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Protective role of Royal Jelly (honeybee) on genotoxicity and lipid peroxidation, induced by petroleum wastewater, in *Allium cepa* L. root tips

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In the present study, the protective effect of Royal Jelly (RJ) on genotoxicity and lipid peroxidation, induced by petroleum wastewater, in *Allium cepa* L. root-tip cells was investigated. For this purpose, we used the malondialdehyde (MDA) level, mitotic index (MI), frequency of micronucleus (MN) and chromosomal aberrations (CAs) as indicators of genotoxicity and lipid peroxidation, and correlated these data with statistical parameters. In additional to the genotoxic analysis, we examined changes in the root anatomy of *A. cepa* seeds treated with the wastewater. Heavy metal concentrations in the wastewater were measured by atomic absorption spectrophotometry. The seeds were divided into six groups as control, wastewater and RJ treatment groups. They were treated with the wastewater alone, RJ alone (25 and 50 μm doses) and RJ + wastewater for 10 consecutive days. As a result, the mean concentrations of heavy metals in the wastewater were observed to be in the order: Pb > Fe > Al > Ni > Cu > Zn > Cr > Cd. The results showed that there was a significant alteration in MI and in the frequency of MN and CAs in the seeds exposed to the wastewater when compared with the controls. The wastewater exposure resulted in a significant increase in CAs and MN formation ($P < 0.05$). The wastewater also caused a decrease in MI ($P < 0.05$). Additionally, there was a significant increase in the MDA levels of the roots exposed to the wastewater ($P < 0.05$). Heavy metals in the petroleum wastewater significantly increased the MDA production, indicating lipid peroxidation. Moreover, light micrographs showed anatomical damages such as an accumulation of chemical compounds in cortex parenchyma, cell death, an unusual form of cell nucleus and unclear vascular tissue. However, the RJ treatment caused amelioration in the indices of lipid peroxidation and MI, and in the frequency of CAs and MN, when compared with the group treated with petroleum wastewater alone ($P < 0.05$). Also, the RJ application caused the recuperation of anatomical structural damages induced by the petroleum wastewater. Each dose of RJ provided protection against the wastewater toxicity, and the strongest protective effect was observed at dose of 50 μm. *In vivo* results showed that RJ is a potential protector against toxicity induced by petroleum wastewater, and its protective role is dose-dependent.

Keywords: *Allium cepa* L.; chromosomal aberration; genotoxicity; wastewater; lipid peroxidation; micronucleus; root anatomy

Introduction

Wastewater pollution is a major problem throughout the world. The wastewaters are complex mixtures that may contain thousands of different pollutants of different origins such as industry, agriculture, municipal and domestic waste. Many of them show cytotoxic and genotoxic effects and are therefore potentially hazardous for humans, animals and the environment [1]. Many wastewaters contain heavy metals such as Cd, Zn, Cu, Pb, Hg, Ni or Co [2].

Heavy metals have long been recognized as major pollutants of both aquatic and terrestrial habitats. They may affect organisms directly by accumulating in the body or indirectly by transferring to the next trophic level of the food chain [3]. Most of these metals are highly toxic substances and have no known role in living organisms. Heavy metals are dangerous because they tend to bioaccumulate. They accumulate in soil, sediment and different tissues of plants and animals [4–6]. They can cause inhibition of photosynthesis in water plants, affect phytoplankton growth in water, cause chromosomal aberrations in terrestrial plants and induce carcinogenesis in humans [7–9]. Despite regulatory measures carried out in many countries, heavy metals continue to increase in the environment.

The use of certain materials may help to decrease the toxicity created by chemical agent such as heavy metals [10]. Recently, biopolymers of various biological

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materials such as *Phyllanthus* fruit extract, *Ocimum sanctum* L. leaf extract, grape seed, lycopene and Royal Jelly have been used for this aim [11–13].

Chemical composition shows that Royal Jelly (RJ) contains many important compounds with biological activity, such as free amino acids, proteins, sugars, fatty acids, minerals, vitamins, aspartic acid, gelatine, sterols, phosphorous compounds, acetylcholine, nucleic acids and numerous trace ingredients, which are all important in RJ's documented therapeutic and nutritional properties [14]. Thus, RJ can be used as a dietary food supplement with functional health-enhancing properties.

Although there are many published clinical studies on RJ in the literature, unfortunately, the protective mechanisms of RJ on heavy metal toxicity in plants are still poorly understood. The aim of the present study was to evaluate the protective role of RJ on toxicity induced by petroleum wastewater in *Allium cepa* root-tip cells.

Materials and methods

A large number of different assays have been recommended for the assessment of the presence of genotoxic contaminants in wastewater. The most frequently applied techniques in wastewater monitoring include the umu-test, ames test, comet or single-cell gel electrophoresis assay, microtox bioluminescence test, chromosomal aberrations (CAs) test and the micronucleus (MN) assay [15]. In the present study, we used the CAs and MN tests for the determination of petroleum wastewater genotoxicity in *A. cepa* root-tip cells.

Products and chemicals

Royal Jelly (60 capsules of 1500 mg freeze dried) was obtained from Health Genesis Corp., Bay Herbor Island, Florida, USA.

Analysis and collection of wastewater

The wastewater samples were collected from a petroleum industry plant located fairly close to the Melet River, Ordu province, Turkey. The wastewater samples were stored in sterile 1000 mL amber-coloured plastic bottles, transported to the laboratory on the same day and stored in the dark at 4 °C until the experimental procedure was started. The samples were filtered through 0.45 μm Millipore filter paper. The measurement conditions were optimized for each metal [16]. The concentrations of Pb, Fe, Al, Ni, Cu, Zn, Cr and Cd were measured by atomic absorption spectrophotometry (AAS).

Preparation of root tips and wastewater exposure

In this study, healthy and approximately equal-sized *A. cepa* seeds were selected. The seeds were sterilized with 2.5% sodium hypochlorite solution for 10 min and washed for 24 h in ultra-distilled water. The seeds in each treatment group were placed into the beaker glass. The seeds were divided into six groups:

- Group I (control) was treated with tap water, for 10 consecutive days.
- Group II was treated with a 25 μm dose of RJ, for 10 consecutive days.
- Group III was treated with a 50 μm dose of RJ, for 10 consecutive days.
- Group IV was treated with petroleum wastewater alone, for 10 consecutive days.
- Group V was treated with a 25 μm dose of RJ + petroleum wastewater, for 10 consecutive days.
- Group VI was treated with a 50 μm dose of RJ + petroleum wastewater, for 10 consecutive days.

For the cytogenetic analysis, when the roots attained a length of approximately 1–2 cm, they were treated with distilled water, and temporary squash preparations were made.

Chromosome analysis and mitotic index

The excised root tips were fixed in Clarke's fixator for 24 h, and kept in 70% alcohol in a refrigerator at 4 °C. These samples were sectioned routinely and stained with Feulgen. For each group, 10 root-tip squashes were prepared, and 500 mitotic cells were counted from each slide [17,18]. Chromosomal analyses were made in anaphase cells in order to identify chromosome alterations such as chromosome bridges, loops and fragments as well as alterations in the centromere and mitotic spindle disturbances, through the appearance of multipolar anaphases.

The cell division intensity of the microscopic preparations was determined by calculating the MI. The latter was determined as the percentage between the number of dividing cells (N') and the total number of cells analyzed (N) according to the formula:

$$MI = N' / N\ 100\%\ [10].$$

Micronucleus (MN) assay

The root-tips were fixed for six hours in a Clarke's fixator (3:1 – glacial acetic acid:distilled water), washed for 15 min in ethanol (96%) and stored in ethanol (70%) in the fridge at +4 °C until later use. The root-tips were

hydrolyzed in 1 N HCl at 60 °C for 20 min, treated with 45% CH_3COOH solution for 30 min and stained for 24 h in acetocarmine. After staining, the root meristems were separated and squashed in 45% CH_3COOH solution [19]. For MN analysis, 1000 cells were scored for each slide. Micronucleated cells were evaluated under a binocular light microscope (Olympus BX51) at ×500 magnification. For the scoring of MN the following criteria were adopted from Fenech *et al*. [20]: (i) the diameter of the MN should be a tenth of the main nucleus, (ii) the MN should be separated from or marginally overlap with the main nucleus as long as there is clear identification of the nuclear boundary, (iii) the MN staining should be similar to that of the main nucleus.

Quantification of lipid peroxidation

Lipid peroxidation was determined by measuring the amount of MDA according to Unyayar *et al*. [21]. About 0.5 g of root tissues from control and treated groups were cut into small pieces and homogenized by the addition of 5 mL of 5% trichloroacetic acid (TCA) solution. The homogenates were then transferred into fresh tubes and centrifuged at 12,000 rpm for 15 min at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution were added into a new tube and boiled at 96 °C for 25 min. The tubes were transferred into an ice-bath and then centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm; 0.5% TBA in 20% TCA solution was used as the blank. The MDA contents were calculated using the extinction coefficient of 155 M^{-1} cm^{-1}. Values of MDA contents were taken from measurements of three independent samples, and the standard deviations of the means were calculated.

Anatomical investigation

For determination of anatomical changes in the root tips, *A. cepa* seeds were cultivated under similar conditions to those of the wastewater toxicity tests. The root tips were then separated and washed twice with distilled water. Cross-sections of the root tips were taken manually. These sections were stained with Methylen Blue and fixed with Entellan [22]. All the photographs were taken with under a binocular light microscope (Olympus BX51).

Statistical analysis

The statistical analysis was carried out using SPSS for Windows version 10.0 (SPSS Inc, Chicago, USA). Statistically significant differences between the groups were compared using one-way analysis of variance (ANOVA) and Duncan's test. The data are displayed as means ± standard deviation (SD), and *P*-values less than 0.05 are considered 'statistically significant'.

Results and discussion

Heavy metal content

Table 1 shows the metal concentrations in petroleum wastewater in the order: Pb > Fe > Al > Ni > Cu > Zn > Cr > Cd.

Chromosomal aberration frequency and mitotic index (MI)

The effects of petroleum wastewater on the CA frequency are given in Table 2. The seeds treated with 25 and 50 µm doses of RJ did not show any significant difference in the total number of CAs and MI compared with the control ($P > 0.05$). In meristematic root-tip cells of seeds in these groups, only a few CAs were found. But, wastewater treatment caused an increase in the frequency of CAs in the root-tip cells. Chromosomal aberrations in anaphase cells were the most common in all the treatment groups. The types of CAs observed included sticky chromosomes, chromatine bridges, condensed chromatine and unequal distribution of chromatine and fragmentations (Figure 1(b)–(f)).

Also, petroleum wastewater caused a decrease in MI. As shown in Table 3, the MI of the wastewater alone and RJ+wastewater treatment groups was significantly different ($P < 0.05$) from the MI of the control group. Also, the MI of group V and VI was higher than that of group IV ($P < 0.05$), while the differences between groups II and III were not significant ($P > 0.05$). These results revealed that the protective effect of RJ on the MI of *A. cepa* root-tip cells is dependent on their doses. Similar observations have also been reported by other authors for *A. cepa* and other plant

Table 1. Heavy metal concentrations (mg/L) in petroleum wastewater.

Heavy metals	Minimum	Maximum	Average
Pb	65.18	80.54	72.36 ± 3.98
Fe	33.48	53.40	44.28 ± 3.95
Al	25.66	39.53	31.72 ± 4.12
Ni	18.24	32.13	25.88 ± 3.24
Cu	14.55	27.16	21.24 ± 2.97
Zn	11.35	23.88	16.65 ± 2.25
Cr	2.18	11.67	5.46 ± 1.92
Cd	0.56	6.30	3.66 ± 1.85

*All values are the mean ± SD.

Table 2. The frequency of CAs, induced by petroleum wastewater, in *A. cepa* root-tip cells.

Group	No. of root-tips	No. of mitotic cells counted in each root-tip	Mean disturbed chromosome	Mean sticky chromosome	Mean chromatine bridge
I	10	500	09.66 ± 3.78[d]	06.63 ± 2.44[d]	04.18 ± 2.82[d]
II	10	500	08.44 ± 4.50[d]	05.84 ± 3.21[d]	04.26 ± 3.41[d]
III	10	500	09.20 ± 3.42[d]	06.12 ± 4.16[d]	03.96 ± 3.66[d]
IV	10	500	120.38 ± 7.65[a]	65.25 ± 6.36[a]	42.24 ± 5.29[a]
V	10	500	96.52 ± 6.63[b]	51.34 ± 5.18[b]	31.38 ± 4.74[b]
VI	10	500	75.88 ± 5.86[c]	40.28 ± 4.63[c]	23.25 ± 3.88[c]

All values are the mean ± SD. Group I (control group) seeds were treated with tap water, group II seeds were treated with 25 μm RJ, group III seeds were treated with 50 μm RJ, group IV seeds were treated with petroleum wastewater, group V seeds were treated with 25 μm RJ + petroleum wastewater, group VI seeds were treated with 50 μm RJ + petroleum wastewater. Five hundred cells were analyzed per root-tip (10 root-tips per group, for a total of 5000 cells/treatment) for CAs. Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test ($P < 0.05$). Means with the same letter within the same column are not statistically different.

systems. Staykova *et al*. [19] investigated the cytogenetic effect of heavy metal and cyanide polluted waters from the region of Panagjurishte, southwest Bulgaria. An *in vivo* system using *A. cepa* was employed. They observed a decrease in cell division rate and deviations from the normal mitosis. In a similar study, El-Shahaby *et al*. [23] evaluated the potential cytotoxic and genotoxic toxicity of industrial wastewater collected from four different sites along the new Mansoura drain in the Sandub area, Dakahlia Province, Egypt. They indicated that all the water samples collected from the drain were highly mutagenic. The heavy metals (Pb, Zn, Co, Cd, Cu) in the wastewater induced an increase in the frequency of CAs. In another study, Inceer *et al*. [18] investigated the cytogenetic effects of 10, 25, 50 and 100 mg/L doses of copper chloride on root-tip cells of *Helianthus annuus*. They found that copper chloride had a marked mitodepressive action on mitosis. Mitotic abnormalities increased and the MI decreased depending on the concentration of the copper chloride applied.

Micronucleus (MN) frequency

Microscopic examination of the squashes of *A. cepa* root-tip meristem cells showed that no MN formation was seen in the control, group II and group III, but a significant increase in MN formation was observed in all the seeds exposed to petroleum wastewater (Figure 1(a)), and the frequency is indicated in Table 4. The maximum frequency of MN was observed in the seeds treated with the wastewater alone. The MN frequency showed a decrease with increasing RJ dose for wastewater-treated seeds; there was a strong dose–effect relationship between the MN frequency and RJ doses. There was a statistically significant difference between the MN frequencies of the control and RJ+wastewater-treated groups ($P < 0.05$).

The 50 μm dose of RJ had a greater effect than the 25 μm dose on the MN frequency for wastewater-treated seeds, which was statistically significant ($P < 0.05$). These findings suggested that heavy metals in petroleum wastewater had cytotoxic activity and induced MN formation in the root tips of *A. cepa*. These observations are also in agreement with genotoxicity data reported by other authors. In most of these studies, the results indicated that heavy metals ions can produce chromosomal or spindle damage, and mitotic apparatus damage, leading to the formation of MN [18]. In particular, the inhibition of spindle formation has been shown to lead to severe abnormalities such as stickiness, unequal distribution, multipolar anaphase, chromosal bridges and laggards [24]. In our opinion, heavy metals may enter the cell nucleus and bind to purine and pyrimidine bases or proteins such as spindle. These interactions may denature spindles and may cause a delay in the formation of the chromosome–spindle complex, which may cause MN formation. The chromosomal analysis results from the our present experiment support these data and show that heavy metals in the wastewater cause serious anomalies in the process of cell division and induce the formation of CAs in *A. cepa* root meristem. We suggest that the existence of the chromosomal damages result from different types of chromosomal aberration, such as sticky chromosomes, chromatin bridges, condensed chromatine, unequal distribution of chromatine and fragmentations, associated with a loss of genetic material. This knowledge is also in agreement with results reported by Staykova *et al*. [19]. They reported a high MN frequency induced by the lagging of whole chromosomes or the immobility of large acentric fragments in *A. cepa*. In a similar study, it was shown that a systematic increase in MN frequency and chromosome aberrations occurred with increased concentration of CrO_3 in *Vicia faba* [17].

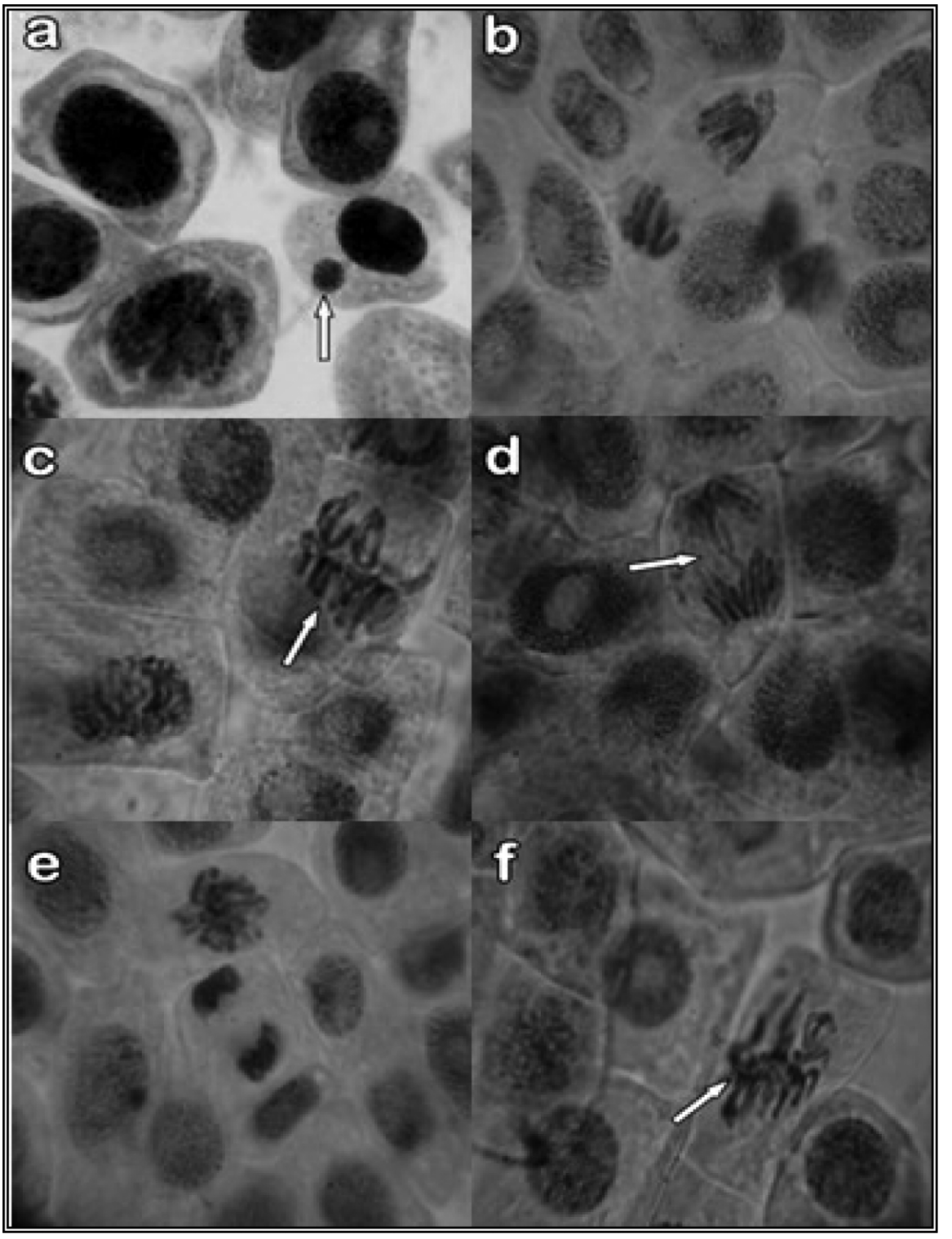

Figure 1. The effects of petroleum wastewater on mitosis of *Allium cepa* root-tip cells, ×500: (a) MN, (b) unequal distribution of chromatine, (c) sticky chromosomes, (d) chromatine bridge, (e) condensed chromatine, (f) disturbed chromosomes.

Table 3. The effect of petroleum wastewater on mitotic cell division of *A. cepa.*

Group	Number of root-tips	MI	%
Group I	10	756.45 ± 13.56	7.56[a]
Group II	10	750.28 ± 14.68	7.50[a]
Group III	10	764.26 ± 12.42	7.64[a]
Group IV	10	510.34 ± 08.54	5.10[d]
Group V	10	577.41 ± 07.75	5.77[c]
Group VI	10	682.19 ± 10.23	6.82[b]

All values are the mean ± SD. Group I (control group) seeds were treated with tap water, group II seeds were treated with 25 μm RJ, group III seeds were treated with 50 μm RJ, group IV seeds were treated with petroleum wastewater, group V seeds were treated with 25 μm RJ + petroleum wastewater, group VI seeds were treated with 50 μm RJ + petroleum wastewater. The MI was calculated by analyzing 1000 cells/root tip (for a total of 10,000 cells/treatment) and percentage of the MI calculated for each treatment group. Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test ($P < 0.05$). Means with the same letter within the same column are not statistically different.

Table 4. The effect of petroleum wastewater on MN frequency of *A. cepa* seeds.

Treatment time (day)	Group	Number of counted cells	Minimum MN	Maximum MN	Average MN
10	I	1000	0	0	00.00 ± 0.00[d]
10	II	1000	0	0	00.00 ± 0.00[d]
10	III	1000	0	0	00.00 ± 0.00[d]
10	IV	1000	47	66	56.41 ± 4.76[a]
10	V	1000	35	52	44.78 ± 3.64[b]
10	VI	1000	18	33	25.96 ± 2.53[c]

*All values are the mean ± SD. Group I (control group) seeds were treated with tap water, group II seeds were treated with 25 μm RJ, group III seeds were treated with 50 μm RJ, group IV seeds were treated with petroleum wastewater, group V seeds were treated with 25 μm RJ + petroleum wastewater, group VI seeds were treated with 50 μm RJ + petroleum wastewater. 1000 cells were counted for each group for MN frequency. Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test ($P < 0.05$). Means with the same letter within the same column are not statistically different.

Lipid peroxidation (MDA content)

It is known that heavy metal stress causes molecular damage to plant cells, either directly or indirectly, through the formation of reactive oxygen species (ROS) such as hydrogen peroxide, and hydroxyl and superoxide radicals [25]. Harmful ROS can damage biological molecules such as lipids, which are altered by peroxidation. Measurement of MDA levels is routinely used as an indicator of lipid peroxidation under stress conditions [26]. The results concerning MDA data are given in Table 5. The results showed that there was a significant increase in the MDA levels of the roots exposed to the wastewater. Heavy metal ions in the wastewater significantly affected the MDA production, indicating lipid peroxidation. There was a statistically significant difference in MDA content between the control and treatment groups ($P<0.05$). The RJ treatment decreased the toxic effects of heavy metals, as manifested by lower lipid peroxidation, lesser production of hydrogen peroxide and reduction in the generation of superoxide

Table 5. The effect of petroleum wastewater on MDA content (μmol g^{-1} fresh weight) of *A. cepa* seeds.

Group	Minimum MDA	Maximum MDA	Average MDA	% of MDA reduction
I	8	15	12.54 ± 2.44[d]	–
II	8	16	12.16 ± 2.18[d]	–
III	9	18	12.45 ± 2.36[d]	–
IV	26	44	35.80 ± 5.89[a]	–
V	20	35	28.66 ± 4.76[b]	19.95
VI	14	26	19.62 ± 3.55[c]	45.20

All values the mean ± SD. Group I (control group) seeds were treated with tap water, group II seeds were treated with 25 μm RJ, group III seeds were treated with 50 μm RJ, group IV seeds were treated with petroleum wastewater, group V seeds were treated with 25 μm RJ + petroleum wastewater, group VI seeds were treated with 50 μm RJ + petroleum wastewater. 0.5 g of root tissues was used for each group for MDA analysis. Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test ($P < 0.05$). Means with the same letter within the same column are not statistically different.

radicals. A decreased MDA content was observed in roots at both dose of RJ. In RJ-treated roots (groups V and VI), the level of MDA was reduced by about 20% and 45% (compared with group IV) at 25 and 50 μm doses of RJ, respectively. These observations are also in agreement with results reported by Unyayar *et al*. [21], Meng *et al*. [26], Hong *et al*. [27], Choudhury and Panda [28], and Pandey *et al*. [29] on the generation of lipid peroxidation products under different heavy metal stresses. These researchers reported a significant increase in the MDA content in the roots treated with different doses of heavy metals.

Anatomical observations

In order to identify anatomical changes, caused by petroleum wastewater, in the root tips, the root tissues were microscopically examined. When seeds were exposed to petroleum wastewater for 10 days, root cells survived, but the root tissues were seriously affected under the presence of heavy metals. Anatomical damage, such as cell death or necrosis, unclear vascular tissue, unusual form (especially flat) of cell nucleus instead of the normal form (annular) and accumulation of some chemical compounds in the cortex parenchyma were observed (Figure 2) when compared with the

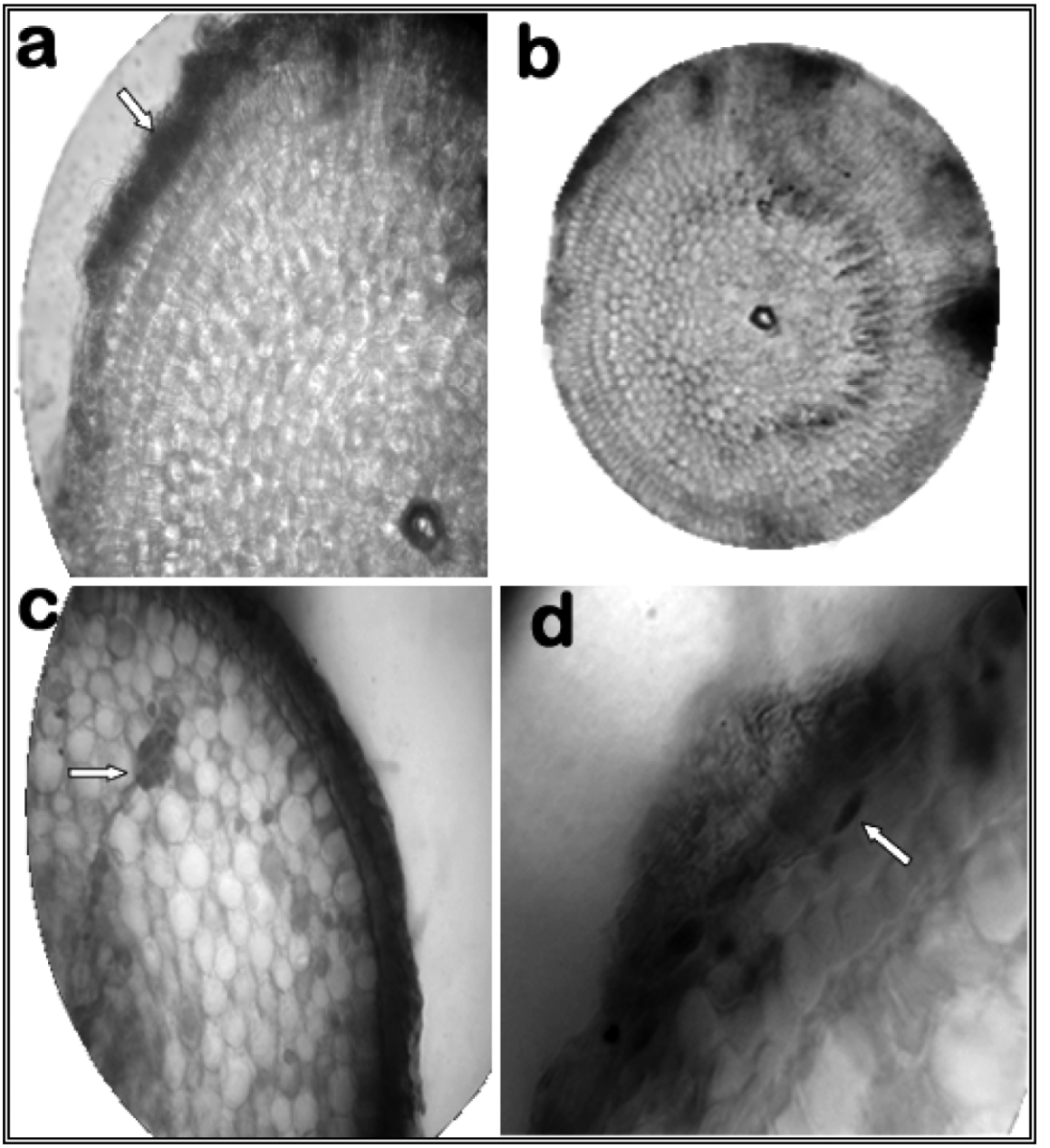

Figure 2. The anatomical alterations induced by petroleum wastewater in *Allium cepa* root-tips, ×500: (a) necrosis, (b) unclear vascular tissue, (c) accumulation of some chemical compounds in cortex parenchyma, (d) unusual form (flat) of cell nucleus.

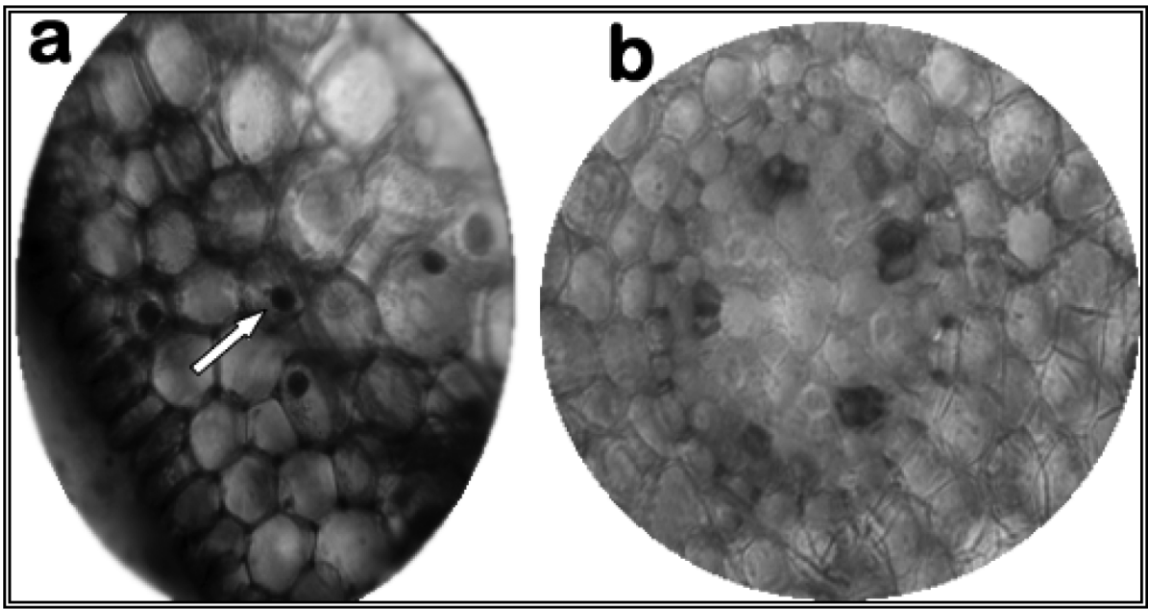

Figure 3. The anatomical appearance of root-tips of control group seeds, ×500: (a) normal form of cell nucleus (annular), (b) clear vascular tissue.

controls (Figure 3). However, RJ application significantly protected the root tissues from wastewater-induced free radical damage, as compared with group IV. In particular, RJ caused significant decreases in the cell necrosis of the root tissues. The effect of petroleum wastewater on the root tip anatomy has also been described by other authors. Suzuki [30] investigated the toxic effects of 50–500 μm doses of Cd on the root-tip anatomy of two-week-old *Arabidopsis* seedlings. As a result, they determined retardation of root growth and increase in cell death at the root elongation zone. Arduini *et al*. [31] investigated the toxic effects of Cd and Cu compounds on the roots of stone pine (*Pinus pinea* L.) and maritime pine (*Pinus pinaster* Ait.) seedlings. They showed that the root density (weight per unit length) and the width of the cortex in both species increased in response to Cd^{2+} exposure. Adamakis *et al*. [32] investigated the developmental effects of sodium tungstate on pea (*Pisum sativum* L. cv. Onmard) and cotton (*Gossypium hirsutum* L. cv. Campo) seedlings. Their results showed that tungstate retarded the seedling growth rate and stopped root elongation in both species. Also, tungstate induced premature vacuolation in cells of the root apical meristem, and tungstate-treated cell nuclei contained spherical nucleoli with a big nucleolar vacuole.

Consequently, RJ had a protective effect on the genotoxicity and lipid peroxidation induced by petroleum wastewater in seeds of *A. cepa*, and its protective effect was dose-dependent. No study so far has been undertaken to examine the protective role of RJ in plant tissues, but several studies on animals have demonstrated that RJ has antioxidant and therapeutic activities. For example, Bincoletto *et al*. [33] demonstrated that RJ prevented the myelosuppression induced by the temporal evolution of a tumour and abrogated the splenic haematopoiesis observed in mice. Fujii *et al*. [34] reported the anti-inflammatory properties of RJ in streptozotocin-diabetic rats. Inoue *et al*. [35] investigated the effect of RJ on the oxidative DNA damage and lifespan of mice. In the latter study, C3H/HeJ mice were fed a dietary supplement of RJ for 16 weeks: the levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress, were significantly reduced in kidney DNA and serum. Also in the same study, these authors determined the effect of dietary RJ on the lifespan of C3H/HeJ mice. The 50% survival rate for mice in the intermediate-dose (about 6 mg/kg weight) and high-dose groups (about 60 mg/kg weight) was reached at a significantly longer time than that of the control group. These results indicated that dietary RJ increased the average lifespan of C3H/HeJ mice, possibly through the mechanism of reduced oxidative damage. The results of Inoue *et al*. [35] agree with our study. From the results of our experiment, we speculated that RJ could retard and prevent damage in *A. Cepa* root-tip cells, owing to a decrease in oxidation damage. But the mechanism for the protective effects of RJ has not been clarified in detail, and future experiments along these lines are planned. Moreover, recent studies in the literature have demonstrated that RJ also has anti-hypercholesterolemic activity, anti-fatigue effects, insulin-like activity, estrogenic effects, hypoglycaemic activity, wound-healing

properties and promotes collagen production. Also, there are a few reports about its antioxidative role in anti-aging processes [36].

Conclusions

The results of the present study indicated that petroleum wastewater caused significant toxic effects in the root-tip cells of *A. cepa*. However, RJ application enhanced the antioxidant status and decreased the incidence of toxicity induced by heavy metals. Supplementation with RJ may decrease toxic damages induced by petroleum wastewater. Therefore, its protective role as 'a toxicity-limiting agent' may be used in the future to reduce the negative effect of toxic agents such as heavy metal ions.

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