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Ultrastructural, physiological and biochemical analyses of chlorate toxicity on rice seedlings

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Abstract

Chlorates are oxidizing substances considered phytotoxic to all green plant parts. In this study the physiological, biochemical and ultrastructural effects of chlorate treatments were studied in rice seedlings grown at two different temperatures (28 and 6 °C). Visual analyses demonstrated that the chlorate treatment provoked severe damage to plant leaves only at 28 °C. The electron microscopy of the chloroplast revealed several changes in the organelle organization, whose integrity of outer membrane and the internal network of thylakoid membranes were almost completely lost. A high level of lipid peroxidation was observed in 28 °C-chlorate-treated leaves in comparison to plants exposed to 6 °C-chlorate treatment. Photochemical efficiency of photosynthesis (F_v/F_m) was drastically affected by the 28 °C-chlorate treatment but, at low temperature, chlorate-treated plants suffered a discrete reduction in F_v/F_m. Root structure and organization were analyzed by light microscopy (LM) and scanning electron microscopy (SEM). The 28 °C-chlorate treatment provoked severe damages to the exodermis, endodermis, cortex cells and root hairs. In the 6 °C-chlorate treatment, damage to root cells was also observed, but at a lower intensity. Nitrate reductase (NR) activity was analyzed and revealed the involvement of this enzyme in the chlorate action, even at a low temperature.
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1. Introduction

Chlorates are highly oxidizing substances that present toxic effects in several animal [1–3] plant [4] and microorganism species [5–9]. Among the majors sources of chlorate input to the environment, are its use as a defoliant and weed control in agriculture [10,11]. Another increasing source of chlorate is its use for chlorine dioxide production used in the bleaching of pulp and paper. Minor sources may be verified in the manufacture of matches and explosives and dye-ing printing [12]. As a consequence increasing levels of this compound have been detected in the environment especially in water ecosystems [9,13].

Chlorates are considered phytotoxic to all the green parts of plants. The absorption of chlorate may occur through both roots and leaves. When sprayed on leaves, the chlorate ions penetrate the cuticle. When absorbed by plant roots, after soil applications, the ions are translocated through the xylem to the leaves [14]. Direct toxicity is thought to be the effect of the high oxidizing capacity of the ion. Indirect toxic effects may result from the decomposition of products such as chlorite, the increase of the catalase activity, and the increased rate of respiration [15]. The stress also induces the plant to produce ethylene, which is known to cause leaf abscission [16,17]. Sodium chlorate treatment on the roots of weeds results in the depletion of their starch reserves [10].

Abbreviations: FW, fresh weight; MDA, malondialdehyde; NR, nitrate reductase; SEM, scanning electron microscopy; TBARS, thiobarbituric acid reactive species; TCA, trichloroacetic acid

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After its absorption, chlorate is reduced to chlorite by the enzyme nitrate reductase (NR) [4,18–20]. The reduction product of chlorate, chlorite, directly oxidizes and causes irreparable damage [20]. Plant cells do not discriminate against chlorate and nitrate, which are taken up at the same rate, acting as competitive inhibitors of each other at the transport site [21] and at the reduction site [4,20].

Although the toxicity of chlorates on plants is evident, the levels of damage caused by the stressor on the ultrastructure, biochemistry and physiology of plants are still poorly characterized. In the present study, the effects of chlorate stress on rice plants were studied. Ultrastructural, physiological and biochemical methods were used to analyze roots, leaves and tissues of plants exposed to $KClO_3$ and grown at two different temperatures.

2. Methods

2.1. Plant material and treatments

Rice seeds (*Oryza sativa* L., cv Pesagro 104) were obtained from Empresa de Pesquisa Agropecuária do Rio de Janeiro (Campos dos Goytacazes, RJ, Brazil). Seeds were germinated and grown in substrate imbibed with hydroponic culture solution, pH 5.6 [22]. Seedlings were grown at 27 °C under a 16 h-light per 8 h-dark regime for 14 days. Plants were transferred to hydroponic culture vessels containing the same nutrient solution with or without the stressing agent $KClO_3$ (16 mM). Triplicates of each treatment (control and $KClO_3$) were grown at 6 °C or 28 °C in plant growth chambers (Heraeus-votsch, Germany).

2.2. Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence measurements were performed on intact leaves with a light-modulated fluorimeter (Mini-PAM, Walz, Germany) on three plants per pot per treatment. Sampled leaves were pre-darkened for 20 min and then minimal fluorescence (F_0) was obtained with the measuring beam set to a frequency of 600 Hz, while maximal fluorescence yield (F_m) was determined after exposure to a 0.8 s saturating flash of light. Photochemical efficiency of PSII (F_v/F_m) was estimated as the ratio of variable ($F_V = F_m - F_0$) to maximal fluorescence yield. F_v/F_m measurements were performed at 0, 2, 4, 8, 12, 36, and 48 h after adding $KClO_3$.

2.3. Determination of lipid peroxidation

Lipid peroxidation was determined by the malondialdehyde (MDA) content, according to [23]. Tissue samples (250 mg) were homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at $10,000 \times g$ for 5 min. The supernatant was collected and 1 ml was mixed with 4 ml of 20% TCA and 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min and quickly cooled on ice. Then the sample was centrifuged at $10,000 \times g$ for 10 min. The absorbance of the supernatant was measured at 535 nm and corrected by subtracting the non-specific absorbance at 600 nm. Due to the limited specificity of the method, the concentration of TBA reactive species (TBARS) was calculated by using the extinction coefficient of $155 mM^{-1} cm^{-1}$, and results were expressed as nmol TBARS per gram FW.

2.4. Light and electron microscopy

For microscopy analysis, the leaf and root samples of control and $KClO_3$-treated plants were fixed in an aqueous solution containing 2.5% glutaraldehyde, 4% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2), for 2 h. Samples were post-fixed with 1.0% OsO_4 in the same buffer, during 1 h, the samples were dehydrated in acetone and embedded in Epoxi resin (Plybed). For light microscopy (LM), semi-thin sections (1.0 μm) were stained with 1% toluidine blue and examined in Axioplan ZEISS microscope. For transmission electron microscopy (TEM), ultra-thin sections (70 nm) were obtained using a Reichert Ultracuts ultramicrotome, stained with uranyl acetate followed by lead citrate and observed in TEM 900 ZEISS microscope. For scanning electron microscopy (SEM), the samples were fixed, post-fixed and dehydrated as above, dried by critical-point method (CPD 080, Balzers), covered with 20 nm of gold (SCD 050, Bal-tec), and observed in DSEM 962, ZEISS microscope.

2.5. In vivo nitrate reductase activity

The in vivo NR activity was measured according to [24]. Leaf samples (200 mg) were cut and introduced in 10 ml of the incubation mixture comprising of 16.5 mM KH_2PO_4 (pH 7.5), 0.7% 1-propanol, 0.1% Triton X-100, and 7.5 mM KNO_3. Samples were submitted twice to vacuum (30 mmHg), 30 s each time, and incubated for 30 min at 30 °C in the dark. One millilitre was collected and mixed with 1 ml of 1% sulfonamide (in HCl 3 N) and 1 ml of ultrapure water. The colorimetric determination of the reaction was achieved by adding 1 ml of aqueous 0.02% *N*-1-naphthyl-ethylene-diamine-dihydrochloride. Activities are expressed as nmol NO_2^- released per gram (FW) per minute.

3. Results

3.1. Dose effect of chlorate on rice plants

The dose-dependent effect of chlorate treatment on rice plants has been previously determined by a time-course assay (0, 6, 12 and 24 h) with 0, 0.5, 7, 16 and 32 mM of $KClO_3$. Analysis of photochemical efficiency revealed a weak damage provoked by 0.5 mM $KClO_3$. The effects of
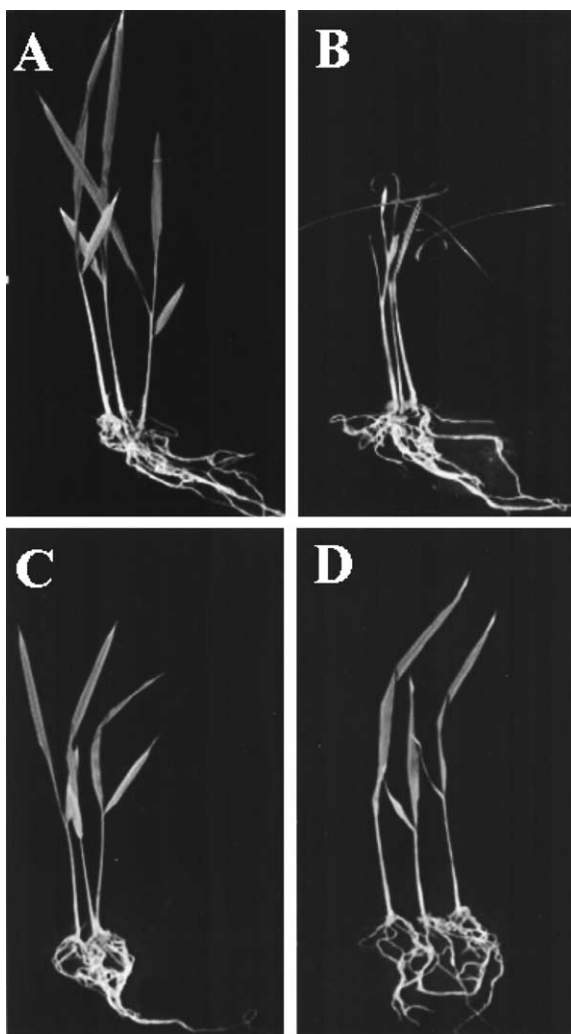
Fig. 1. Phenotypes of 15-day-old plants of rice, grown in the presence of potassium chlorate at different temperatures. (A) Plants grown at 28 °C, in the absence of the herbicide; (B) plants grown at 28 °C, in the presence of potassium chlorate 16 mM; (C) plants grown at 6 °C in the absence of the herbicide; (D) plants grown at 6 °C, in the presence of 16 mM potassium chlorate.

higher concentrations were severe, however the damage levels were similar for 7, 16 and 32 mM of $KClO_3$, after 24 h of stress (data not shown). Based on such information, the concentration of 16 mM chlorate was established for all the subsequent experiments.

3.2. Visual effects of chlorate stress

We compared the visual effects of chlorate (16 mM) on 15-day-old rice seedlings after 24 h treatment, at 6 and 28 °C, respectively (Fig. 1A–D). Chlorate treatment at 28 °C (Fig. 1B) provoked severe damage to plant leaves, which became distorted, chlorotic and dehydrated, when compared to control plants (Fig. 1A). In contrast, chlorate treatment at 6 °C presented no visual damage (Fig. 1D), compared to control plants exposed to the same temperature (Fig. 1C).
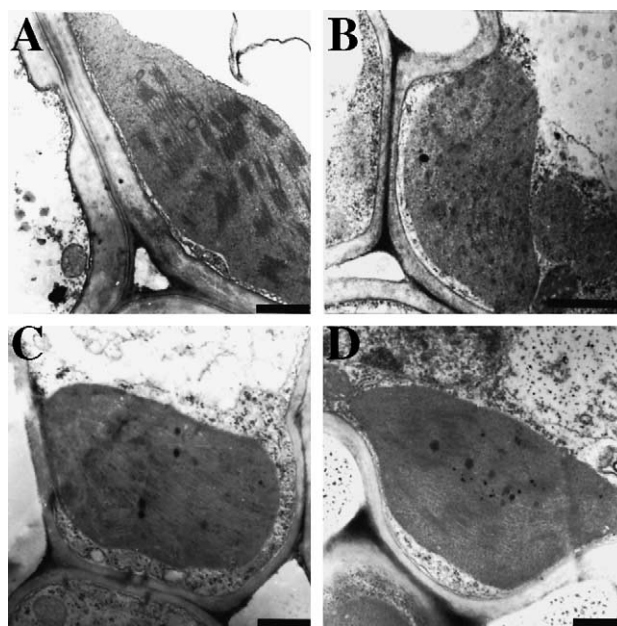

Fig. 2. Transmission electron microscopy of chloroplasts of mesophyll cells of control or herbicide-treated rice plant leaves, grown at two different temperatures. (A) Organelle from a plant grown at 28 °C in the absence of the herbicide; (B) chloroplast of plant grown at 28 °C in the presence of 16 mM potassium chlorate; (C) chloroplast of plants grown at 6 °C in the absence of the herbicide; (D) chloroplast of plants grown at 6 °C in the presence of 16 mM potassium chlorate.

3.3. Chloroplast ultrastructure

Leaf sections were studied by electron microscopy, focusing on the chloroplast structure. Chloroplasts from the control leaves exhibited a typical chloroplast structure, ellipsoidal shape, with well displayed thylakoid membranes organized in granal and stromal membranes (Fig. 2A). In the corresponding tissues of the plants submitted to chlorate treatment, at 28 °C, the chloroplasts suffered severe damage (Fig. 2B). Two types of changes could be distinguished: (1) the chloroplasts became more rounded instead of an ellipsoidal shape; (2) there was disruption of the membra-
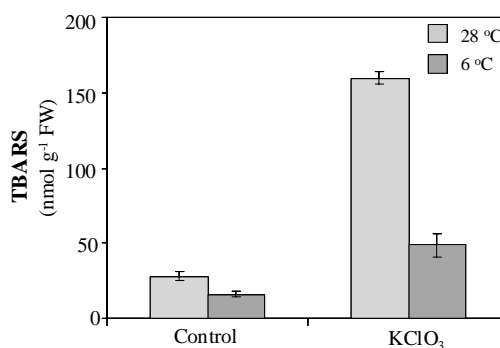

Fig. 3. Effect of chlorate treatment on lipid peroxidation of leaf extracts from rice plants exposed to different temperatures. Lipid peroxidation rates were expressed as nmol TBARS per gram FW. Vertical bars represent the mean ± S.E. of five independent measurements.

nar structure and the integrity of the outer membrane and the internal network of thylakoid membranes was almost completely lost.

The chloroplasts of plants treated with chlorate, at 6 °C (Fig. 2D), were structurally similar to chloroplasts from untreated plants exposed to the same temperature (Fig. 2C). In both conditions, they were more rounded than that of the chloroplasts observed in the control plants (28 °C) (Fig. 2A), and presented a considerably reduced system of stacks and stroma thylakoids.

3.4. Lipid peroxidation

Membrane lipid peroxidation in the leaves was assessed by determining the accumulation of TBARS. The effect of chlorate treatment, at 28 and 6 °C, on the TBARS formation in the leaves is shown in Fig. 3. Under chlorate treatments, at 28 °C, an elevated level of lipid peroxidation was observed in leaves as compared to control-grown seedlings (28 °C). A lower lipid peroxidation level was induced in plants exposed to chlorate, at 6 °C.

3.5. Photochemical efficiency

The physiological effects of chlorate treatments were evaluated by estimating the damage caused in the photochemical efficiency of PSII (F_v/F_m). Fig. 4A shows that chlorate stress, at 28 °C, severely affected the photochemical efficiency, with minimum levels reached after 23 h of treatment. At 6 °C, however, the chlorate-treated plants suffered a discrete reduction in F_v/F_m in comparison with the untreated plants exposed to the same temperature(Fig. 4B).

3.6. Anatomical and micromorphological effects

Anatomical effects of chlorate treatments on roots were analyzed by LM of cross-sections (Fig. 5A–D). The chlorate treatment, at 28 °C, provoked severe damage to root structure and organization (Fig. 5B), in comparison to the control plants at the same temperature (Fig. 5A). The two layers making up the exodermis are highly disorganized, similar to the innermost part of the root consisting of the endodermis and vascular cylinder. The cortex, between these two regions contains large, irregularly shaped air spaces. At 6 °C, however, chlorate treatments resulted in more discrete effects, with irregular shape in cells of cortex and endodermis (Fig. 5D), in comparison with the control plants at the same temperature (Fig. 5C).
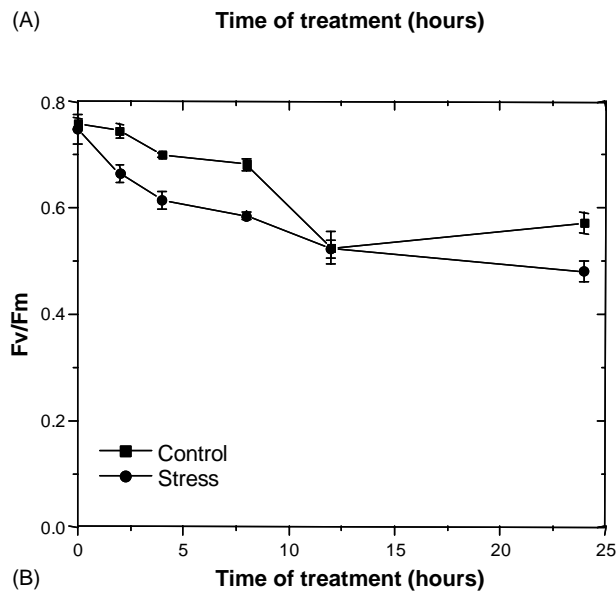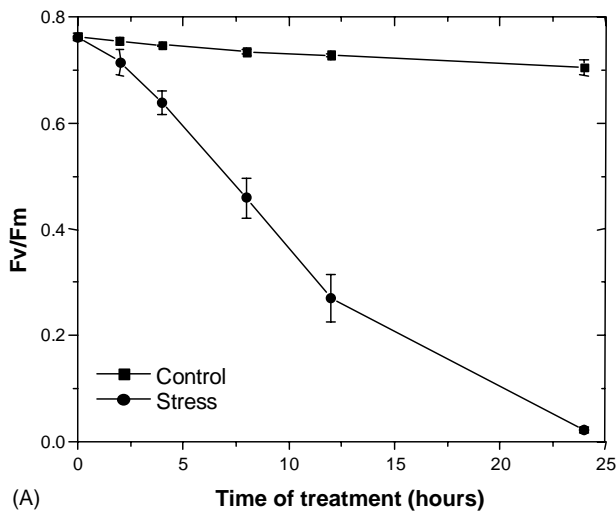

Fig. 4. The effect chlorate treatment on photochemical efficiency in rice plants exposed to 28 and 6 °C. Time-courses of the changes in the maximal photochemical efficiency PSII (F_v/F_m) of control and KClO$_3$-treated plants, at 28 °C (A) and at 6 °C (B). Each point is the mean value of two independent experiments in which three measurements were made for each treatment.
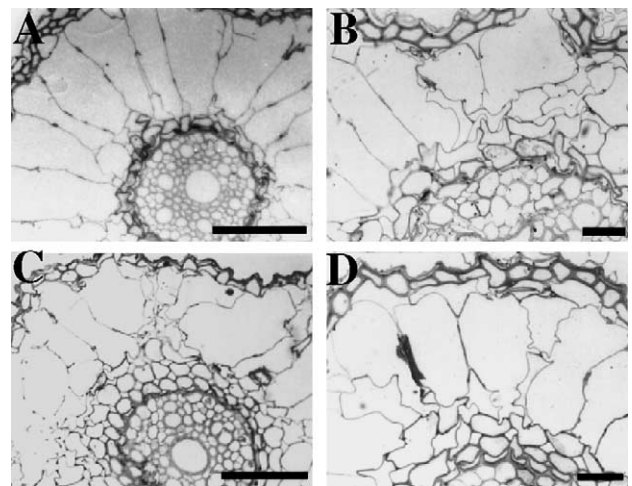

Fig. 5. Light microscope photography showing transverse sections of rice roots from normal and chlorate-treated plants, grown at two different temperatures. (A) Plants grown at 28 °C in the absence of the herbicide; (B) plants grown at 28 °C in the presence of 16 mM potassium chlorate; (C) plants grown at 6 °C in the absence of the herbicide; (D) plants grown at 6 °C in the presence of 16 mM potassium chlorate. Bars represent 50 μm in panels (A) and (C) and 10 μm in panels (B) and (D).
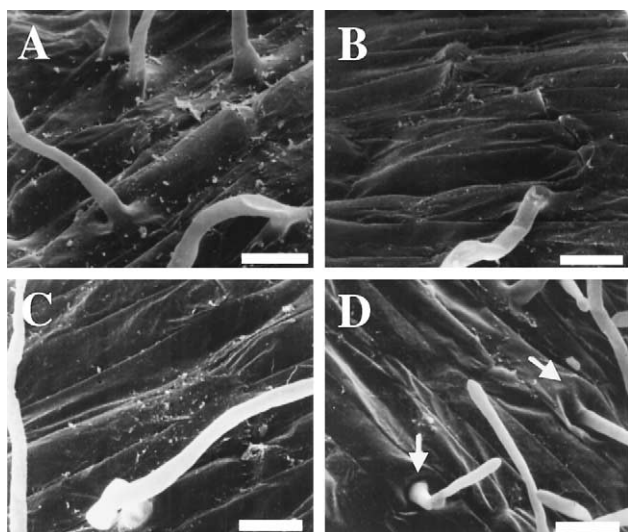
Fig. 6. Scanning electron microscopy micrographs showing the effect of 16 mM KClO₃ on surface of roots from rice plants grown at two different temperatures. (A) Plants grown at 28 °C, in the absence of the herbicide; (B) plants grown at 28 °C in the presence of 16 mM potassium chlorate; (C) plants grown at 6 °C in the absence of the herbicide; (D) plants grown at 6 °C in the presence of 16 mM potassium chlorate. Arrows indicate invaginations on the basis of root hairs. Bars represent 20 μm.

SEM examination of chlorate-treated roots, at 28 °C (Fig. 6B), showed completely withered cells and root hairs, in comparison with roots from untreated plants, at the same temperature (Fig. 6A). The chlorate effect at 6 °C, however, resulted in slightly withered cells. The basis of root hairs appeared to be folded or invaginated (Fig. 6C). Roots from control plants, grown at 6 °C, showed slightly withered cells but with apparently normal root hairs.

3.7. Nitrate reductase (NR) activity

NR activity was analyzed at 28 and 6 °C. Fig. 7 shows that approximately 40% of the enzyme activity remains in plants exposed to 6 °C (0.17 μmoles NO_2^- h^{-1} g^{-1} MF), in comparison with the activity observed in control plants (28 °C) (0.45 μmol NO_2^- h^{-1} g^{-1} MF).
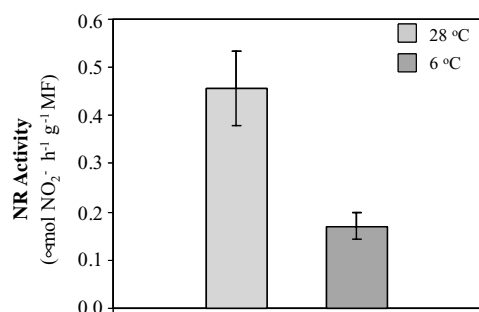

Fig. 7. Effect of low temperatures on nitrate reductase activity. NR activities were determined in leaves from rice plants, exposed to 28 or 6 °C, for 24 h. Vertical bars represent the mean ± S.E. of five independent measurements.

4. Discussion

In the present work, the effect of ClO_4 on rice plants exposed to different temperatures has been investigated. The morphological and physiological characteristics were emphasized. Morphological analyses of chlorate-treated plants revealed that, at 28 °C, the herbicide caused severe damage to leaves, which became distorted, chlorotic and dehydrated. Such changes have been described earlier by Mackown et al. [25] in wheat plants. At 6 °C, however, it was not possible to verify any important damage to the exposed plants by the stressor, using visual analyses. Such results were expected because at low temperatures, the activity of the NR, responsible for the reduction of the anion to chlorite and hypochlorite, was severely reduced.

The effect of the chlorate on the chloroplast ultrastructure at 28 °C was severe, including changes in organelle shape and disruption of membranar structure. At low temperature, no structural damage was observed. These results were corroborated by analyses of the photochemical efficiency of photosynthesis, which was severely affected by the stress at 28 °C. The treatment at low temperatures resulted in a discrete reduction of the photosynthesis efficiency, demonstrating that the anion is still absorbed and processed at this temperature.

Several stresses have been described as inducers of reactive oxygen species (ROS) in plants [26–28]. The ROS are chemically aggressive species and attack the polyunsaturated fatty acid components of membrane lipids resulting in lipid peroxidation [29]. This process promotes the disruption of physical and functional properties of membranes [30]. The chlorate treatment, at 28 °C, resulted in strong lipid peroxidation in rice leaves. At low temperatures, the chlorate effect was severely reduced, but presented higher levels in comparison with control plants, at the same temperature.

The analyses of root morphology from chlorate treated rice plants revealed that the anion promotes damage in tissue structure and organization. SEM analyses of root surface showed withered cells and root hairs. The damage observed in the superficial cells of roots may result from both the conversion of chlorate to chlorite and hypochlorite as the direct effect of the anion on membranes.

The involvement of NR in the reduction of chlorate to chlorite and hypochlorite has been reported in several studies [31,32]. Chlorate is a nitrate analog that can be taken up by nitrate transporters. The reduction of chlorate to chlorite by NR was proposed by [19]. This hypothesis was later investigated and confirmed with studies on *Escherichia coli* [33], *Chlorella vulgaris* [20] and tomato plants [4]. Nowadays, several plant mutants, defective for nitrate uptake, have been isolated by means of their resistance to chlorate treatments [32,34,35]. In almost all aspects studied in this work, the effects of chlorate treatments at 28 °C were stronger than those observed at low temperatures (6 °C). We hypothesized that the differences might result from the inhibition of the NR activity at low temperatures, which is responsible for the

conversion of chlorate to the active toxic forms: chlorite and hypochlorite. The evaluation of NR activity in leaves from rice plants exposed to 28 and 6 °C demonstrated that the low temperatures reduce the NR activity to 40%, confirming our previous expectations.

Acknowledgements

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