table 1. Selected properties of spent drilling fluids (SDFs) and soil prior to application.

Properties	Drilling Fluids	Soil	
		Aeolian	Loessal
pH (unitless)	9.93	9.21	8.86
EC (dS m^{-1})	7.66	0.16	0.24
SAR (unitless)	1110.76	3.21	4.36
Soluble Na (mg kg^{-1})	29920.00	16.13	19.76
Soluble Mg (mg kg^{-1})	1435.75	11.47	15.23
TN (g kg^{-1})	1.96	0.58	0.67
AK (mg kg^{-1})	1145.27	76.51	93.37
AP (mg kg^{-1})	1.59	5.38	5.64
TOC (g kg^{-1})	115.17	0.22	4.73
Cl^- (mg kg^{-1})	950.55	5.74	20.22
SO_4^{2-} (mg kg^{-1})	1551.65	3.64	8.08
NO_3^- (mg kg^{-1})	12.44	3.09	4.72

EC: electrical conductivity, SAR: sodium adsorption ration, TN: total nitrogen, TOC: total organic carbon, AK: available potassium, AP: available phosphorus, Cl^-: chloride, $SO4^{2-}$: sulfate.

Table 2. Heavy metals of spent drilling fluids determined by ICP-OES.

Elements		Zn	Cu	Cr	Cd	Ni	Pb	Hg
Content (mg kg^{-1})		506.7	23.5	99.83	0.11	15.8	34.4	-
CHN Regulation *	I	1200	500	500	3	100	300	3
	II	3000	1000	1300	4	200	1000	15

* Maximum permissible limits of two class risk control standards for soil contamination of agricultural land, according to the regulation of the Ministry of Ecology and Environment (China/GB 15618-2018).

3.2. Responses of Seed Germination to Spent Drilling Fluids

The maize germination parameters for all treatments are shown in Figure 1. Analysis of the maximum germination (A) and germination rates (μ) showed that the treatment with SDFs significantly ($p < 0.0001$) inhibited germination in a dose-dependent manner, compared with control conditions (no treatment) (F = 25.551 for A; F = 39.783 for μ; Figure 1A,B). Treatment with 8% SDFs caused 10.37% and 25.64% inhibition of A in loessal soil and aeolian soils, respectively, compared with the controls. Treatment with 8% SDFs caused a 35.81% and 65.07% reduction in μ in loessal and aeolian soils, respectively. Spent drilling fluids significantly increased the length of the lag phase (λ) (F = 62.887, $p = 0.0001$; Figure 1C). The length of the lag phase was found to be ~2.5 d for both soils treated with 8% SDFs. Spent drilling fluids significantly increased the time for 50% germination ($T_{50\%}$) (F = 20.813, $p < 0.0001$; Figure 1D) from 3.5 and 3.3 days under control conditions to 5.4 and 6.9 days after treatment with 8% SDFs in loessal and aeolian soils, respectively. There was no significant difference in A, λ, or $T_{50\%}$ of maize seeds ($p = 0.071$, $p = 0.916$ and $p = 0.072$,

respectively) between the two soil types, whereas the μ of maize seeds was significantly greater in loessal soil than in aeolian soil (F = 21.251, p = 0.0002; Figure 1B).

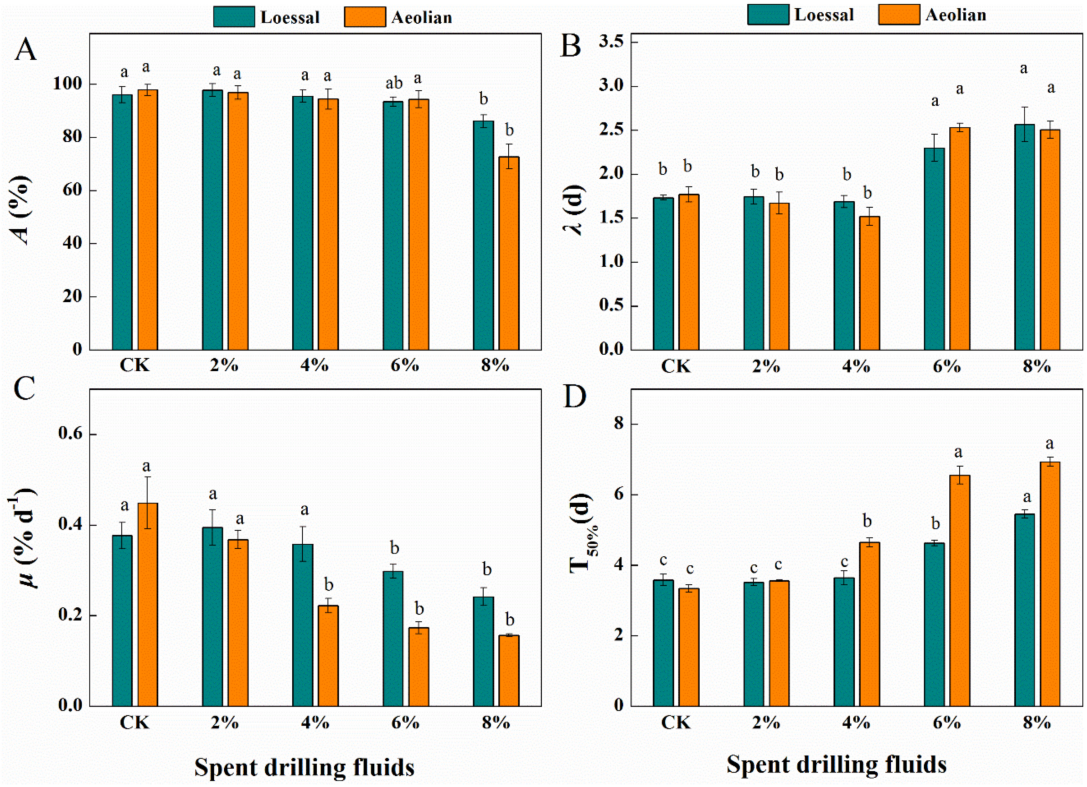

Figure 1. Germination kinetics of maize seeds exposed to spent drilling fluids. Maximum germination (**A**); the length of the lag phase (**B**); germination rates present by germination percentage per day (**C**); the time for 50% germination (**D**). Each data is presented as mean ± SD (n = 5). Different lowercase letters in each column represent significant differences within the same soil at the $p < 0.05$ level, based on Tukey's Honestly Significant Difference (HSD) Test.

3.3. Responses of Root Development to Spent Drilling Fluids

3.3.1. Root Growth

The SDFs treatment significantly retarded the growth of maize roots in a concentration-dependent manner in both soils. The morphological parameters, that is, the primary root lengths, and the lengths and numbers of adventitious roots, were used to monitor morpho-toxicity related to the inhibition of root growth (Figure 2A). The SDFs clearly inhibited hypersensitive growth response to the primary root length (F = 243.077; $p < 0.0001$; Figure 2B), and the inhibitory effects were more pronounced in aeolian soil than in loessal soil (F = 230.776, $p < 0.0001$; Figure 2B). Treatment with 8% SDFs caused 53.53% and 59.79% inhibition of primary root length in loessal and aeolian soil, respectively. The number of adventitious roots did not differ between the two soils ($p > 0.05$; Figure 2C), whereas the lengths of adventitious roots were significantly affected ($p < 0.0001$) by SDFs and soil type (F = 54.651, F = 332.433; respectively, Figure 2D). Treatment with 8% SDFs caused 58.09% inhibition of adventitious root length in loessal soil, while 76.81% inhibition was detected in aeolian soil.

3.3.2. Biochemical Responses

Spent drilling fluids significantly increased ($p < 0.0001$, Table 3) ascorbate peroxide (APX; F = 10.993), peroxidase (POD; F = 48.438), superoxidase (SOD; F = 26.316) activity in primary roots. Soil type significantly affects the POD and SOD activity but not the APX activity. POD and SOD activity in primary roots grown in loessal soil was lower than in aeolian soil (F = 18.236, p = 0.0004; F = 12.195, p = 0.0023; respectively), whereas APX

activity did not differ between the two soils (F = 0.009; p = 0.9246). SDFs significantly increased the content of proline in root samples (F = 131.357, $p < 0.0001$), and the proline content was lower in samples grown in loessal soil than in those grown in aeolian soil (F = 22.625, p = 0.0001; Table 3). The root proline content increased by 3.02-fold in loessal soil and by 3.69-fold in aeolian soil after exposure to 8% SDFs, compared with untreated samples.

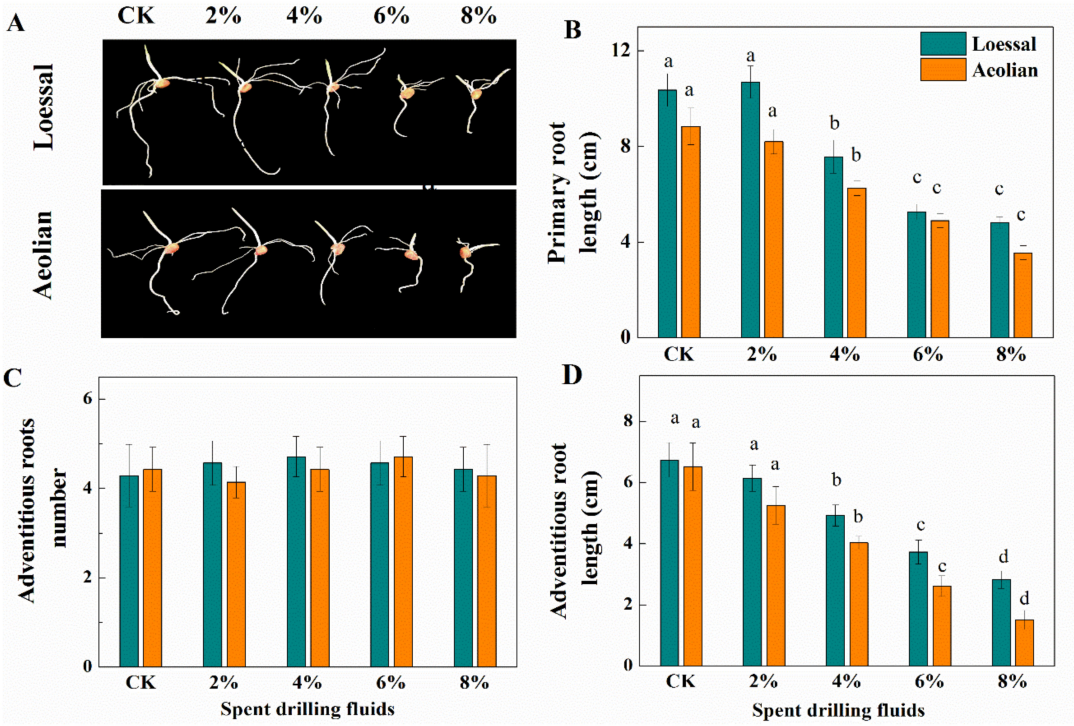

Figure 2. Root development of maize seedlings exposed to SDFs at two soils. Growth response (**A**), measurement of primary root length (**B**), adventitious roots numbers and lengths (**C**,**D**) of 4-day-old seedlings. Each data is presented as mean ± SD (n = 5). Different lowercase letters in each column represent significant differences within the same soil at the $p < 0.05$ level based on Tukey's HSD test.

Table 3. Effects of SDFs on hydrogen peroxide (H_2O_2, μM g^{-1} FW) and proline (μg g^{-1} FW) content, and superoxidase (SOD, U g^{-1} FW), peroxidase (POD, U g^{-1} FW), ascorbate peroxidase (APX, U g^{-1} FW) activity in maize roots. Each data is presented as mean (n = 5). Different lowercase letters represent significant differences within the same soil at the $p < 0.05$ level, based on Tukey's HSD test.

Soil	SDF	H_2O_2	Proline	SOD	APX	POD
Loessal	CK	3.37 ^{c}	9.48 ^{c}	88.07 ^{c}	154.62 ^{b}	122.29 ^{c}
	2%	3.32 ^{c}	10.78 ^{c}	105.61 ^{b,c}	165.01 ^{b}	124.79 ^{b,c}
	4%	3.58 ^{c}	13.89 ^{c}	122.28 ^{b,c}	184.68 ^{b}	141.67 ^{b,c}
	6%	5.23 ^{b}	19.83 ^{b}	130.89 ^{b}	226.32 ^{a}	168.75 ^{a,b}
	8%	7.27 ^{a}	38.13 ^{a}	169.58 ^{a}	222.47 ^{a}	207.72 ^{a}
Aeolian	CK	4.25 ^{d}	10.54 ^{c}	107.47 ^{c}	152.41 ^{b}	126.67 ^{c}
	2%	4.64 ^{c,d}	11.36 ^{c}	119.94 ^{b,c}	161.42 ^{a,b}	149.17 ^{b,c}
	4%	6.05 ^{c}	18.54 ^{b,c}	136.19 ^{b,c}	173.57 ^{a,b}	164.16 ^{b,c}
	6%	7.95 ^{b}	27.95 ^{b}	155.56 ^{a,b}	230.57 ^{a}	182.08 ^{b}
	8%	9.81 ^{a}	49.48 ^{a}	197.41 ^{a}	244.44 ^{a}	275.83 ^{a}
$p > F$	SDF	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Soil	<0.0001	0.0001	0.0023	0.9246	0.0004
	SDF × Soil	0.0207	0.0223	0.9160	0.9731	0.0247

3.3.3. ROS Generation in Maize Roots

The distribution and accumulation of superoxide anion ($O_2 \cdot^-$) formation in excised apex roots (~0.5 cm) is shown in Figure 3. Histochemical staining showed that SDFs significantly affected the distribution of $O_2 \cdot^-$ in root tips after four days (Figure 3A). Superoxide anion in root tips treated with a high concentration (≥6%) of SDFs was localized mainly to the apical meristem, whereas in those tips treated with a moderate concentration (≤4%) or in the control (no treatment),$\cdot O_2 \cdot^-$ localized to the apical meristem or the elongation or differentiation zone, and the distribution of staining was uniform in both soils. The $O_2 \cdot^-$ was quantified by measuring the absorption at OD_{560} in roots. SDFs significantly decreased the OD_{560} value ($F = 8.276$; $p = 0.0061$; Figure 3B), and the reduction of $O_2 \cdot^-$ accumulation was greater in root tips grown in aeolian soil than those grown in loessal soil ($F = 99.616$, $p < 0.0001$; Figure 3B). Treatment with 8% SDFs caused 43.52% and 52.17% reductions in absorption at OD560 in loessal and aeolian soils, respectively. SDFs significantly affected the hydrogen peroxide (H_2O_2) concentration in maize roots ($F = 14.514$, $p < 0.0001$; Table 2), and H_2O_2 accumulation was higher in root tips grown in aeolian soil than those grown in loessal soil ($F = 8.917$, $p = 0.006$; Table 2). Root samples treated with 8% SDF caused a 2.38-fold increase in the H_2O_2 content in loessal soil and a 2.15-fold increase in aeolian soil, compared with untreated samples.

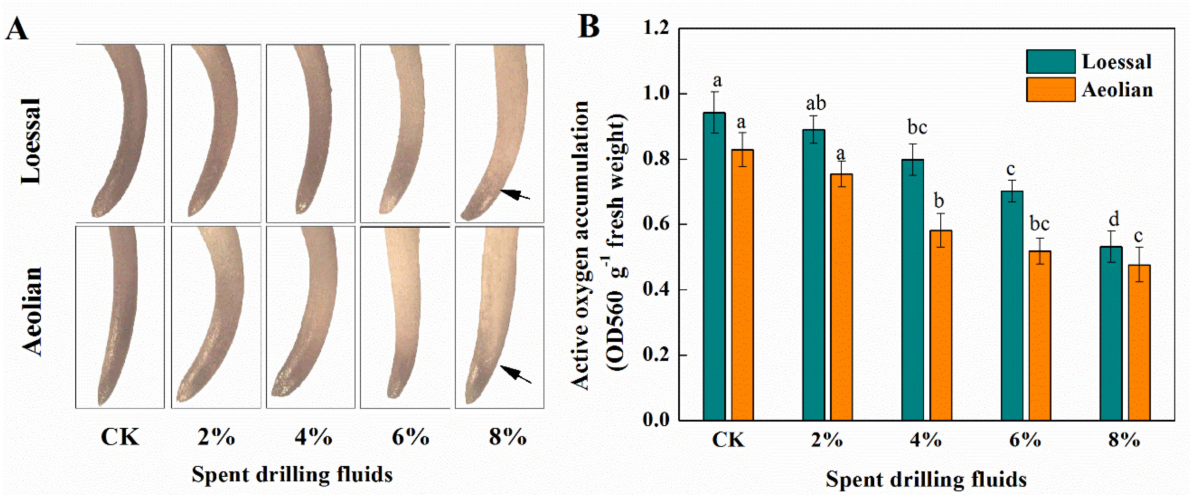

Figure 3. Induction of oxidative stress by SDFs at 4-day-old seedlings. Localization of $O_2^{\cdot -}$ generation in primary roots after incubation with nitroblue tetrazolium (NBT) (superoxide anion localization is shown in blue patches (arrows)) (**A**); quantitative value of $O_2^{\cdot -}$ in SDF-treated roots by the absorption value of OD_{560} (**B**). Each data is presented as mean ± SD (n = 5). Different lowercase letters in each column represent significant differences within the same soil at the $p < 0.05$ level based on the Tukey's HSD test.

3.3.4. Membrane Stability and Root Cell Viability

Lipid peroxidation, as revealed by malondialdehyde (MDA) content, in root samples increased significantly following the increased concentration of SDFs ($F = 27.536$, $p < 0.0001$; Figure 4A), and the MDA content was lower in root samples grown in loessal soil than those grown in aeolian soil ($F = 7.706$, $p = 0.0117$). Treatment with 8% SDF increased the MDA content by 1.49-fold and 1.61-fold in loessal and aeolian soils, respectively. SDFs dose-dependently disrupted membrane permeability, as indicated by increased membrane conductivity (leakage) ($F = 69.597$, $p < 0.0001$; Figure 4B). The membrane conductivity of the roots was significantly lower in loessal soil than in aeolian soil ($F = 20.637$, $p = 0.0002$). Lower concentrations of SDFs (≤4%) had no effect on membrane conductivity compared with the corresponding control in the two soils. Lipid peroxidation indirectly affected cell viability by disrupting membrane integrity. The results of trypan blue staining showed that SDFs distinctly compromised cell viability at higher concentrations in the two soils

(Figure 4C). Approximately, a 57.52% decrease in cell viability was found in treatment with 8% SDFs, compared to control seedlings in loessal soil, while up to a 66.44% loss of cell viability was detected in aeolian soil (Figure 4D). There was no significant difference in cell viability of maize seeds (F = 1.029, p = 0.315; Figure 4D) between the two soils.

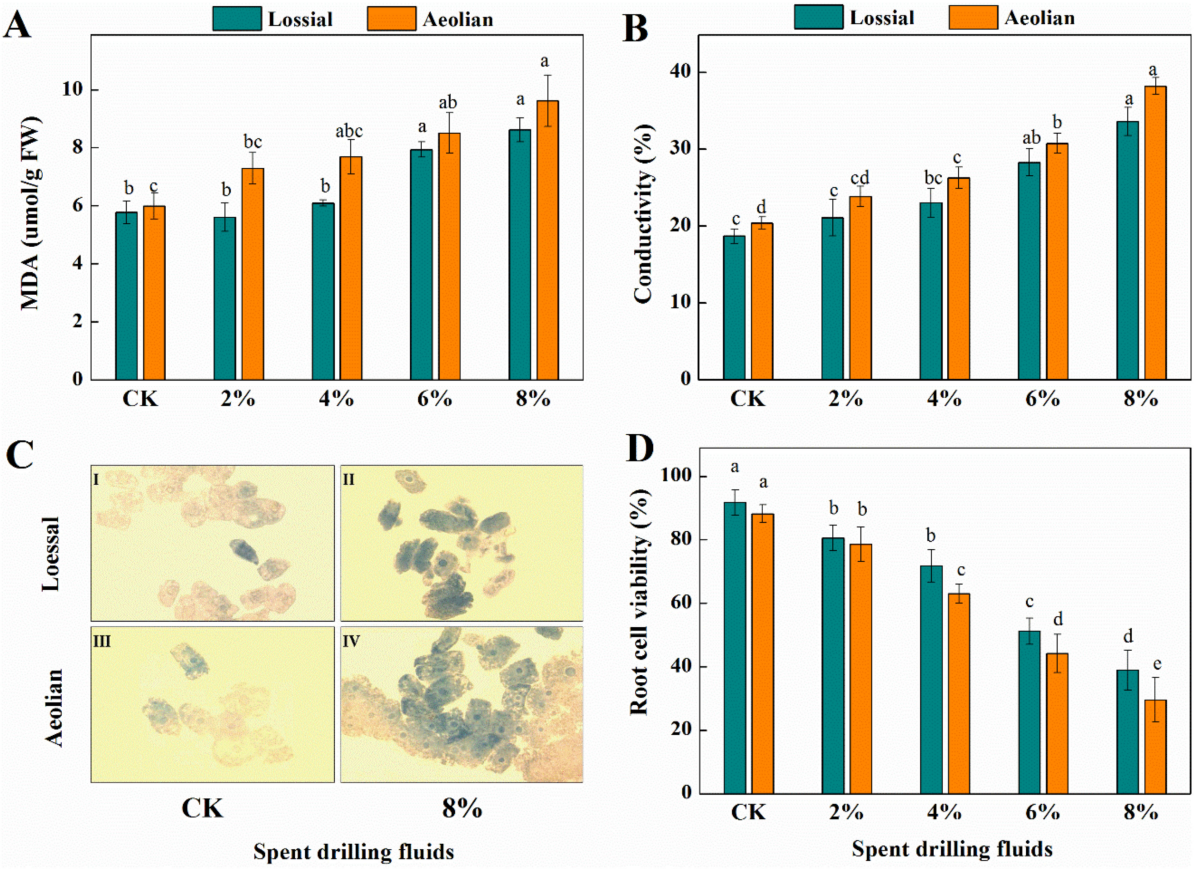

Figure 4. Analysis of lipid peroxidation (**A**) and membrane conductivity (**B**) in 4-day-old seedlings exposed to SDFs. Root cell viability assay using a trypan blue test in roots tips from 4-day-old maize seedlings exposed to SDFs (**C**) and the quantification of cell viability data (**D**). Different lowercase letters in each column represent significant differences within the same soil at the $p < 0.05$ level based on Tukey's HSD test.

3.3.5. Cytogenetic Effects

The cytogenetic effects of SDFs are listed in Figure 5. Spent drilling fluids caused strong inhibition in the mitotic index (MI), with a statistically significant difference in relation to the control, and the decrease in the MI was correlated with the increasing concentrations of SDFs (F = 10.167, p = 0.0001; Figure 5I). Treatment with 8% SDFs caused a 29.42% and 26.24% decline in MI in roots grown in aeolian and loessal soils, respectively. Chromosomal anomalies, including stickiness and bridges, were observed at most stages of mitosis in the presence of SDFs (Figure 5A–H). Chromosomal abnormality in roots increased significantly in response to SDFs (F = 4.352, p = 0.008; Figure 5J). The abnormality index (AI) was 33.77% and 38.95% for loessal and aeolian soils, respectively, in roots treated with 8% SDFs. Untreated root tips showed <6% abnormalities in both soils. There were no significant differences in AI between SDFs-treated samples in the two soil types (F = 1.258, p = 0.275).

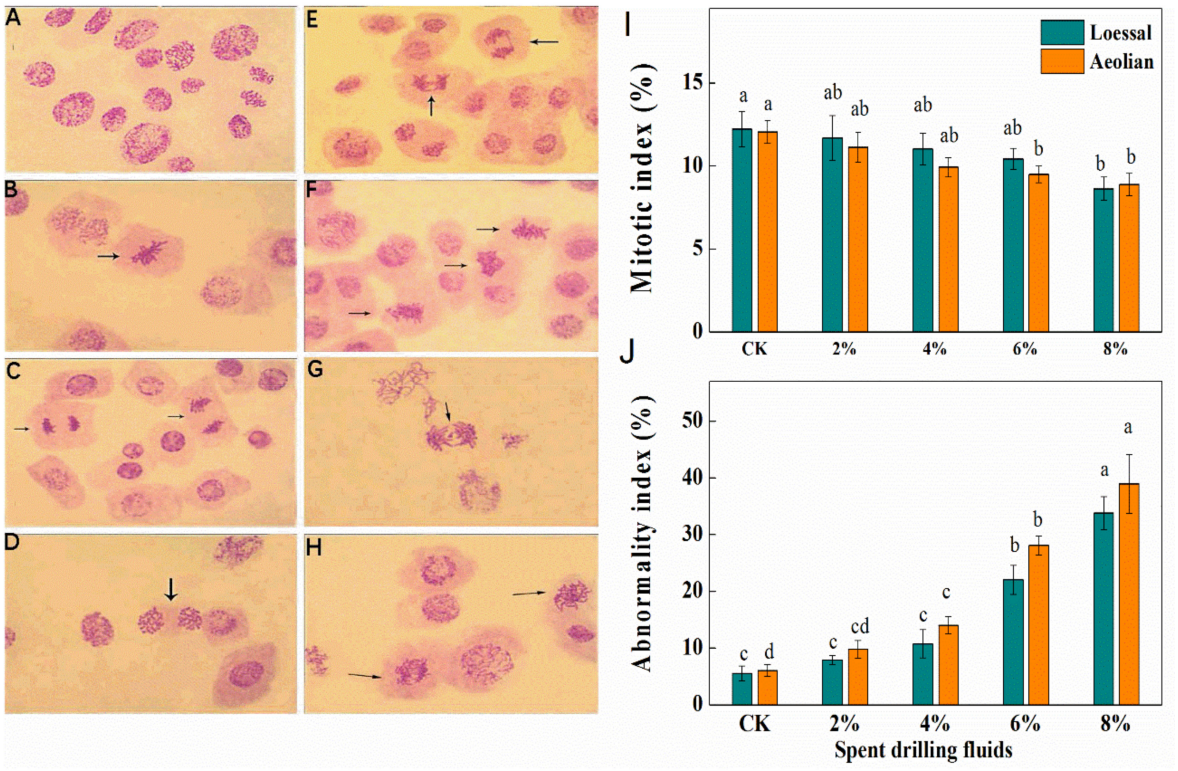

Figure 5. Assessment of chromosomal abnormality (CA) and mitotic index (MI). Images of stages of mitosis from the control (**A**–**D**) and SDF-treated (**E**–**H**) root tip samples. Various abnormalities in maize root tip cells: anaphase with chromosomal bridge (**E**), metaphase with anaphase stickiness (**F**), metaphase with chromosomal break (**G**), telophase with chromosomal stickiness and multipolarity (**H**). Determination of mitotic and abnormality indices (%) (**I**,**J**) were obtained from the control and SDF-treated root tips. Different lowercase letters in each column represent significant differences within the same soil, respectively, at the $p < 0.05$ level, based on Tukey's HSD test.

4. Discussion

In the current study, we investigated the phytotoxic effects of SDFs on maize germination and root growth. The results indicate that SDFs may be phytotoxic to maize, as suggested by their effects of disrupting germinability and root growth. High levels of SDFs decrease A and μ, and increase λ and $T_{50\%}$ in the two soils (Figure 1). SDFs caused growth retardation of maize roots in a concentration-dependent manner, compared with the controls (Figure 2B,D). The phytotoxicity of SDFs, which affected the germination and early growth, was due to salt-induced seed mortality or other unfavorable external conditions. For example, high levels of Na^+, Cl^-, and SO_4^{2-}, and/or other soluble salts in SDFs may produce deleterious effects on maize seedlings alone or in combination (Table 1). Salt stress can cause ion toxicity and ion imbalance, leading to osmotic and oxidative stress. The primary roots are the first organs to come into contact with toxic ions, and plant growth depends on cell division in the meristematic activity. Salinity affects cell division by inhibiting mitotic activity and decreasing the soluble proteins in seeds during germination.

In response to environmental stressors such as salt stress, plants have evolved mechanisms to protect themselves from adverse conditions [33]. Reactive oxygen species (ROSs) play a key role in the acclimation process of plants to abiotic stresses and are maintained at low steady-state levels under normal conditions [34]. This dynamic balance between the generation and scavenging of ROSs can be altered in plant cells under stress conditions [35]. In the present study, SDFs increased H_2O_2 accumulation in root tissues. This could be a response to the abiotic stress caused by the chemical agents in SDFs, such as high levels of salt ions. ROSs are primarily signal transduction molecules that regulate many biological processes. Recent studies showed that H_2O_2 appears to be involved in growth restriction,

whereas $O_2 \cdot^-$ seems to be necessary for root elongation [36,37]. ROSs produced in response to abiotic stress directly affect the level and function of different plant hormones, such as auxins and gibberellin (GA) [38,39]. For example, the auxin-independent increase in apoplastic $O_2 \cdot^-$ facilitates cell wall modifications during cell elongation, and the alteration in cellular redox status caused by auxin regulates the plant cell cycle [40,41]. Alterations of GA levels and signaling in response to abiotic stress affect plant growth by modulating cell division and cell elongation [42]. The role of ROSs in programmed cell death during germination and seed development was demonstrated in the aleurone layer of cereal grains, and is related to interactions with hormones, such as GA and abscisic acid [43]. The deleterious effects of SDFs on germination and seedling development of maize at concentrations ≥6% in both soils observed in the present study may be associated with the alteration of plant hormones and the changes in redox status in plant roots, such as an increased accumulation of H_2O_2 and reduction of $O_2 \cdot^-$ in root tissues (Table 2 and Figure 3B).

In addition to their role as signal transduction molecules, ROSs are considered toxic byproducts of stress metabolism and can oxidize lipids, thereby causing cell death. The radical-induced peroxidation of the lipid membrane is both a reflection and a measure of stress-induced damage at the cellular level [44]. Lipid peroxidation (degradation) increases in plants under stress conditions because of the rapid generation of ROSs [45]. Increased membrane lipid damage and the concomitant increase in MDA content can alter the intrinsic properties of the membrane, such as fluidity and permeability, and the alteration of membrane integrity may lead to a loss of cell viability [46]. In the present study, higher concentrations of SDFs (≥6%) significantly increased the MDA content of plants grown in both soils (Figure 4A). Membrane permeability, as indicated by electron conductivity, increased considerably in maize roots treated with 8% SDFs in both soils (Figure 4B). The root cell viability, which was determined using the trypan blue exclusion method, decreased by 40.78% and 50.31% after exposure to 8% SDFs, compared with untreated roots, in loessal and aeolian soils, respectively (Figure 4D). The increased MDA and membrane conductivity and decreased cell viability in samples may be related to the enhanced lipid peroxidation caused by SDFs-induced ROS generation.

Plants respond to oxidative stresses by stimulating enzymatic and non-enzymatic antioxidant defense mechanisms. Therefore, monitoring the response of antioxidant enzymes and non-enzyme systems in plants exposed to abiotic stresses is a useful indicator of their adaptability to stress. The simultaneous expression of multiple antioxidant enzymes is more effective than a single or double expression to increase the tolerance of plants to multiple environmental stresses [33]. For example, H_2O_2 is a byproduct of the activity of SOD to prevent cellular damage, and must be eliminated by conversion to H_2O in a subsequent reaction involving APX and POD, which regulate H_2O_2 levels in plants [43]. In the current study, the main antioxidant enzymes, including SOD, POD, and APX showed increased activity in maize seedlings treated with high concentrations of SDFs (≥6%) compared with untreated seedlings in both soils (Table 2). Increased activity of ROS-scavenging enzymes may prevent the signaling effects of ROSs and regulate root growth. Proline, an important non-enzymatic antioxidant defense molecule, plays a crucial role in mitigating the harmful effects of ROSs and oxidative damage caused by environmental stress [47]. Proline accumulation is a common phenomenon in plants exposed to various stressors, including salt, metal ions, and other oxidative stresses [48]. In this study, high concentrations of SDFs (≥6%) caused notable increases in the proline content of samples, compared with the corresponding controls in both soils (Table 2). Taken together, these results indicate that SDFs activate some of the major components of enzymatic and non-enzymatic antioxidant defense systems to neutralize the harmful effects of ROSs.

Root growth is regulated by a series of independent events that lead to cell division in the mitotically active meristematic zone and cell elongation in the proximal region of the root tip. Inhibition of root development and the appearance of stunted roots are indicators of growth retardation. In the present study, the growth retardation observed in maize roots exposed to SDFs can be explained by cytotoxic and genotoxic effects, such as

chromosomal aberrations (CA). The MI is a measure of the cytotoxic potential of agents, and alterations in MI are used as indicators of cytotoxicity in environmental monitoring studies [49]. In the present study, SDFs decreased the MI in a concentration-dependent manner in maize roots grown in both soils. The cytotoxicity of SDFs in root meristem cells may be related to the mito-depressive action of chemicals such as salt stress (Table 1). Salt stress decreases the rate of root elongation by reducing mature cell size and lowering the number of dividing cells, resulting in a shortened merism and arrested cell division. Hossion et al. (2004) reported that treatment with Na^+ significantly decreased the mitotic activity in the root meristems of *Chrysanthemum morifolium* [50]. Gerrit et al. (1996) reported that Na^+ significantly reduced cell production by decreasing the number of dividing cells in *Arabidopsis thaliana* [51]. Richardson et al. (1996) reported a similar effect of salinity-induced changes in meristematic cells in *Solanum tuberosum* [52]. The cytotoxicity of chemicals can be determined by measuring the MI, and a decline in the MI to <22%, compared with the negative control, can have a lethal impact on the organism. The analysis of the current study showed that 8% SDFs had lethal effects on maize by significantly decreasing the MI in loessal and aeolian soils (26.24% and 29.42%, respectively; Figure 5I). A decrease in the rate of cell division, which is referred to as a mito-depressive effect, is a common effect of oxidative stress caused by chemical agents. The present results indicate that SDFs are mito-depressive, and this effect was concentration-dependent in both soils. The reduction in mitotic activity could be due to a block in the G2-phase of the cell cycle, which prevents the cell from entering mitosis, or to the inhibition of nuclear protein synthesis essential for the normal mitotic sequence [53]. This can also be achieved by inhibiting DNA synthesis at the S phase, or by altering the relative duration of the mitotic stages [54,55].

In addition to its mito-depressive effect, the cytological analysis of the root meristems revealed an increase in the AI with increased concentrations of SDFs (Figure 5J). Analysis of chromosomal aberrations covering most stages of mitosis provided a better overview of the effects of SDFs on the cell cycle. Chromosome aberrations are the consequence of DNA double-strand breaks that are repaired improperly [56]. The most common aberrations observed in maize cells in the current study were chromosome stickiness and bridge formation (Figure 5). Stickiness is an irreversible chromosomal aberration that is considered to be an indicator of toxicity and a cause of cell death. In the current study, the occurrence of stickiness after SDFs exposure may be a response to the toxicity of chemicals in SDFs, which may lead to the depolymerization or degradation of chromosomal DNA, or condensation of DNA, and/or a function failure of non-histone chromosomal proteins [57,58]. This can also be explained as the physical adhesion of the chromosomal proteins [59]. In addition to stickiness, we observed a significant increase in chromosome bridges, which could be related to chromatin dysfunction. Bridges originate from dicentric chromosomes resulting from a failed repair of the DNA double-strand breaks or the fusion of telomere ends. The induction of bridges in maize cells could also be attributed to chromosome stickiness, which prevents the separation of daughter chromosomes or disturbs the replication of chromosomes. This could be caused by defective or less active replication enzymes or late-replicating DNA sequences of telomeric heterochromatin [60]. These bridges can also result from chromosome or chromatid breakage induced by chemical agents in SDFs, which can occur during an unequal chromatid exchange or the presence of a dicentric chromosome [61].

5. Conclusions

The present study provides comprehensive information on the phytotoxic and cytotoxic effects of SDFs collected from a natural gas field and tested at elevated concentrations in maize (*Zea may* L.). The results show that high levels of SDFs (≥6%) have negative effects on seed germination and root development by promoting H_2O_2 accumulation and decreasing $O_2^{\cdot -}$ in root tissues. The oxidative stress induced by SDFs increased lipid peroxidation (as revealed by MDA content) and electrolyte leakage, thereby impairing cell viability. To reduce the deleterious impact of SDFs-induced oxidative stress, the activities

of different antioxidants (SOD, POD, APX, and proline) were observed to increase in maize roots. Among the two soils tested, the aeolian soil was more responsive than the loessal soil in terms of membrane damage and antioxidant defense against SDFs-induced oxidative stress. The occurrence of chromosomal abnormalities, such as stickiness and bridges, indicates that SDFs are a potent clastogen with direct destructive effects on the chromosomes. Based on the parameters measured, waste-based drilling fluids have potential as a soil amendment for land reclamation and have no adverse effects on plants when applied to aeolian and loessal soil at rates $\leq$ 40 g/kg.

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