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Selected Physiological and Molecular Responses of *Arabidopsis thaliana* and *Nicotiana tobacum* Plants Irrigated with Perchlorate-containing Water

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Abstract: Perchlorate is a significant contaminant of concern in surface and ground water. It has been extensively investigated for uptake and accumulation by land and aquatic plants. Although, the uptake and accumulation of perchlorate by plants is well investigated, no study was undertaken to investigate how perchlorate may affect plants's physiological and metabolic processes. *Arabidopsis thaliana* and *Nicotiana tobacum* plants were used as model organisms to investigate the effects of perchlorate to some selected physiological and molecular processes of the plants. Six-week-old Arabidopsis and tobacco plants were exposed to varying concentrations of perchlorate (ClO_4^-) in irrigation water and the rates of the light reactions of photosynthesis of their chloroplasts along with the activities of the Superoxide Dismutase (SOD) and of the Ascorbate Peroxidase (APO) were measured. Chlorosis and necrosis were noted in all plants irrigated with perchlorate solutions but not in the control plants. Measurement data indicated that perchlorate concentration as low as 1 mM was enough to induce a 2.15 fold decrease in the rate of the light reactions of photosynthesis and to induce a 1.2 folds increase in the total protein expressed, to cause a 2.0 folds increase in SOD activity and 1.7 folds increase in APO activity in Arabidopsis; while in tobacco plants, the same concentration of perchlorate was enough to induce 3.12 folds decrease in the rate of the light reactions and 1.7 fold increase in SOD activities and a 1.3 fold increase in APO activities, indicating that perchlorate in soil or irrigation water will not only interfere with plants growth and development but will also induce oxidative stress to plants.

Key words: Arabidopsis, tobacco, perchlorate, photosynthesis, superoxide dismutase (SOD), ascorbate peroxidase (APO)

INTRODUCTION

Environmental pollution is a frequent phenomenon in the industrialized world. When released in the environment, pollutants find their ways in aquatic and terrestrial ecosystems. One such pollutant is perchlorate (ClO_4^-). Perchlorate is a salt derived from perchloric acid ($HClO_4$). It occurs naturally as well as artificially. It is commonly associated with ammonium, potassium and sodium salts. Perchlorate salts can undergo mild explosive reactions in presence of oxidizable substances. Because of this property, perchlorate has been used as oxidizer in manufacturing propellants, ballistics and rocket and missile fuel. Perchlorate is also used in manufacturing munitions and fireworks and used in other applications such as roadside flares, airbag inflators and matches. Lakes, rivers and underground waters near rocket-assembly plants, military bases and fertilizer industries are at high risk of perchlorate contamination. Perchlorate is a risk factor for human health. It affects human health by inhibiting iodide uptake which in turns reduces thyroid hormone production and cause abnormal growth and development (USEPA, 1998). In the mid-1980's, perchlorate was detected in drinking water supplies in Nevada, Utah and California. Since first reported, perchlorate has been detected in ground water of more than 20 U.S. states (Smith *et al*., 2001; USEPA, 2002; Yu *et al*., 2004). Perchlorate is a significant contaminant of concern in surface and ground water in recent years. Since first discovered in drinking water in the USA, it has been detected in several parts of the world including South Korea (Her *et al*., 2010) where dairy milk samples and milk-based powdered infant formulas were found to contain elevated levels of perchlorate. The occurrence of perchlorate was also reported in Japan (Kosaka *et al*., 2007), in China (Shi *et al*., 2007, 2011), where elevated concentrations of perchlorate were reported in drinking water sources, in the atmosphere, rice, bottle water and milk. In China, the problem is even more accentuated following the spring festival. Perchlorate pollution is a global problem, an enigma for the new millennium (Dasgupta, 2006). Because of its high water solubility, perchlorate may seep into the aqueous phase of soils and may become available for uptake by plants. Susarla *et al*. (2000), Urbansky *et al*. (2000) and Smith *et al*. (2001) evaluated perchlorate uptake in several

plants species including vegetable crops, trees, grasses and forbs. They found that all plants tested, with few exceptions, were capable of rapid uptake of perchlorate. Other plant species tested and found to uptake and accumulate perchlorate include tobacco *Nicotiana tobacum* (Ellington *et al*., 2001; Sundberg *et al*., 2003), cucumber *Cucumis sativus*, lettuce *Lactuca sativa*, soybean *Glycine max* (Yu *et al*., 2004), poplar trees *Populus deltoide* (Van Aken and Schnoor, 2002) and salt cedar *Tamarix ramosissima* (Urbansky, 1998). Tests conducted on water, soil, vegetation and rodents collected from 3 areas along the Las Vegas Wash (Smith *et al*., 2004), showed that plants uptake and accumulate perchlorate from soil or water sources. These authors also found that with the exception of cockleburs (*Xanthium strumarium*), all the plant species contained elevated concentrations of perchlorate. Sanchez *et al*. (2006) analyzed perchlorate in leaves and peels of lemon, grapefruit and orange trees and found that citrus trees do accumulate perchlorate. With the exception of the published comparative study of perchlorate and chlorate modes of action in bean plants (Weaver, 1942), no physiological or molecular investigation on plants' responses to perchlorate has been reported. Weaver (1942) observed that the symptoms associated with perchlorate toxicity to plants include chlorosis and necrosis which may be mistaken for water or nutrient deficiency symptoms. Perchlorate may cause physiological, molecular stresses to plants by interfering with the structures and functions of biological molecules. Being an oxidizer, perchlorate may also cause oxidative stress to plants as the result of a build-up of Reactive Oxygen Species (ROS) such as the superoxide radical O_2^-, the singlet oxygen $^1O^-$, the hydroxyl OH^-. In this study, the oxidative stress responses of *Arabidopsis thaliana* and *Nicotiana tobacum* plants to perchlorate along with the effects of perchlorate to the plants light reactions of photosynthesis are investigated.

MATERIALS AND METHODS

This research was conducted between 2008 and 2010 in the Department of Biology of Jacksonville State University, Jacksonville, Alabama, USA. Seeds of wild type *Arabidopsis thaliana*, Ecotype Columbia Wt-02, were purchased from LEHLE SEED Company (Round Rock, TX, USA). The seeds of *Nicotiana tobacum* (var. K-139) were donated by NC State University. The seeds were sown in 10 inch pots containing a mixture of vermiculite and potting soil. The germinated tobacco plants were thinned to 1 plant per pot and the Arabidopsis plants to 2 plants per pot. All plants were reared and maintained in a growth chamber programmed at 18 h light, 6 h dark, 24°C for a period of 6 weeks. The plants were watered every other day and fertilized once a week with Miracle Gro® plant food. At the start of perchlorate treatment, the Arabidopsis plants were at 8-rosette-leaves stage and the tobacco plants were at 3-fully-expanded-leaves stage. Perchlorate treatment consisted of irrigating the experimental plants every other day for 7 days, with 50 mL solutions containing 1.0, 5.0 and 10 mM $NaCLO_4^-$. The control plants were watered distilled water. On the eight day of the experiment, leaves were harvested randomly and used for chloroplasts and native enzymes extraction.

Chloroplasts were extracted by lightly grinding leaf tissues in citrate-borate buffer (0.45 M sucrose, 50 mM boric acid, 30 mM Na-Citrate, 10 mM EDTA, pH 7.2) The homogenates were filtered through 2 layers of cheesecloth. The filtrates were filtered again through 4 layers of cheesecloth and then centrifuged at 300 g for 5 min in a refrigerated centrifuge. The supernatants were collected and further centrifuged at 4,000 g for 25 min. The chloroplast-containing pellets were resuspended in 1/10th volume of the extraction buffer and used to investigate if watering with perchlorate has an effect on the plants chloroplasts to perform the light reactions of photosynthesis.

Native enzymes were extracted from the experimental and from the control plants as total soluble proteins, by grinding leaf tissues at the ratio of 0.3 g per 1.0 mL of enzyme extraction buffer (50 mM Tris-HCl pH 6.8, 1 mM EDTA and 0.5% PVP) in mortar and pestle and then homogenizing in a Teflon glass homogenizer. The homogenates were filtered through 2 layers of cheesecloth. The filtrates were centrifuged at 25,000 g in a refrigerated centrifuge for 20 min. The resulting supernatants were retained as total native soluble proteins (enzymes). The total protein yields of the experimental treatments were assessed according to Bradford (1976) method using Bovine Serum Albumin (BSA) as the standard protein. Proteomic profiles of the experimental treatment were established using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) by electrophoresing 5 μg total soluble proteins in 12% SDS poly acrylamide gels at 120 volts and then staining the gels with Coomassie blue R-250.

Effects of perchlorate to the light reactions of photosynthesis: The effects of perchlorate to the light reactions of photosynthesis were investigated spectrophotometrically, in the isolated chloroplasts, according to Bregman (1990).

Oxidative stress responses of Arabidopsis and Tobacco plants to Perchlorate: The plants oxidative stress responses to perchlorate were investigated by measuring the activities of the oxidative enzymes Superoxide Dismutase (SOD) and of the Ascorbate Peroxidase (APO) in the protein extracts. Superoxide Dismutase (SOD; EC 1.15.1.1) activity was measured spectrophotometrically at 560 nm in the protein extracts according to Beauchamp and Fridovich (1971) with a minor modification. The modification involved generating a standard curve of commercial chloroplast SOD (cpSOD, SIGMA, St. Louis, MO., USA) to allow for quantification of SOD units of activities. The SOD isozymes of the plants treated with and without perchlorate were visualized by electrophoresing 5 μg total proteins extract of each treatment in 10% native poly-acrylamide gels at 4°C. Three units of activity of commercial cpSOD (SIGMA, St. Louis, MO., USA) were used as positive control and the extraction buffer was used as the negative control. After electrophoresis, the gels were incubated for 45 min in the dark in a solution containing 50 mM KPO_4 pH 7.5, 0.5 mM riboflavin, 1.6 mg mL^{-1} NBT, 1.2 mg mL^{-1} BCPIP and 0.4% (v/v) TEMED. The gels were rinsed in a solution containing 100 mM KH_2PO_4 pH 7.5 and 1 mM EDTA in the light (Beauchamp and Fridovich, 1971; Vallejos, 1983). SOD isozymes were observed in the gels as zones of clearing against a purple background. Ascorbate peroxidase (APO; EC 1.11.1.7) activity was measured spectrophotometrically according to modified procedures of Nakano and Asada (1981) and of Asada and Takahashi (1987). The modification involved generating a standard curve of O_2 evolved through the breakdown of H_2O_2 by a commercial peroxidase enzyme solution to allow for quantification of O_2 evolved. The reaction mixtures consisted of 50 mM Na-phosphate buffer, pH 7.0, 0.5 mM Ascorbate, 0.1 mM EDTA and 1.2 mM H_2O_2. 5 μg total proteins extracts from each treatment were used for each assay.

The experiment was repeated 5 times, giving consistent results. The data were grouped, averaged and analyzed using the randomized block design.

RESULTS

The 5 and 10 mM applications of perchlorate had pronounced effects on both Arabidopsis and tobacco plants. Chlorosis and leaf desiccation were visible in Arabidopsis and tobacco plants (Fig. 1a-d). The chlorotic spots were larger on tobacco leaves than on Arabidopsis leaves but both plant species experienced severe curling

Fig. 1 (a-d): Chlorsis and necrosis on the leaves and shoot apical meristems of *Arabidopsis thaliana* and *Nicotiana tobacum* plants watered with varying concentration of perchlorate. The concentrations were a: 0 mM, b: 1 mM, c: 5 mM and d: 10 mM; plants were irrigated every other day for 7 days

and necrosis of the shoot apices as the result of perchlorate treatment. Neither chlorosis nor desiccation was observed in the control plants.

Effects of perchlorate to the light reactions of photosynthesis: The data from the measurements of the light reactions of photosynthesis, measured in terms of the reduction of DCPIP to DCPIPH2 by the isolated chloroplast, are as follows: plants of the control treatment, in Arabidopsis, averaged 3.22 μmol. $DCPIPH_2$/min compared to 1.5 μmol. $DCPIPH_2$/min recorded in plants of the 1 mM perchlorate treatment. The average rates of photoreduction of Arabidopsis plants from the 5 and 10 mM perchlorate treatments were 0.92 μmol. $DCPIPH_2$/min and to 0.67 μmol. $DCPIPH_2$/min, respectively (Fig. 2) Plants of the control treatment, in tobacco, averaged 12.5 μmol. $DCPIPH_2$/min compared to 4.0 μmol. $DCPIPH_2$/min recorded in plants of the 1 mM perchlorate treatment. The average rates of photoreduction of tobacco plants from the when the 5 and 10 mM perchlorate treatments were 2.70 and 2.4 μmol and $DCPIPH_2$/min, respectively.

Effects of perchlorate to plants protein expression: The total protein yield of control Arabidopsis plants was 5.187 μg mg^{-1} fresh weight compared to 8.483 μg mg^{-1} fresh weight recorded in plants of the 1 mM perchlorate treatment. Plants of the 5 and the 10 mM treatment averaged 12.193 μg mg^{-1} fresh weight and 12.10 μg mg^{-1} fresh weight yield, respectively (Fig. 3) In tobacco plants, the average yield of total proteins in control tobacco plants was 4.487 μg mg^{-1} fresh weight compared to 6.243 μg mg^{-1} fresh weight recorded in plants of the 1 mM perchlorate treatment. The plants of the 5 mM and 10 mM treatments averaged 11.860 μg mg^{-1} fresh weight and 14.043 μg mg^{-1} fresh weight protein yields, respectively (Fig. 3).

SDS-PAGE analyses of the total proteins revealed 2 densely stained protein bands of 55 and 14 KD in both Arabidopsis and tobacco treatments The intensity of the 14 KD band decreased when plants were watered with increasing concentration of perchlorate but the intensity of the 55 KD band was not (Fig. 4) In Arabidopsis plants, 6 other protein bands of 180, 150, 75, 60, 40 and 35 KD stained denser in the control plants than in perchlorate-treated plants while in tobacco plants, 3 bands of 145, 75 and 60 KD stained denser in the control plants than in the perchlorate-treated plants.

Oxidative stress responses of Arabidopsis and tobacco plants exposed to perchlorate: The oxidative stress responses of the Arabidopsis and the tobacco

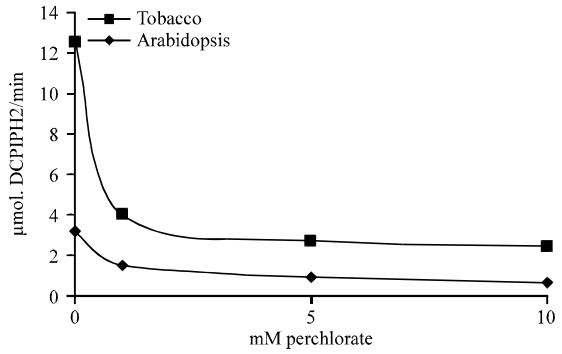

Fig. 2: Averages rates of photoreduction (μmol. DCPIPH2/min) by isolated chloroplasts of *Arabidopsis thaliana* and *Nicotiana tobacum* plants subjected to perchlorate treatment. The plants were irrigated every other day with 0, 1, 5 and 10 mM perchlorate for 7 days before isolating their chloroplasts

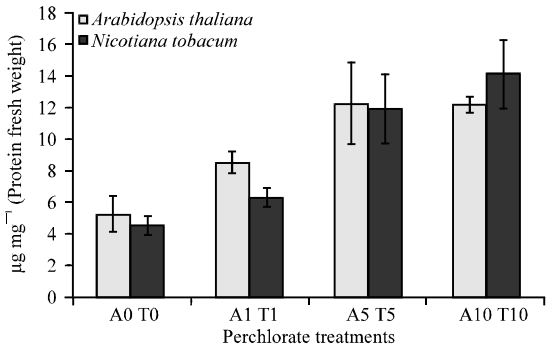

Fig. 3: Averages of protein yields (μg mg^{-1} fresh weight) of *Arabidopsis thaliana* and *Nicotiana tobacum* plants in response to perchlorate treatment. Letters "A" and "T" denote Arabidopsis and tobacco plants, respectively and the numbers next to the letters indicate the concentrations of perchlorate (mM) used. The plants were irrigated every other day with 0, 1, 5 and 10 mM perchlorate for 7 days and proteins were isolated from their leaves

experimental plants were measured as the activities of the anti-oxidative enzymes SOD and APO.

The results of the SOD activities are presented in (Table 1). An average of 1.98 units of SOD activity per microgram total proteins (ua μg^{-1} prot) were recorded in control Arabidopsis plants compared to 3.95 ua μg^{-1} recorded in plants of the 1 mM perchlorate treatment. Arabidopsis plants of the 5 and the 10 mM treatment averaged 5.10 and 7.64 ua μg^{-1}, respectively. The control

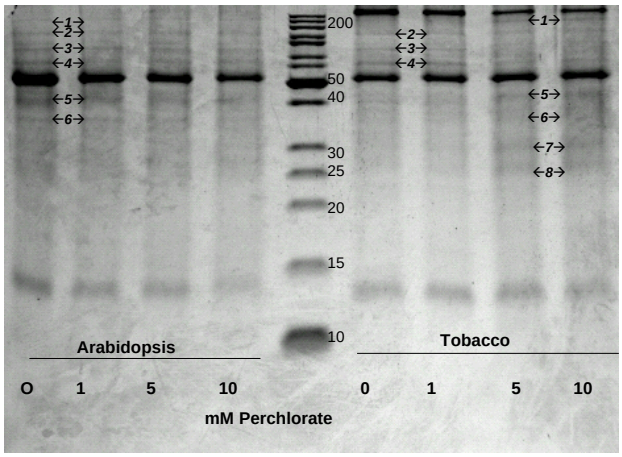

Fig. 4: SDS-PAGE profiles of *Arabidopsis thaliana* and *Nicotiana tobacum* proteins expressed in response to perchlorate treatment. The plants were watered every other day with 0, 1, 5 and 10 mM perchlorate for 7 days. The gel is obtained by resolving 5 μg total protein in 12% SDS-polyacrylamide gels and then stained with Coomassie blue R-250. Numbers and arrows are pointing at protein bands affected by perchlorate treatment

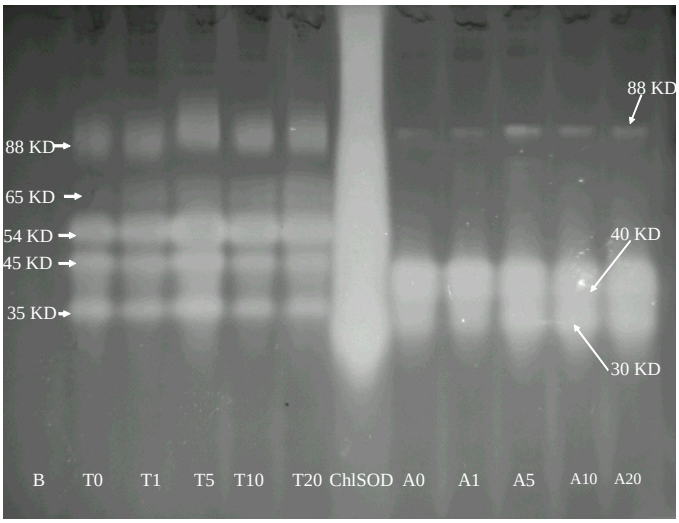

Fig. 5: SOD isozymes profiles of *Arabidopsis thaliana* and *Nicotiana tobacum* in response to perchlorate treatment. SOD activities are shown as zones of clearing. The enzymes were extracted in native form; 5 μg total proteins were resolved in 10% native polyacrylamide gels at 4°C. Letters "A" and "T" denote Arabidopsis and tobacco plants, respectively and the numbers next to the letters indicate concentrations of perchlorate (mM) used. The letter "B" denotes the extraction buffer used as a negative control and ChlSOD denotes commercial Chloroplast SOD used as a positive control

tobacco plants averaged 1.90 ua μg^{-1} compared to 3.24 ua μg^{-1} recorded in plants of the 1 mM perchlorate treatment. The averages of SOD activity were 5.56 and 9.08 ua μg^{-1} in tobacco plants of the 5 and 10 mM treatments, respectively. Investigation of the SOD isozymes in Native Acrylamide Gel Electrophoresis (NAGE), (Fig. 5), revealed 3 SOD isozyme bands of 88, 40 and 30 KD in Arabidopsis plants and 5 isozyme bands of 88, 65, 54, 45 and 35 KD in tobacco plants.

Table 1: Average SOD units of activities per microgram total soluble proteins (ua μg^{-1}) recorded in *Arabidopsis thaliana* and *Nicotiana tobacum* plants irrigated with perchlorate solutions

Treatments (mM)	Arabidopsis	Tobacco
0	1.987[a]	1.901[c]
1	3.954[a]	3.241[c]
5	5.103[ab]	5.556[cd]
10	7.643[b]	9.076[d]

Means followed by the same letter, within columns, do not differ significantly at the 5% probability level (n = 5)

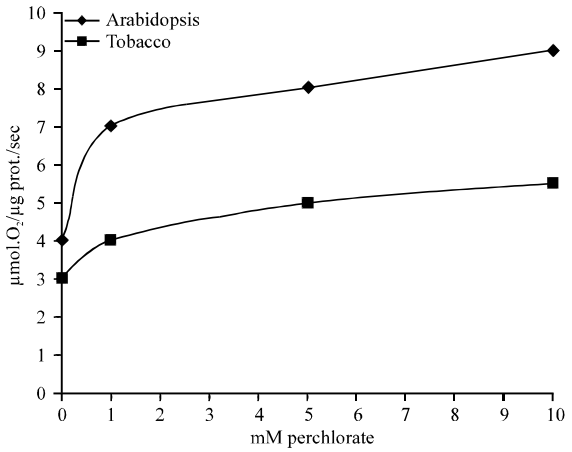

Fig. 6: Averages APO activity (µmol. O_2) recorded in *Arabidopsis thaliana* and *Nicotiana tobacum* plants in response to perchlorate treatment. Letters "A" and "T" denote Arabidopsis and tobacco plants, respectively and the numbers next to the letters indicate the concentrations of perchlorate (mM) used. The plants were irrigated every other day with 0, 1, 5 and 10 mM perchlorate for 7 days and native enzymes were isolated from their leaves

The activity of the APO enzyme was expressed as µmol.O_2 per microgram total protein per second (µmol.O_2/µg Prot./sec) and presented in Fig. 6. An average of 4.0 µ µmol.O_2/µg Prot./sec was recorded in control Arabidopsis plants compared to 7.0 µmol.O_2/µg Prot./sec recorded in plants watered with 1 mM perchlorate, 8.0 µmol.O_2/µg Prot./sec for the 5 mM treatment and 9.0 µmol.O_2/µg Prot./sec for the 10 mM treatment. The control tobacco plants averaged 3.0 µmol.O_2/µg Prot./sec compared to 4.0 µmol.O_2/µg Prot./sec. recorded in plants watered with 1 mM perchlorate. Tobacco plants of the 5 and 10 mM treatments averaged 5.0 µmol.O_2/µg Prot./sec 5.1 µmol.O_2/µg Prot./sec, respectively.

DISCUSSION

This research provided evidence that perchlorate induced chemical damage to Arabidopsis and tobacco plants by interfering with the plants' physiological processes. Perchlorate caused damage to both Arabidopsis and tobacco plants. The plants were visibly injured by perchlorate. Concentration as low as 1 mM, was sufficient to cause leaf chlorosis and necrosis. Perchlorate caused heavy leaf curling and desiccation to both Arabidopsis and tobacco plant, indicating that stress caused by perchlorate to plants may be similar to that caused by water deficiency. These conclusions are in agreement with those of Weaver (1942). Leaf curling and desiccation in plants cause reduction in leaf areas that could otherwise be used for photosynthesis. Indeed, this research observed an exponential decrease in the rates of $DCPIPH_2$ formation by both Arabidopsis and tobacco plants as the result of perchlorate application. Fifty three percent decrease in the rate of photoreduction was recorded between the control Arabidopsis plants and plants of the 1 mM perchlorate treatment and 79% reduction was recorded between the control plants and plants of the 10 mM perchlorate treatment. In tobacco plants, the decrease in photoreduction between the control plants and the 1 mM treatment was 68 and 81% between control plants and plants treated with 10 mM perchlorate. This indicates that perchlorate significantly decreases plants abilities to efficiently carry the light reactions of photosynthesis.

The total protein yields produced in response to perchlorate application increased linearly as the concentration of perchlorate was increased in both Arabidopsis and tobacco plants. This indicated that plants under environmental stress will experience an increase in protein expression so as to provide the necessary defense against the stress. In this case, most of the proteins are expressed for the sake of defense against the stress caused by perchlorate. SOD activity in both Arabidopsis and tobacco plants increased exponentially with increasing concentrations of perchlorate. Arabidopsis plants exposed to 1 mM perchlorate had twice the amount of SOD of the control plants, 2.5 times when applied 5 mM and 3.85 times when applied 10 mM perchlorate. Similar trend was observed in tobacco with plants exposed to 1 mM perchlorate having 1.7 times the amount of SOD of the control plants, 2.92 times when exposed to 5 mM and 4.80 times when exposed to 10 mM. The APO activity recorded also showed similar trends to the SOD activity, indicating that oxidative stress will occur to plants as direct consequences of exposure to perchlorate in irrigation water. Unlike all the literature reviewed in which uptake and accumulation is heavily investigated, this research is one of the first types investigating the physiological effects of perchlorate to plants. With no other published literature to compare with, it is safe to say that this research has laid down the

basis for further investigations into the physiological and molecular responses of plants to perchlorate in order to find sources of tolerance or resistance.

CONCLUSIONS

The results of present study suggest that perchlorate in the soil or irrigation water is a potential health hazard to plants. Indeed, all tobacco and Arabidopsis plants irrigated with perchlorate containing water showed significant chlorosis and necrosis. The data also demonstrated that perchlorate interferes with the light reactions of photosynthesis and induces oxidative stress to plants. Indeed, significant reduction in the rate of DCPIPH2 formation and an increase in the activities of SOD and APO were recorded in all plants irrigated with perchlorate containing water.

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