Nickel and ozone stresses induce differential growth, antioxidant activity and mRNA transcription in *Oryza sativa* cultivars

Amel Tammama, Reem Badra, Hanan Abou-Zeida, Yaser Hassanb and Aida Baderc

*Department of Botany and Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt; bDepartment of Air Pollution, National Research Centre, Giza, Egypt; cFaculty of Science, Mergib University, Koms, Libya*

**ABSTRACT**

This study evaluates the influences of nickel and ozone exposure individually and/or in combination on growth performance, antioxidant activity and genes up regulation of two rice cultivars Sakha 101 and Giza 178. Ni treatment at high doses (100 μM Ni) and 75 ppb O₃ alone reduced the fresh weight, mineral content, membrane integrity and Rubisco content. There was an increase in SOD and APX activity in the shoots of rice cultivars, whereas, there was a decrease in GR and GST in the roots and shoots. In contrast combined stresses appeared to be stimulatory for growth and mineral contents, Rubisco content and antioxidant enzymes. Nonspecific lipid transfer protein and transmembrane protein genes were up regulation in rice cultivars in response to Ni stress. Carotenoid cleavage dioxygenase is upregulated in response to ozone stress; RNA pseudouridine synthase, heat shock proteins and cytochrome P450-like were upregulated in response to combined stresses. The results revealed that the interactive effects of both stresses were antagonistic on growth parameters and antioxidant activities to overcome the adverse effects of both pollutants.

**INTRODUCTION**

Nickel is required for iron absorption, seed germination and its deficiency leads to the production of viable seeds in plants. Application of nickel to crops protects them from certain yield limiting diseases, thus potentially reducing pesticide usage and improving crop yield. Nickel (Ni²⁺) also acts as a biocontrol agent for microbial pests. It is a key factor affecting the production of secondary plant metabolites and thus influencing plant resistance to diseases (Wood and Reilly 2007). The increased level of Ni in the environment, as a result of anthropogenic pollution, is a growing concern for its impact on the food chain and the ecosystem; in the past decades increasing efforts have been made to develop technologies to reduce Ni²⁺ concentrations in contaminated soils and waters (Iori et al. 2013). Recently, maize could be used as the indicator plant for the environment quality assessment for Ni, lettuce is the most tolerant to increasing nickel doses, whereas maize is the most sensitive (Antonkiewicz et al. 2016).

Tropospheric ozone (O₃) is one of the main pollutant gasses that cause air pollution. The photochemically induced reaction between the hydrocarbons and nitrogen oxide released from motor vehicle exhaust (Manning and Feder 1976). Many studies have shown that average concentrations of O₃ in many regions have increased; the changes may come from climate changes and human-emitted precursors (Oltmans et al. 2006). It was anticipated that many factors will contribute to O₃ increase by 20–25% between 2015 and 2050 and 40–60% at 2100 (Singh et al. 2010). Exposure of plants and crops to O₃ usually results in loss of physiological functions and consequently reduce the yield. It was estimated that O₃ alone or in combination with other pollutants, accounts for approximately 90% of air pollution induced crop loss in the USA (David et al. 1994).

Pollution of the environment with both ozone (O₃) and heavy metals has been steadily increasing. An understanding of their combined effects on these plants, especially crops, is limited (Guo et al. 2012). The prevailing view is that elevated (O₃) causes an up regulation of antioxidant metabolism in plants (Olbrich et al. 2009). However, the direct evidence for this up-regulation is variable, dependent on the duration and method of O₃ fumigation, and the species and components of antioxidant metabolism investigated (Iglesias et al. 2006). Ormrod (1977) reported that after pea plants were treated with Ni²⁺ and exposed to 50 ppm/h O₃ for 6 h in a cube plexi glass chamber, higher Ni concentrations were found in the plants than in the controls not exposed to O₃. In poplar, O₃ and Cd stresses seem to share common toxicity pathways at physiological and molecular levels (Pietrinì et al. 2010), by affecting synergistically or antagonistically whole plant responses when they act concomitantly. On the other hand, a reduction in stomatal conductance and, consequently, water (and metal) uptake might provide a physiological basis for cross protection afforded against excess Cd by O₃ treatment, and vice versa. Guo et al. (2012) recorded that elevated O₃ led to higher concentrations of Cd in wheat tissues (shoots husk and grains) with respect to contaminated soil. Combined exposure to Cd and elevated O₃ levels strongly affected the antioxidant isoenzymes APX, CAT and POD and accelerated oxidative stress in wheat leaves and suggest that elevated O₃ levels cause a reduction in food quality and safety.

The current study was appraised to evaluate the impact of ozone and different concentrations of Ni used separately on changes in fresh weight, RUBISCO activity, microanalysis of different elements content, membrane permeability,
specific activities of key enzymes of antioxidant system and expression of up and down regulated genes in two rice cultivars. In addition, the possibility that O₃ exposure may somehow interact with metal accumulation in leaves was evaluated in order to understand whether this combined effect could antagonistically interact and affect growth, biochemical and molecular aspects of two rice cultivars.

**Materials and methods**

**Plant materials and experimental condition**

Seeds of a number of rice (*Oryza sativa*, five agricultural cultivars, Sakha 101, Sakha 103, Sakha 106, Giza 178 and Giza 179) were obtained as a pure variety from the Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. Seeds were soaked in distilled water to test their viability, and then the precipitated grains were air dried at room temperature. Healthy grains of all cultivars were disinfected with 0.1% HgCl₂ solution for 5 min and washed several times with distilled water to remove its traces. The effects of different concentrations (0, 10, 50, and 100 µM) of Ni²⁺ upon *O. sativa* plants were studied using as a substrate medium for the germinating grains. Measurements of germination percentage, plunule and radicle lengths were recorded daily along 7 days; to choose two extreme cultivars. Seeds of rice plant (*O. sativa L* cv. (1) Sakha101 and (2) Giza178) were selected for uniformity of size, shape, viability and were surface sterilized by immersion for two minutes in 0.1% w/v HgCl₂. After rinsing several times with sterile distilled water, grains were soaked overnight in continuously aerated distilled H₂O and allowed to germinate in Petri dishes (10 × 10 cm) on a double layer of filter paper saturated with half strength Hoagland solution supplied with the soil. All pots were irrigated with half strength Hoagland solution fumigating with ozone (75ppb/h) generated from ozonator (Tonbridge, Kent, UK). The fourth sets of pots supplied with ozone (75ppb/h) and was irrigated with half strength Hoagland solution supplied with 10, 50, and 100 µM NiSO₄ (H₂O)₆. The pots (in triplicates) were taken and placed in Open-Top Chambers (OTCs) in the botanical garden of Faculty of Science, Alexandria University.

**Ozone fumigation to plants**

Open-Top Chambers (OTCs) of the design of Treshow and Stewart (1973) were used. They were constructed that could be placed over the plots under field conditions. An iron hexagonal shape framework 100 cm side length × 200 cm height was covered with plastic. Polluted air was distributed into chambers of the third and fourth sets of pots by means of tubes with small pores at heights 40 cm above ground attached to each corner inside each chamber (Badr 2014). Polluted air was distributed into chambers by means of pipes with small pores at heights 40 cm above ground attached to each corner in each chamber; each chamber contains 16 pots of two cultivars rice plants. There was 8 pots/species/chamber (16 pots for both plants), pots were distributed in a randomized completely block design. They were rotated within each chamber every week. The seedling harvest was carried out 28-days after sowing, whole plant samples were removed and washed carefully with sterile distilled H₂O and blotted dry gently between filter paper. Just after harvest, fresh weight was measured for characterizing the plant growth. The whole plants or dissected organs (root and shoot) were weighed carefully for fresh weight determinations (FW).

**Protein extraction and relative amount of Rubisco enzyme estimation**

In every plot, the second leaf of 10 plants was used for protein extraction and subsequent activity and protein amount measurements. Leaves were coarsely ground in a mortar under liquid nitrogen. Then, 600 mg of this coarsely ground leaf tissue were reduced to powder in a mortar with 6 ml of buffer consisting of 0.1 M (tris-hydroxymethyl - amino-methane)-HCl (Tris–HCl, pH 7.5), 5 mM ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM pepstatin, 2 mM dithiothreitol (DTT), 5 mM MgCl₂, 10 mM NaHCO₃, 1.5% (w/v) polyvinylpolypyrrolidone, and 5 µg ml⁻¹ leupeptin. Soluble protein was determined according to Bradford (1976).

Soluble and insoluble fractions were separated by centrifugation at 17,600g at 48°C for 15 min. Then, 2.5 ml of protein-containing supernatant was desalted on a Sephadex G25 column (Pharmacia PD-10, Orsay, France) in darkness at 4°C, with elution buffer composed of 0.1 M Tris-HCl (pH 7.5) and 2 mM DTT. The desalted protein extract was immediately used for determination of enzyme activities. Proteins present in the remaining supernatant (about 1 ml) were then precipitated. After centrifugation (17,608g, 4°C, 10 min), pellets were resuspended in 200 µl of extraction buffer. Relative amounts (%) of Rubisco (SSU and LSU) were measured using HPLC analysis (C₁₈ column) (Leitao et al. 2003).

**Damage signals (oxidative damage)**

**Estimation of lipid peroxidation**

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction (Wang et al. 2009). One gram of fresh leaf samples was homogenized in 5 ml 0.6% thiobarbituric acid in 10% trichloroacetic acid (TCA). The mixture was heated at 100°C for 15 min in a water bath. After cooling in ice, the mixtures were centrifuged at 5000g for 10 min. The absorbance of supernatants were read at 450, 532 and 600 nm and MDA content was calculated on a fresh weight basis using the following formula: MDA (nmol g⁻¹FW) = 6.45 (OD₅₃₂ – OD₆₀₀) – 0.56 (OD₄₅₀) × 1000.
Estimation of membrane integrity (MI)

Membrane integrity was determined by recording the electrical conductivity of leaf leachates in double distilled water at 40°C and 100°C (Deshmukh et al. 1991). Leaf samples (0.1 g) were cut into discs of uniform size and placed in two sets of test tubes each containing 10 ml distilled deionized water. One set of the tubes was incubated in a water bath at 40°C for 30 min and the other set was incubated at 100°C in boiling water bath for 15 min and their respective electric conductivities C1 and C2 were measured by a conductivity meter. Membrane Integrity (MI) was calculated by using the formula:

\[
MI = \left[1 - \frac{(C1/C2)}{2}\right] \times 100.
\]

Elements analysis

Elements content (Ni²⁺, K⁺ and Ca²⁺) were measured in shoots and roots of both O. sativa L. cultivars following the method previously described by Chapman and Pratt (1961). Shoots and roots of O. sativa cultivars were washed by tap water and dried in an oven at 70°C for 72 h and then weighted. After drying, one g of each sample was placed into porcelain crucible and heated in a furnace. The furnace temperature was slowly increased from room temperature to 550°C in 1 h. Samples were ashed for 3 h. The residue was dissolved in 5 ml HCl (2N) and the total volume was adjusted to 50 ml by adding distilled water. The metal content was then analyzed by atomic absorption spectroscopy. Elements were determined using an atomic absorption spectrophotometer Perkin-Elmer, 2380 (Association of Official Analytical Chemists 1984) and expressed on the basis of dry weight.

Estimation of antioxidant

Preparation of enzyme extract and assay of enzyme activity.

Two gram of fresh rice shoots and roots were ground to a fine powder in liquid N₂ and then homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM D-isoscorbic acid, 2% (w/v) polyvinylpyrrolidone(PVP) and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar. The homogenate was centrifuged at 20,000 g for 10 min at 4°C and the supernatants were collected and used for the assays of ascorbate peroxidase, glutathione reductase, and glutathione S- transferase.

For assay of superoxide dismutase (SOD, EC 1.15.1.1) activity, fresh shoots and roots (1 g) were homogenized in 8 ml potassium phosphate buffer (50 mM, pH7.8) containing 0.1 mM Na₂EDTA and 1% insoluble PVP with a chilled pestle and mortar. The homogenate was centrifuged at 20,000 g for 20 min. The supernatant was collected and used for the assay of SOD following the method of Beyer and Fridovich (1987). The reaction mixture was prepared by mixing 27 ml of 50 mM potassium phosphate, pH 7.8, 1.5 ml of 12 mM L-methionine, 1 ml of 50 µM nitroblue tetrazolium salt, and 0.75 ml of Triton X-100. Aliquots (1 ml) of this mixture were delivered into small glass tubes, followed by 20 µl of enzyme extract and 10 µl of 10 µM riboflavin. The cocktail was mixed and then illuminated for 7 min in an aluminum foil-lined box, containing two 20 W fluorescent tubes. A control tube in which the sample was replaced by 20 µl of buffer was run in parallel and the A560 was measured in all tubes.

The test tubes containing the reaction mixtures were exposed to light immersing the glass tubes in a cylindrical glass container three-fourth filled with clean water and maintained at 25°C and placed in between two 20 W fluorescent tubes. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per min. Fifty percent of inhibition was taken as equivalent to 1 unit of SOD activity.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed as described by Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM H₂O₂. The reaction was started at 25°C by the addition of H₂O₂ after adding the enzyme extract containing 50 µg of protein. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient (Δε) 2.8 mM⁻¹ cm⁻¹.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined at 25°C by measuring the rate of NADPH oxidation as the decrease in absorbance at 340 nm (Δε = 6.2 mM⁻¹ cm⁻¹) according to the method of Halliwell and Foyer (1978). The reaction mixture (1 ml) consisted of 100 mM Tris-HCl (pH 7.8), 21 mM EDTA, 0.005 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and the enzyme. NADPH was added to start the reaction. GR, one unit of enzyme was defined as the amount of enzyme necessary to decompose 1 μmol of substrate per min at 25°C.

Glutathione S-transferase (EC 2.5.1.18) activity was measured by following the changes in the absorbance at 340 nm in a mixture containing 0.17 mM sodium phosphate buffer, pH 6.5, 1 mM, glutathione reduced form (GSH) 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol and enzyme extract. U=μl/mg of enzyme which catalyses the formation of 1 μmol of S-2, 4-dinitrophenylglutathione mg⁻¹ protein h⁻¹ (Mannervik and Guthenberg 1981).

Extraction of RNA

Total RNA was extracted from rice leaves using GStract™ RNA Isolation Kit according to the manufacturer’s instructions (QIAGEN, Germany). The RNA was dissolved in diethylpyrocarbonate DEPC-treated water, quantified spectrophotometrically and analyzed on 1.2% agarose gel.

Reverse transcription-polymerase chain reaction (RT–PCR)

Reverse Transcription (RT) first stand reaction for converting mRNA to complementary DNA (cDNA) is made from an mRNA template using DNTPS and reverse transcriptase. The components are combined with a DNA primer in a reverse transcriptase buffer for an hour at 42°C was carried out with the RT–PCR system and the obtained cDNA was used as a template for the second amplification.

Reverse transcription reactions were performed using primer oligo dT primer (5’T-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT). Each 25 µl reaction mixture containing 2.5 µl of 5× buffer with MgCl₂, 2.5 µl of 2.5 mM dNTPs, 1 µl of 10 pmol primer, 2.5 µg RNA of 2 mg ml⁻¹ and 200 U reverse transcriptase enzyme. RT–PCR amplification was performed in a Master cycler Personal (Eppendorf, Germany) programmed at 42°C for 1 h, 72°C for 10 min to inactive the reverse transcriptase, this
method was modified from Carginale et al. (2004). cDNA was then stored at −20°C until used.

**PCR amplification**

Different arbitrary four primers were used in the differential display analysis one of them is specific for 18S (Table 1). The reaction mixture for differential display PCR was carried out in total volume of 25 µl containing 2.5 µl from each RNA sample was mixed with 5 µl of 2× reverse transcription mixture containing 50 mM Tris–HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 20 mM dithiothreitol, 2.5 µl dNTPs (each at 4 mM), 1 µl oligo dT primer (Promega), 13 µl of RNase free water and 1 µl (50 unit/µl) of reverse transcriptase and then incubated at 37°C for 1 h. After this reaction, 23 µl of Taq DNA polymerase reaction mixture containing 10 mM Tris (pH 8.3), 2.25 mM KCl, 4 mM MgCl₂, and 1 unit of Taq polymerase (AmpliTaq, Perkin-Elmer) were added to the product of reverse transcriptase and cycled first in a 9700 thermal cycler (Perkin-Elmer) programmed at 94°C for 5 min, 56°C for 5 min, and 72°C for 5 min, followed by 40 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The reaction was then incubated at 72°C for 10 min for final extension. Two microliters of loading dye was added prior to loading of 10 µl per gel pocket. Electrophoresis was performed at 80 Volt with 0.5× TBE buffer in 1.5% agarose gel. The gel was stained in 0.5 g/l ethidium bromide solution and destained in deionized water. Finally, the gel was visualized and photographed by using gel documentation system.

**DNA sequencing and sequence analysis**

The expected PCR-amplified product was extracted and eluted from the gel using a Gel Extraction Kit (Koma biotechnology Korea). The PCR products were sequenced by using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Analysis of the obtained nucleotide sequences was carried out using DNA and Protein Blast. DNA sequences were translated into amino acid sequences online at Expsay the website: (https://www.expasy.org/old_links). After that, amino acid sequences were analyzed and compared to the Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI). The amino acid sequences were compared to protein database using a protein query (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similarities to sequences from prokaryotic microbe such as *E. coli* or *Shigella flexneri* were eliminated from the following analyses. These DNA fragments were deposited in the EMBL-Bank the accession numbers of LT 622240, LT 622241, LT 622242, LT 622243, LT 622244 and LT 622245, respectively.

**Statistical analysis**

Statistical analysis of the results was carried out according to Duncan’s multiple range tests using SPSS-20. Data were subjected to one-way ANOVA (Sokal and Rohlf 1995). Differences between treatment-means were considered statistically significant at *p* ≤ 0.05.

**Results**

**Plant growth**

The data indicated that rice responses to simultaneously imposed stress conditions were distinctly different from the responses displayed by plants when the stresses were applied individually. In both cultivars, Ni applied alone generally reduced the fresh weight of the roots and shoots of Sakha 101 and Giza 178. However, a difference in response could be noted, in roots, the reduction of fresh weight was 39% and 61%, in comparison with their corresponding controls for Sakha 101 and Giza 178, respectively. It is well noted that fumigation by 75 ppb/h ozone showed a significant reduction in the fresh weight of shoots by 42% and 53% of the relevant control values for Sakha 101 and Giza 178 (Table 2). These observations might indicate that the Giza 178 cultivar was relatively more sensitive to nickel and ozone stresses than the Sakha 101 cultivar. When Ni and O₃ stress were applied together the effects were less reduced in comparison with the injurious effects of a single stress factor: the reduction of fresh weight was considerably shifted to some extent in comparison with the corresponding controls for both cultivars.

**Influence of experimental treatments on Ribulose 1, 5 bisphosphate carboxylase (Rubisco) amount**

Nickel and or/ ozone impact on the both Rubisco-SSU and Rubisco-LSU are displayed in Figure 1.

In spite of a trend towards a decrease in both Ni²⁺ and ozone -enriched atmospheres as compared to the control, there was a differential response in Rubisco-SSU and Rubisco-LSU amounts between two cultivars. These two cultivars different in average amounts of Rubisco-LSU: about 57% and 44% for Sakha 101 and Giza 178 respectively under control condition. Indeed, 100 µM Ni²⁺ declined Rubisco-SSU and Rubisco-LSU proteins of *O. sativa* cultivars with varying degree. The amount of both Rubisco subunits were consistently disturbed by ozone, indeed ozone significantly reduced both SSU and LSU proteins of both tested cultivars. On the average, protein accounted for 31% and 19% for Rubisco-SSU and Rubisco-LSU for Sakha 101 and Giza 178, respectively.

**Table 2. The influence of different concentrations of Ni²⁺, ozone (75 ppb/h) and their combinations on (A) the fresh weights of the roots and shoots of 28-day-old *O. sativa* cultivars Sakha 101 and Giza 178**

| Treatment | Root FW (µg plant⁻¹) | Shoot FW (µg plant⁻¹) |
|-----------|----------------------|-----------------------|
| Sakha 101 | Giza 178             | Sakha 101             | Giza 178             |
| Control   | 38.33 ± 3.41         | 26.66 ± 2.37         | 120 ± 10.69          | 103 ± 9.17           |
| 10 µM Ni  | 28.33 ± 2.20         | 25.0 ± 1.95          | 72 ± 5.60            | 65 ± 5.06            |
| 50 µM Ni  | 23.33 ± 2.01         | 17.0 ± 1.46          | 62 ± 5.33            | 45 ± 3.87            |
| 100 µM Ni | 23.33 ± 2.08         | 10.33 ± 0.92         | 57 ± 5.08            | 32 ± 2.85            |
| Ozone     | 23.33 ± 1.72         | 15.0 ± 1.10          | 70 ± 5.15            | 48 ± 4.22            |
| Ozone + 10 µM Ni | 21.66 ± 1.76 | 20.0 ± 1.63 | 70 ± 5.70 | 55 ± 4.46 |
| Ozone + 50 µM Ni | 25.00 ± 1.80 | 18.33 ± 1.32 | 66 ± 4.76 | 53 ± 3.82 |
| Ozone + 100 µM Ni | 30.00 ± 2.55 | 20.5 ± 1.74 | 85 ± 7.22 | 72 ± 6.11 |
| *P*       | <0.05                | <0.05                | <0.001**             | <0.001**             |

Note: Values are the means ± SD based on triplicate independent determinations, and different letters indicate significant differences as evaluated by Duncan’s multiple comparison test.
shoots under unstressed condition, as Ni increased in nutrient solution, a gradual decline in the concentration of Ca$^{2+}$ and K$^+$ level in both rice cultivars was recorded but the reduction was more obvious in Giza 178 than Sakha 101 (Figure 2). Combined treatment showed a higher ability to accumulate Ca$^{2+}$ and K$^+$ in both roots and shoots of rice cultivars in comparison to the relevant controls (Figure 2).

**Malondialdehyde and membrane integrity**

Nickel acted in a more destructive manner than O$_3$ stress and caused considerable membrane damage in the roots and shoots of both rice cultivars as assessed either by lipid peroxidation measured as MDA equivalents (Figure 3(A,B)) or by the membrane integrity (Figure 3(C)). In the 100 µM Ni$^{2+}$-treated Sakha 101 plants, the MDA content in the roots and shoots was 2.0- and 2.4-fold higher compared to the control value, respectively. The corresponding values in Giza 178 plants were 2.3- and 3.3-fold, respectively. Concerning the treatment with O$_3$ alone, the membrane integrity in leaves was 78% and 55%, for Sakha 101 and Giza 178, respectively. The application of Ni$^{2+}$ together with O$_3$ seemed to have a protective effect in terms of these parameters in both plant cultivars by lowering the intensity of lipid peroxidation and increasing the membrane integrity caused by Ni$^{2+}$ alone.

**Antioxidant enzyme activities**

SOD activity increased in the roots and shoots of both rice cultivars subjected to Ni$^{2+}$ stress (Figure 4). Ni$^{2+}$ at 100 µM increased SOD activity in the roots and shoots of Sakha 101 by 2.4- and 3.0-folds compared to corresponding control value respectively. The corresponding value for Giza 178 was 2.2- and 1.9-fold compared to the corresponding control. Ozone fumigation increased the activity of SOD. The combined application of both stress factors leads to a significant increase in SOD enzyme activities in comparison with the Ni$^{2+}$ application alone.

The change in APX activity in roots was irregular, as a slight change was recorded at lower Ni$^{2+}$ concentrations (up to 50 µM Ni$^{2+}$), and then decreased significantly below control levels at the highest Ni$^{2+}$ concentration in both cultivars. In shoots, activity increased significantly in the low and high Ni$^{2+}$ treatments to above the control level (Figure 4). The mean activity of APX increased over the control level in both rice cultivars after ozone exposure. When 75ppb O$_3$ and Ni$^{2+}$ were applied together they had a positive effect on the APX activity of rice shoots as it increased over the control value; On the other hand; there was an insignificant change for APX activity in roots upon the interaction of both stresses.

The level of GR activity was reduced by Ni$^{2+}$ - treatment in the roots and shoots of both rice cultivars compared to their relevant controls. The 100 µM Ni-treated plants showed a significant decrease in GR activity in roots by approximately 27% and 51% for cultivars Sakha 101 and Giza 178 respectively compared to their corresponding controls (Figure 4). The activity of the enzyme was reduced to levels below the control under ozone fumigation (15.96 and 11.58 in the control to 10.00 and 9.36 unit mg$^{-1}$ protein min$^{-1}$ at 75ppb O$_3$) in the shoots of both rice cultivars Sakha 101 and Giza 178 respectively. However, the combined application of both stresses had a positive effect on the GR activity of the roots and shoots as it increased by 26% and 62%, respectively, as

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**Elements analysis**

Results indicated that rice grown in Ni$^{2+}$-enriched soils led to a significant increase in Ni$^{2+}$ content with increasing Ni$^{2+}$ concentrations, maximum Ni$^{2+}$ content was recorded in Giza 178 (72.0 and 29.5 for roots and shoots) and minimum in Sakha 101 (66.0 and 17.5 for roots and shoots, respectively). In O$_3$-treated rice plants, the metal accumulation showed step decrease in Ni$^{2+}$-treated samples and did not further augment at the highest treatment, somewhat showing a trend to decrease (particularly in 100 µM Ni$^{2+}$). Accordingly, O$_3$ exposure in the presence of Ni$^{2+}$ led to approximately 23% decrease of Ni$^{2+}$ accumulation in roots and shoots of both cultivars as compared to their corresponding-treated rice cultivars.

The two rice cultivars exhibited insignificant difference in the endogenous Ca$^{2+}$ and K$^+$ concentration in roots and shoots under unstressed condition, as Ni increased in nutrient solution, a gradual decline in the concentration of Ca$^{2+}$ and K$^+$ level in both rice cultivars was recorded but the reduction was more obvious in Giza 178 than Sakha 101 (Figure 2). Combined treatment showed a higher ability to accumulate Ca$^{2+}$ and K$^+$ in both roots and shoots of rice cultivars in comparison to the relevant controls (Figure 2).

![Figure 1. The influence of 100 µM Ni$^{2+}$, ozone (75 ppb O$_3$/h) and their combinations on the amounts of Rubisco-LSU (A) and Rubisco-SSU (B) protein amounts were expressed as percentage of soluble protein determined in leaves from two rice cultivars Sakha 101 and Giza 178. Values are means based on triplicate independent determinations, means indicated with the same letter are not significantly different and different letters means significant difference as evaluated by Duncan’s multiple comparison test.](#)
compared to the Ni-treated rice plant for Sakha 101 (Figure 4).

Ni stress was an effective treatment resulting in a 39% decrease in GST activity in the roots and shoots of 28-days old Sakha 101 plants. The corresponding values for Giza 178 were 55% and 47% in the roots and shoots respectively. The specific activity of this enzyme was reduced to levels below the control under ozone stress (20.75 and 15.77 in control to 13.35 and 9.24 unit mg\(^{-1}\) protein min\(^{-1}\) at 75ppb ozone) in the shoots of the rice cultivars Sakha 101 and Giza 178, respectively (Figure 4). The combined application of Ni and ozone up-regulated the activity as anticipated in plants supplemented with Ni concentrations in both tested organs compared with metal- treated rice cultivars.

**Differential display analysis**

The differential expression of nickel and/or ozone interaction related genes in rice cultivars Sakha101 and Giza 178 under 100 µM Ni\(^{2+}\) and ozone stress was stable and significant based on the results of the mRNA differential display under stress conditions and non- stress using four primers.

Differential display analysis for *O. sativa* cultivars using the C2 primer showed that 100 µM Ni\(^{2+}\) enhanced the expression of 272, 209, 165 and 125 bp fragments for Sakha101 (lane 2) and expression of the fragment with 362 bp along with the disappearance of the fragment with 209 bp in Giza 178. Rice samples treated with 75 ppb/O\(_3\) enhanced expression of fragments with 272 and 209 bp for Sakha 101 (lane 3), as well as fragments with 362, 272 and 165 bp in Giza 178 (7). Samples treated with 100 µM Ni and fumigated with 75 ppb/h O\(_3\) (lane 4) enhanced the expression of 272 and 209 fragments for leaves of Sakha 101 and 362, 229, 141 and 125 bp fragments in the leaves of Giza 178 (lane 8, Figure 5).

Differential display analysis for *O. sativa* cultivars Sakha 101 and Giza 178 using the C1 primer showed that Ni\(^{2+}\) at 100 µM enhanced expression of 423, 320, 253, 209 and 157 bp fragments in Sakha 101(lane 2) and fragments with 297, 226 and 202 bp as well as disappearance of 209 bp in Giza 178 (lane 6), the highest intensity band was 320 bp. Rice samples fumigated with 75 ppb/h O\(_3\) enhanced expression of 423, 350, 320, 297, 253, 226, 209, 202 and 157 bp fragments in both rice cultivars (lanes 3 and 7); samples treated with 100 µM Ni\(^{2+}\) and fumigated with 75 ppb/h O\(_3\) (lane 4) exhibited the same expression as the highly intense samples of 202 bp (lane 8, Figure 6).
Differential display analysis using the NAR-47 primer showed an increase in polymorphism, and the band pattern ranged in molecular size from 1500 to 100 bp. The 100 µM Ni²⁺ revealed six bands with 650, 565, 503, 329, 309 and 212 bp in Sakha 101; the highly intense bands were 329, 309, 235 and 212 bp (lanes 2) and expression of 235 bp and disappearance of 650 bp in Giza 178 (lane 6). The rice sample fumigated with 75 ppb/h O₃ alone enhanced the expression of 309, 297 and 212 bp fragments; samples treated with 100 µM Ni²⁺ and fumigated with 75 ppb/h O₃ (lanes 4 and 8) enhanced the expression of 650, 565, 503, 329, 309, 297 and 212 bp fragments (Figure 7).

Differential display analysis using the At1-primer showed polymorphism among the examined treatments, rice treated with 100 µM Ni²⁺ showed that Ni²⁺ enhanced the expression of 492, 407, 335, 285 and 215 bp fragments; the highly intense bands were 335 and 285 bp (lanes 2 and 6). Overall 75 ppb/h O₃ stress enhanced the expression of 492, 407, 335, 285 and 215 bp fragments (lanes 3 and 7); combined stresses of 100 µM Ni²⁺ and ozone showed a similar pattern as ozone stress alone (Figure 8).

**Nucleotide sequence and sequence analysis**

After the differential screening of cDNA bands of Ni²⁺-treated and/or ozone-treated rice cultivars and their interaction, six cDNA fragments were sequenced. cDNA fragments No. 1 (GenBank accession No. LT622240) was upregulated under 100 µM Ni²⁺ stresses in Sakha 101. It was found to be 56% homologous to genes for nonspecific lipid transfer protein complexes from *O. sativa* under stress by BLAST analysis from GenBank. cDNA fragments No. 4 (GenBank accession No. LT622243) is upregulated under 100 µM Ni²⁺ stress in Sakha 101. It was found to be 89% homologous to the expression of genes for transmembrane protein, putative from *Medicago truncatula*. cDNA fragments No. 6 (GenBank accession No. LT622245) is upregulated under ozone stress in Sakha 101. It was found to be 90% homologous to genes for probable carotenoid cleavage dioxygenase 4, chloroplastic in *Cucumis sativus*. cDNA fragments No. 2 (GenBank accession No. LT622241) is upregulated under combined stress of Ni and ozone stress in Giza 178. It was found to be 86% homologous to genes for heat shock proteins in *O. sativa*. cDNA fragments No. 3 (GenBank accession No. LT622242) is upregulated under the combined stress of Ni and ozone stress in Sakha 101. It was found to be 73% homologous to genes for RNA pseudouridine synthase in *C. sativus*. cDNA fragments No. 5 (GenBank accession No. LT622244) is upregulated under combined stress of Ni and ozone stress in Sakha 101. It was found that it was 88% homologous to the expression of genes for cytochrome P450 –like in *Glycine max* (Table 3).
Discussion

With the development of urbanization, the concentration of tropospheric ozone and the increase in heavy metals have been considerably increased over the past several decades, and their phytotoxicity has attracted serious attention as a widespread pollution problem. As a highly oxidative pollutant, ozone affects terrestrial plants in a variety of ways (Gan-wen and Li 2014). Plant responses to heavy metals (and Ni in particular) or to O₃ stress has been extensively studied, although little is known about the effects of the two stress factors acting in combination. The current study showed that growth of *O. sativa* cultivars was significantly reduced in response to Ni treatments, which was evidenced by the reduction in fresh weight of the cultivars Sakha 101 and Giza 178 (Table 1). This reduction may be the result of decreased water uptake or enhanced water loss, both of which may occur as a result of membrane damage. This effect on cell division and/or cell expansion may occur through effects on DNA and RNA synthesis. Furthermore, the reduction in growth by Ni²⁺ toxicity results from metabolic disorders as a result of Ni accumulation, altered nutrients and a decrease in the rate of net photosynthesis that reduces the supply of carbohydrates or proteins and consequently decreases the growth of the rice plant. These results were confirmed in seedlings of *Phaseolus mungo* L. (Vijayarengan and Dhanavel 2005). The growth reduction in this study was more pronounced in the roots of rice cultivars than in the shoots because roots were in direct contact with the stress factor and might have accumulated more Ni than the shoot (Gajewska and Sklodowska 2008).

Under the prevailing experimental conditions, O₃-fumigation reduced the biomass of rice cultivars because respiration and transpiration had decreased due to stomatal closure, with a possible reduction in photosynthesis and gas exchange. This reduction may be due to decreased translocation of fixed carbon to edible plant parts because of either reduced availability at the source or lowered phloem transport capabilities; additionally, decreased carbon transport to the roots reduces nutrient and water uptake and affects plant stability by the reduced photosynthetic rate and stomatal conductance (Wilkinson et al. 2012).
In the current study, it is difficult to separate the effect of O₃ on growth from Ni²⁺ stress because the latter alone can produce severe osmotic stress and growth reductions. Nevertheless, we speculated that mechanism by which O₃ stress increases plant tolerance to Ni may be due to the effect of O₃ on Ni²⁺ uptake and accumulation, Ni decreased the water potential and transpiration rate, resulting in wilting and necrosis of leaves (Pandey and Sharma 2002). Additionally, there is a reduction in stomatal conductance (under publication). The significant reduction in stomatal conductance and gas exchange in the present study could be responsible for this antagonistic effect, as this would result in less O₃-
induced reductions in both ROS and MDA content under Ni stress. However, the final growth response to both stresses may depend on the balance between benefits (e.g., the reduction in O₃ absorption and Ni²⁺ uptake in leaves) and detriments (maintenance of membrane integrity). Any factor, including heavy metal that increases water stress in the plant, can increase both stomatal and mesophyll resistances to gas exchange and presumably reduce O₃ flux into the leaves (Wittig et al. 2007). Since Ni transport to the shoots occurs via the xylem, a reduction in Ni²⁺ content as a consequence of O₃ exposure and water transport could be a possible explanation for such a result. However, if O₃-induced a reduction in Ni²⁺ uptake, the translocation of other minerals in the xylem streams should have been affected which was not the case in the present experiment. It is, therefore, reasonable to hypothesize that other mechanisms may participate.
Nevertheless, the negative effect of O3 on Ni^{2+} translocation to the above ground organs may represent an advantage for the plant limiting metal accumulation in the photosynthetically active tissues.

Rubisco is a holoenzyme made of eight nucleus-encoded small subunits (SSU) and eight chloroplast-encoded large subunits (LSU). Its activity is partly regulated by another protein, Rubisco activase (Spreitzer 1999). The obtained results revealed a decrease in the Rubisco protein subunits in rice cultivars which is more obvious in Giza 178 than in Sakha 101 and may be due to (1) differences in the Ni^{2+} uptake and the tolerance mechanism (Figure 2), (2) the replacement of Mg^{2+} with Ni from Rubisco enzyme (Sheoran et al. 1990), (3) a reduction in PS II, (4) a decrease in chlorophyll content (under publication), and (5) inhibition of electron transport (Srivastava et al. 2012). The blockade of the Calvin cycle reactions would lead to the accumulation of ATP and NADPH which in turn creates a high pH gradient across the thylakoid membrane and boosts the PSI activity (Seregine and Kozhevnikova 2006). The loss in Rubisco protein that has been extensively reported in C3 species exposed to ozone is often regarded as a consequence of an enhancement in proteolysis activity related to an ozone-accelerated senescence process (Dizengremel 2001) and an inhibition of protein synthesis (Bohler et al. 2007). The decrease in Rubisco protein subunits can be interpreted from damage by ROS to chloroplasts and photosynthetic electron transport (under publication). In contrast to the results from Eckardt and Pell (1994) who found that LSU was less responsive to ozone than SSU in Solanum tuberosum but matching previous results gained from Phaseolus vulgaris (Junqua et al. 2000) and Zea mays (Leitao et al. 2007), we showed that both subunits of Rubisco in rice cultivars were equally reduced by an ozone dose. Such similar reductions might reflect distinct ozone impact.

Our results imply that improvement of Rubisco protein subunits by ozone fumigation under Ni^{2+} stress might be at least partly related to the beneficial effect of chlorophyll concentration, the efficiency of PSII and the integrity of chloroplast membranes (under publication). An increase in membrane stability and a decrease in lipid peroxidation (Figure 3) and Ni^{2+} uptake (Figure 2).

The obtained results illustrated that Ni^{2+} accumulation was found to increase with an increasing concentration of the metal in the nutrient solution in both rice cultivars. Reduction in shoot growth associated with Ni toxicity may also be attributed to Ni^{2+} -induced Fe deficiency (Kukier and Chaney 2004). Chen et al. (2009) proposed two distinct mechanisms of nickel toxicity to plants: (i) competitive interference with other essential metal ions, and (ii) induction of oxidative stress. In the present study, Ni has some similar characteristics to Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}, therefore, Ni^{2+} may compete with these metals in absorption and transpiration processes. Ni^{2+} treatment caused an increase in the efflux of K^+ and Ca^{2+} from the roots and shoots of both rice cultivars. Such damage could result from various mechanisms including the oxidation by reactive oxygen species or the changes in cell permeability that increased nonselective conductance and inhibition of the H^+-ATPase activity of the plasma membrane fraction in rice (Sharma and Dhiman 2013).

The elemental analysis confirmed the importance of K content in the leaves as it decreased after ozone exposure to support guard cell swelling and thus activate stomatal widening upon fumigation. Ozone stress also affects the Ca^{2+} contents of the tested rice cultivars. The reduction of biomass allocation to the roots might be due to the increased demand for carbohydrates in the shoot which is required to support the maintenance of respiration, therefore, led to a reduction in root biomass and causing the fewer uptakes of nutrients by plants (Andersen 2003). In the current study, reductions in concentrations of mineral nutrients could be correlated with the inhibition of translocation of mineral nutrients between the source (leaf) and the developing roots.

The combined treatment between 100 µM Ni and 75 ppb /h ozone revealed that the concentrations of K^+ and Ca^{2+} showed a significant increase compared to their corresponding Ni-treated rice cultivars. This suggests that ozone exposure moderates the effects of Ni stress by maintaining membrane stability and efficient membrane function. There is an increase in K content that regulates stomatal opening. There is an early increase in Ca^{2+} that leads to the activation of a mitogen-activated protein kinase (MAPK) and NADPH oxidase. This enzyme is responsible for the spreading of the signal to other cells. The second peak of Ca^{2+} is concurrent with the expression of glutathione S-transferase (Figure 4). Abscisic acid (ABA)-induced closure of the stomata is the result of Ca^{2+} influx activation in the guard cells (Baier et al. 2005).

Nickel-treated rice plants induce a decrease in membrane integrity from roots and leaves of the current study, which could be due to the destructive effect of Ni on membranes and might be associated with increased lipid peroxidation.

### Table 3. Identification and functional classification of up and down regulated cDNAs fragments of O. sativa under 100 µM Ni^{2+} and/or 75 ppb/h ozone fumigation in two rice cultivars Sakha 101 and Giza 178.

| cDNA No. | Accession Number | DD product | Length (bp) | Gene similarity | Percentage | Function |
|----------|------------------|------------|-------------|-----------------|------------|----------|
| 1        | LT622240         | 100 µM Ni Sakha 101 | 171         | Nonspecific Lipid Transfer Protein Complexes From O. sativa | 56%        | Plant defense and antimicrobial |
| 2        | LT622241         | 100 µM Ni + 75 ppb/h ozone Giza178 | 314         | Heat shock proteins O. sativa | 86%        | Protein fold, repair proteins, maintenance of normal conformation |
| 3        | LT622242         | 100 µM Ni + 75 ppb/h ozone Sakha 101 | 372         | RNA pseudouridine synthase C. sativus | 73%        | Initiation of translation, and protection from radiation |
| 4        | LT622243         | 100 µM Ni Sakha 101 | 650         | transmembrane protein, putative M. truncatula cytochrome P450 -like G. max | 89%        | Signaling pathway and putative ion channel |
| 5        | LT622244         | 100 µM Ni^{2+} 75 ppb/h ozone Sakha 101 | 565         | probable carotenoid cleavage in dioxygenase 4, chloroplastic C. sativus | 88%        | Defensive signaling molecule |
| 6        | LT622245         | 75 ppb/h ozone Sakha 101 | 290         | probable carotenoid cleavage in dioxygenase 4, chloroplastic C. sativus | 90%        | ABA biosynthesis |

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as well as changes in the fatty acid profile, resulting in decreased lipid unsaturation (Gajewska et al. 2012). In addition, Ouzounidou et al. (2006) indicated that membrane structure and functioning might also be affected by displacement of Ca2+ from the membrane ligands by Ni2+ ions. Lipid peroxidation is an effective indicator of cellular oxidative damage and the results showed that the MDA content increased significantly in the roots and shoots of the two rice cultivars under O3 stress separately, the tolerant cultivar Sakha 101 maintained lower value than sensitive one Giza 178. Supplementation of the Ni-containing medium with O3 fumigation significantly reduced lipid peroxidation and increased membrane integrity in both rice cultivars. We suggest that O3-induced protection of cell membranes under conditions of Ni2+, stress is related mainly to augmentation of antioxidant capacity. Such a supposition is in agreement with the obtained results documenting decreases in lipid peroxidation and increases in antioxidant enzyme activities. This means a better membrane stability and more efficient membrane function and presumably ascribed to the decrease in Ni2+ uptake and decreased lipid peroxidation which was displayed as antagonistic under the combined stress.

Ni induced a significant promotion of SOD activity in both roots and shoots of rice cultivars which is more pronounced in Giza 178 than in Sakha 101. This might be due to an elevated content of superoxide radicals, with the activation of SOD enzymes. In pigeon pea seedlings, SOD activity has been shown to be increased in response to Ni treatment (Maheshwari and Dubey 2009). Other studies have shown an inhibition of SOD activity on Ni exposure in barley and pea plants (Lu et al. 2010). High levels of SOD activity can act as a systemic intracellular signal for the induction of protective enzymes and the cleaning of H2O2 from the shoots of Ni2+-stressed rice plants (Gajewska et al. 2001). Herein, the current study observed that Ni2+ decreased the GR activity in the roots and shoots of rice cultivars, this reduction could be the result of direct binding of Ni2+ to the sulphhydryl group of the enzyme, influencing the glutathione cycle. GR was inhibited by Ni2+, shifting the redox equilibrium of GSH to a more oxidative form (GSSG, data not shown). Chuttan et al. (2016) recorded that unchanged GR and SOD activities in plants grown under elevated O3 could indicate that constitutive activities in these two soybean cultivars were sufficient to allow normal functioning of the AsA–glutathione cycle. The interaction of 75 ppb/h O3 +100 μM Ni2+ had a positive effect on the GR activity of roots and shoots as it increased GR activity as compared to Ni2+-treated rice cultivars probably through GR modulating GSH levels by regulating its biosynthesis or by protecting the reactive cysteine residue of GSH or through the efficient functioning of GR. The elevated levels of GR activity in photosynthetic organs perhaps may increase the ratio of NADPH/ NADP+, thereby ensuring the availability of NADPH to accept electrons from the photosynthetic electron transport chain. Under such a situation, the flow of electrons to O2 and therefore, the formation of O2− can be minimized.

Glutathione-S-transferases (GST) catalyze both GSH-dependent conjugation and reduction thereby playing an active role in detoxification because GST mRNA responds very quickly to oxidative stress (Conklin and Lasat 1995). GST is clearly reduced upon Ni2+ and O3 exposure in rice cultivars; this may be due to its inhibition by peroxidation of GST protein or inactivation of GST genes. GSTs with peroxidase activity can use GSH as a reductant for scavenging the reactive oxygen species formed upon Ni2+ and O3 stress. GST conjugates itself to these products formed by the oxidative degradation of lipids and nucleic acids and prevents them from propagating further damage. The conjugates are subsequently transported to the vacuole and protect plant cells from their harmful effect (Nagalakshmi and Prasad 2001). The combined interaction between ozone and Ni stress clearly restored the lost thiol pools, with a simultaneous decrease in the oxidized GSSG, or probably modulated GSH levels by regulating its biosynthesis or by protecting the reactive cysteine residue of GSH (data not shown) or through the efficient functioning of GR and GST, thus increasing the activities of antioxidant enzymes to strengthen the ROS scavenging activity or maintain ROS homeostasis.

The mRNA differential display - polymerase chain reaction (DD-PCR) is a powerful method for screening and identifying differentially expressed genes (Liang and Pardee 1992). Many differential fragments related to Ni and / or ozone stress were found in the current study. There was a different level of similarity among the examined treatments and variation was generated due to the appearance of down-regulated genes (turned off) and upregulated genes (turned on). It could be also inferred that some similar mechanism in different plants may exist for stress tolerance under different abiotic stresses.
The sequence analysis of upregulated cDNA fragment 1 with 171 bp derived from the differential display of 100 µM Ni²⁺ of cultivar Sakha 101 showed 56% similarity to the non-specific lipid binding transfer protein complexes (NsLTPs) from *O. sativa*. NsLTPs are involved in the formation of a protective hydrophobic cutin and suberin layers over plant surfaces, which prevent fungal attacks (Trevino and O’Connell 1998). Up regulation of this gene in the Ni²⁺ tolerant cultivar Sakha 101 could be an adaptive response to Ni²⁺ stress.

The sequence analysis of upregulated cDNA fragment 4 with 650 bp in rice cultivar Sakha 101 treated with 100 µM Ni²⁺, revealed the upregulation of genes involved in transmembrane protein, recognized with 89% similarity to *Medicago truncatula*. These membrane proteins were categorized into four functional classes based on putative cellular roles for genes in plants (Ane et al. 2004) including energy and transport, protein destination/storage, and unclassified protein categories. Riely et al. (2007) have identified genes in this signaling pathway including *M. truncatula* DM1 which encodes a putative ion channel. DM1 may play a direct role in responding to Nod factors by triggering the initial calcium flux. The increased expression level of this protein under abiotic stress, in the rice cultivar Sakha 101 might indicate a possibility that these proteins do indeed function in signal transduction as calcium is a second messenger that regulates the transcription of several genes. It modulates several physiological functions; nevertheless, the fact that it regulates the expression of several genes makes it an interesting putative gene for Ni²⁺ stress tolerance in cultivar Sakha 101.

The sequence analysis of upregulated cDNA fragment 6 with 290 bp in the rice cultivar Sakha 101 seedlings treated with 75 ppb/h ozone fumigation, revealed the upregulation of genes with 90% similarity to carotenoid cleavage dioxygenase 4, chloroplastic. The CCD family includes the 9-cis epoxy-carotenoid dioxygenase (NCED) enzyme subfamily, which cleaves neoxanthin during ABA biosynthesis (Tan et al. 2003). The first gene encoding a carotenoid cleavage dioxygenase was isolated from the *Z. mays* ABA-deficient viviparous mutant, VP14. VP14 catalyzes the first step in ABA biosynthesis, ABA can induce stomatal closure when produced endogenously or exogenously supplied to the soil, when injected into the stem, or when supplied to the xylem of explants via the cut petiole (Wilkinson and Davies 2008). Ozone always induces stomatal closure and will have important consequences for water use in natural and agricultural systems in our drying world. Ahmad et al. (2015) recorded that upregulation of cDNA encoding a novel phytocystatin gene (CC14) was observed after 20 days of ozone stress in maize leaves; they concluded that the CC14 gene could have a protective effect on preventing cellular damage.

The sequence analysis of upregulated cDNA fragment 2 with 314 bp in rice cultivar Giza 178 seedlings treated with 100 µM Ni²⁺ and 75 ppb ozone fumigation, revealed upregulation of genes involved in heat shock protein-related with 86% similarity to *O. sativa*. Heat shock proteins (HSPs) are expressed under a variety of stress conditions, including the exposure to an excess of heavy metals. Hsps as mainly contribute to protein transportation, protein folding, protein repair, nucleic acids and bio membrane function from injury and the maintenance of normal conformation, molecular chaperones in dimers regulated by ATP and preventing aggregation with ATP (Wang et al. 2004). A study on *Catharanthus roseus* showed that HSP70s, HSP20 and HSP90, were up regulated by Pb stress (Kumar et al. 2011). The increased expression level of the proteins under abiotic stress, in the rice cultivar Giza 178 might indicate their role in protein folding, translocation, degradation, and assembly under stress conditions.

The sequence analysis of upregulated cDNA fragment 3 with 372 bp in rice cultivar Sakha 101 treated with 100 µM Ni²⁺ +75 ppb/h O₃, revealed upregulation of genes involved in RNA pseudouridine synthase with 73% similarity to *C. sativus*. Pseudouridine (abbreviated by the Greek letter psi-Ψ) is the C-glycoside isomer of the nucleoside uridine, and it is the most prevalent of the over one hundred different modified nucleosides found in all species in many classes of RNA, it is formed by enzymes called Ψ synthases, which post-transcriptionally isomerize specific uridine residues in RNA in a process termed pseudouridylation (Schwartz et al. 2014). It is expected to play a role in the association with aminocyl transferases during their interaction with tRNA, and hence in the initiation of translation. Other studies suggest that it may offer protection from radiation (Monobe et al. 2003).

The sequence analysis of upregulated cDNA fragment 5 with 565 bp in rice cultivar Sakha 101 seedings treated with 100 µM Ni²⁺ and 75 ppb ozone fumigation, revealed upregulation of Cytochromes P450s dependent with 88% similarity with one of the cytochrome P450s present in soybean. Several P450s are involved in the biosynthesis and catabolism of plant hormones (Mizutani 2012). Species-specific P450 families are essential for the biosynthetic pathways of phytochemicals such as terpenoids and alkaloids, the 4-hydroxylation of cinnamic acid, which is an early and obligatory step in the biosynthesis of most phenolic compounds such as lignin monomers, flavonoids, coumarins, stilbenes and lignans that are related to disease resistance in plants (Dixon 2001). Therefore, flavonoids confer Ni²⁺ tolerance via the dual mechanisms of Ni chelation and antioxidation by maintaining membrane integrity by preventing the access of deleterious molecules to the hydrophobic region of the bilayer (Arora et al. 2000). In summary, differential display polymerase chain reaction (DD-PCR), a competent, profound and a powerful tool used to investigate the genes whose expression is modulated by Ni²⁺ and/or O₃ interaction in rice. The interaction between Ni²⁺ and O₃ resulted in the joint action of transcriptional, translational, and post-translational levels which may lead to a comprehensive understanding of the molecular basis of rice tolerance and will be helpful for the organization of genes that are involved in stress tolerance and crop improvement.

**Conclusion**

In light of the above findings, it is concluded that Ni²⁺ and ozone stress provoked severe damages when applied separately, as evaluated by changes in the amounts of stress markers. The interactive effects of both stresses seem to bring out some adaptive effects to reduce the damage experienced by rice cultivars. However, the antagonistic effect of most of the studied parameters increases growth and increases antioxidant activities to overcome the adverse effects of both pollutants. These outcomes suggested that exploration of
Ni$^{2+}$ and ozone together display a high potential for the improvement of antioxidant activities with the expression of defense genes to overcome the adverse effects of both pollutants.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

The mRNA sequence datasets generated during the current study are available in the gene bank under their corresponding accession number.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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