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Full Length Research Paper

Effects of NaCl treatment on the antioxidant enzymes of oilseed rape (*Brassica napus* L.) seedlings

Dai Qi-lin[1], Chen Chen[3], Feng Bin[2], Liu Ting-ting[2], Tian Xia[2], Gong Yuan-ya[2], Sun Ying-kun[2], Wang Jin[1, 2] and Du Shi-zhang[1]*

[1]Plant Genomics Research Center, Mianyang Normal College, Mianyang 621000, Sichuan, China.
[2]Key Laboratory for Nuclear Waste Treatment and Environmental Safety (SWUST), Commission of Science, Technology and Industry for National Defence, Southwest University of Science and Technology, Mianyang 621000, China.
[3]School of Pharmacy, Fudan University, Shanghai 200030, China.

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The effects of NaCl treatment on the activity of antioxidant enzymes in leaves of oilseed rape seedlings (*Brassica napus* L.) were studied. The results showed that the relative water content from leaves of oilseed rape seedlings was gradually decreased and the electronic conductivity was increased during 0 - 24 h under 200 mmol.l^{-1} NaCl treatments. The activity of peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) was gradually increased during 0 - 24 h under 200 mmol.l^{-1} NaCl stress. After 24 h, the activities of these antioxidases were maximum and subsequently decreased. Quantitative real-time PCR analysis revealed that they were salt-inducible genes and their transcript levels were gradually increased during 0 - 24 h and most abundant after 24 h treatment with 200 mmol.l^{-1} sodium chloride. Therefore, these results from above indicated that the expressions of POD, SOD and CAT genes were induced by NaCl; the activities of POD, SOD and CAT were increased, which enhanced the tolerance of oilseed oilseed rape plants against NaCl stress.

Key words: Salt stress, *Brassica napus* L., anti-oxidant enzymes, quantitative real-time PCR.

INTRODUCTION

Salinity is a major abiotic stress reducing the yield of a wide variety of crops all over the world and NaCl is the predominant salt in most saline environments (Bohnert and Jenson, 1996). In saline environment, membrane disorganization, reactive oxygen species (ROS) increase, metabolic toxicity, inhibition of photosynthesis and regulation of saline metabolism attenuated nutrient acquisition are factors that initiate more catastrophic events (Hasegawa et al., 2000). Salinity mainly causes both hyper-osmotic stress and hyper-ionic toxic effects and the consequence can be plant demise (Hasegawa et al., 2000). Osmotic stress mainly brings about a water deficit which results in the relatively high solute concentrations in osmotic stress (Zhu, 2002). The increasing evidences also suggest that high salinity induces oxidative stress (Gosset et al., 1996; Góemez et al., 1999; Savouré et al., 1999) which is a key underlying component of most abiotic stresses (Mittler, 2002; Apel and Hirt, 2004). Metabolic change is a key part of the response to oxidative stress in microbes and higher plants (Charles et al., 2007). Reactive oxygen species (ROS; e.g. O_2^- and H_2O_2) are generated as by-products of plant cellular metabolism (Kumaresan et al., 2008) and are also important as signaling molecules (Mittler, 2002). The production of ROS in cells increases during abiotic and biotic stresses, as does the level of ROS-induced damage. Elevated production of ROS can seriously disrupt cellular homeostasis and normal metabolisms through oxidative damage to lipids, protein and nucleic acid (Charles et al., 2007).

To avoid these cell death scenarios, plants invoke a molecular response that allows them to cope with and adapt to the oxidative stress situation (Charles et al., 2007). Plant cells can synthesize organic compounds, some soluble small-molecular-weight osmolytes which prevent from water efflux and maintain cellular expansion to keep osmotic balance and eliminate reactive oxidative specials (ROS) to increase plant cells salt-tolerance

*Corresponding author. E -mail: wjdsz@vip.sina.com.cn.

under salt conditions by metabolic change. Frequently, the organic compounds include betaine (glycine betaine, praline betaine, β-alanine betaine, vitriol and dimethyl sulfonium propionate, DSMP), sugars (mainly sucrose and fructose), sugar alcohol (glycerol, ononitol, ectoine and methylated inositols), complex sugars (trehalose, rafinose, fructans), mannitol and amino acid (proline) (Paul et al., 2000; Bohnert and Shen, 1999; Hare and Cress, 1997; Jain and Selvaraj, 1997; Nelson et al., 1998). Therefore, antioxidant resistance mechanisms may provide this strategy to entrance salt tolerance (José et al., 2001). Moreover, plants have evolved efficient antioxidant systems that can protect them from the damaging effects of oxidative stress (Asada, 1999). These mechanisms employ ROS scavenging enzymes which detoxify plant by scavenging oxygenic radical. Peroxidase is one of such small molecular antioxidant enzymes, which plays a key role in defense of plant against biotic and abiotic stress (Mark et al., 1987). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are the most important protective enzymes to remove reactive oxygen species. SOD catalyzes the dismutation of superoxide radical to generate O_2 and H_2O_2 and POD and CAT catalyze the conversion of the produced H_2O_2 to H_2O and O_2 (Asada, 1999).

Brassica napus L. belongs to *Brassica* in Cruciferae. As one kind of the most important oilseed crops all over the world, *B. napus* L. are very sensitive to salt stress throughout the growth and development cycle. Therefore, we selected *B. napus* L. as research materials for 0 - 48 h under 200 mmol.l^{-1} NaCl stress. The activity of antioxidant enzymes such as peroxidase, superoxide dismutase and catalase was detected after 0, 6, 12, 24 and 48 h. The expression pattern of these three antioxidant enzymes' genes in leaves of *B. napus* seedlings under 200 mmol.l^{-1} NaCl stress was also investigated by quantitative real-time PCR.

MATERIALS AND METHODS

Plant material and growth conditions

B. napus L. seeds (84100-18) were provided by Genetic Lab in Sichuan University. The seeds were sterilized in 5% sodium hypochlorite solution for 10 min followed by 3 rinses with sterile distilled water and then sown randomly in thoroughly washed sand in plastic containers (40 × 30 × 6 cm) with drainage holes in the bottom. The seedlings were irrigated on alternative days with full-strength Hoagland nutrient solution. After 15 d, the seedlings were transplanted into bigger plastic pots (22.5 cm in diameter and 22.5 cm in depth) and continued irrigation with the same fresh nutrient solution. Twenty-eight days later, NaCl were added into the nutrient solutions until the final concentrations of 200 mmol.l^{-1}.

Measurement of relative water content (RWC) in leaves of *B. napus*

The relative water content (RWC) was measured according to Zhang (1990). The RWC was calculated by the formula: RWC (%) = [(fresh weight - dry weight)/ (turgid weight - dry weight)] × 100 %. Turgid weight of leaf discs having floated on distilled water for 12 h at 4℃ was determined gravimetrically and then dry weight were determined gravimetrically after oven-drying at 100 - 105℃ to a constant mass.

Survey of relative electronic conductivity in leaves of *B. napus*

Relative electrical conductivity from leaves of *B. napus* was surveyed by electrical conductivity meter at 25℃ according to Li et. al., (1983). Two gram leaves taken from the oilseed rape under 200 mmol.l^{-1} NaCl treatments were washed twice with distilled water and then dried with clean filter paper. The leaves were cut into about 1 cm and put into 25 ml tube with plug and the volume was added to 25 ml with distilled water. The air in tube was removed by vacuum pump for 2 h. The electrical conductivity (R_1) in leaves of *B. napus* was measured by the electrical conductivity meter at 25℃. The conductivity (R_2) was measured after boiled for 30 min at 100℃ to kill the plant tissue and then cooled for 10 min at 25℃. The relative electrical conductivity (R_0) was calculated by the formula: R_0 (%) = (R_1/ R_2) × 100%.

Preparation of crude enzyme solution from leaves of *B. napus*

Subjected to 200 mmol.l^{-1} NaCl treatment for 0, 6, 12 , 24 and 48 h, about 0.5 g fresh leaves of *B. napus* were grinded with pre-chilled mortar and pestle at 0℃ and total proteins were extracted with 0.05 mol.l^{-1} pH 7.0 ice-cold phosphate buffer (1 mmol.l^{-1} EDTA, pH7.8, 0.1% Triton X-100, 1 mmol.l^{-1} PMSF, 2% PVP). Homogenate was centrifuged with 4000 rpm for 20 min at 4℃. The supernatant was the crude enzyme extract used for parallel tests of protein quantity and enzyme activity. Protein content of samples was measured using Coomassie brilliant blue G-250 dye-binding assay with bovine serum albumin as a standard (Bradford, 1976). The crude enzyme extract were stored at - 80℃.

Determination of antioxidant enzymes activity

SOD activity was determined according to Beauchamp and Fridovich (1971), where one unit of SOD was defined as the amount required to inhibit the photoreduction of nitroblue tetrazolium by 50% and the specific activity of SOD was expressed as units.mg^{-1}.protein.min^{-1}. Peroxidase (POD) activity was measured according to Omran (1980), where one unit of POD was defined as the protein amount that oxidized 1 mol of diaminobenzidine tetrahydrochloride as a substrate and was expressed as units.mg^{-1}.protein.min^{-1}. Catalase (CAT) activity was measured according to Beers and Sizer (1952) and calculated by the amount of H_2O_2 decomposed and expressed as mg H_2O_2.g^{-1}.protein.min^{-1}.

Expression of POD, SOD and CAT genes under NaCl stress

Total RNA were extracted from young leaves of *B napus* using trizol reagent (GIBCO-BRL). RNase-free DNase I (Takara, Japan) was used to digest the genomic DNA in the total RNA preparation. One hundred microgram of total RNA was then synthesized into 1st strand cDNA using a Reverse-Transcription Kit (Takara, Japan). The expression levels of the POD, SOD and CAT genes were determined using the iCycler iQ Real-time PCR Detection System (Bio-Rad) according to the manual of Two-step Quanti^{Tect} SYBR Green PCR Kit. Data processing was done by iCycler real-time detection system software (version 2.0). The primers used for quantitative real-time PCR amplifications were designed with Primer 12 Express 2.0 (Applied Biosystems) and their PCR products in length

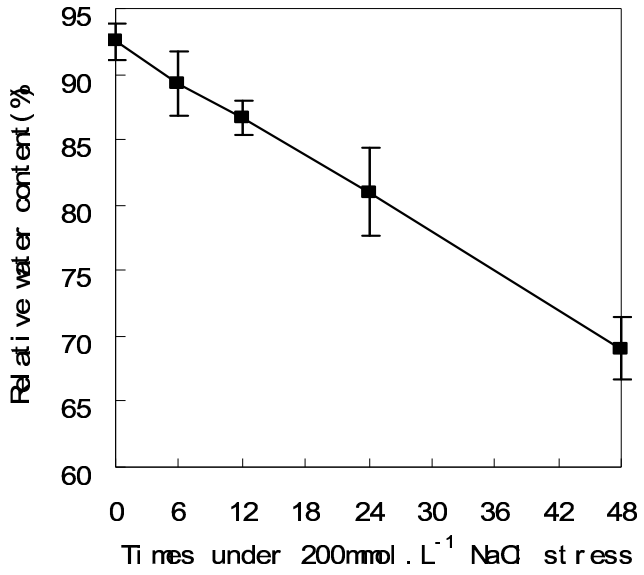

Figure 1. Changes of relative water content in leaves of *Brassica napus* seedlings at 200 mmol.l^{-1} for 0, 6, 12, 24 and 48 h. The data represents the means and standard errors of three replications.

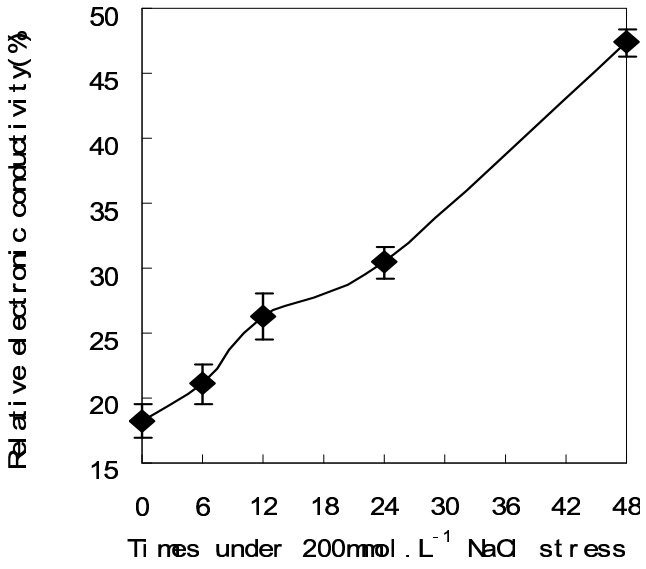

Figure 2. Changes of relative permeability of membrane in leaves of *Brassica napus* seedlings at 200 mmol.l^{-1} for 0, 6, 12, 24 and 48 h. The data represents the means and standard errors of three replications.

were 100~300 bp. The primers of these genes were as follows, *POD* F: 5'-GGCATGTATTATGTTTCGTGCGTCTC-3' and R: 5'-GCGTCACAACCATTGACAAAGCAG-3'; *SOD* F: 5'-GTTCAAC GGCGGAGGTCA-3' and R: 5'-AACATCAATACCCACCAGAGGA-3'; *CAT* F: 5'-GAAGGTTTCGGCGTCCACA-3' and R: 5'-TTGGTC ACATCAAGCGGGTC-3'; *β-actin* F: 5'-ACTGTGCCAATCTACG AGGG TT-3' and R: 5'-TCTTACAATTTCCCGCTCTGCT-3'. Expression levels of these genes were normalized using the house keeping gene (*β-actin*). All real-time quantitative PCR were performed in duplicate in three independent experiments.

RESULTS

Changes of relative water content (RWC) in leaves of oilseed rape seedlings

Under 200 mmol.l^{-1} NaCl stress, the relative water content in leaves of oilseed rape seedlings gradually decreased (Figure 1) and the electronic conductivity gradually increased (Figure 2) after 0, 6, 12, 24 and 48 h. It showed that rape seedling leaves lose more water from 0-48 h under 200 mmol.l^{-1} NaCl stress. Plant cell membrane plays an important role for the maintenance of its microenvironment and normal metabolism. Under normal circumstances, the cell membrane has the permeability to restrict the movement of a substance. When the plant were impacted by abiotic and biotic stress such as high temperature or low temperature, drought and salinity, damaged membrane and increasing permeability enabled electrolyte leakage in plant cell, which resulted in the electronic conductivity increased. Plant cell membrane permeability was related to the degree of stress and the strength of resistance in plant (Asada, 1999). As can be seen from Figure 2, the relative electrical conductivity gradually rose from 0-24 h. It showed that cell membrane of oilseed rape leaves was damaged and the solute was exuded from plant cell in *B. napus* leaves during 0-48 h.

Analysis of expression of three antioxidant enzymes' genes under NaCl stress

Quantitative real-time PCR analysis was carried out to establish the expression profile of POD, SOD and CAT of *B. napus* plants under salt stress. The results showed that the genes of these three antioxidant enzymes expressed at different level during various times (0, 6, 12, 24 and 48 h) under the 200 mmol.l^{-1} NaCl stress. There were little expression of three genes with no NaCl treatment, while their expression increased after 6 and 12 h; their transcript levels were most abundant after 24 h under 200 mmol.l^{-1} NaCl stress and then began to decrease (Figure 3). This result indicated that the expression of these three genes were all salt-inducible.

Changes of three antioxidant enzymes' activity under 200 mmol.l^{-1} NaCl stress

Subjected to NaCl stress, the soluble extracts in plant leaves were completed at 0℃. The effect of NaCl stress on total POD, SOD and CAT activity were examined in leaves of *B. napus* (Figures 4, 5 and 6). The result showed that there were a gradual increase in total POD, SOD and CAT activity from 0 to 24 h, with their lowest

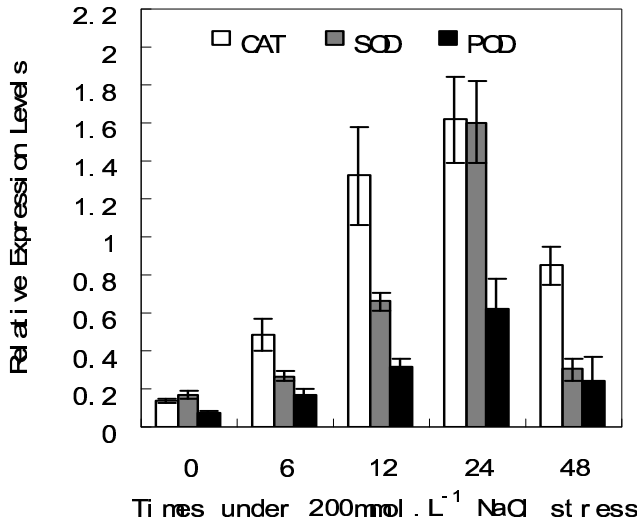

Figure 3. The expression profile of CAT, POD and SOD gene at 200 mmol.l^{-1} for 0, 6, 12, 24 and 48 h determined by quantitative RT-PCR (actin gene as internal standard). Fold inductions are the ratios of the copies of *CAT*, *POD* and *SOD* genes and actin in *Brassica napus.*

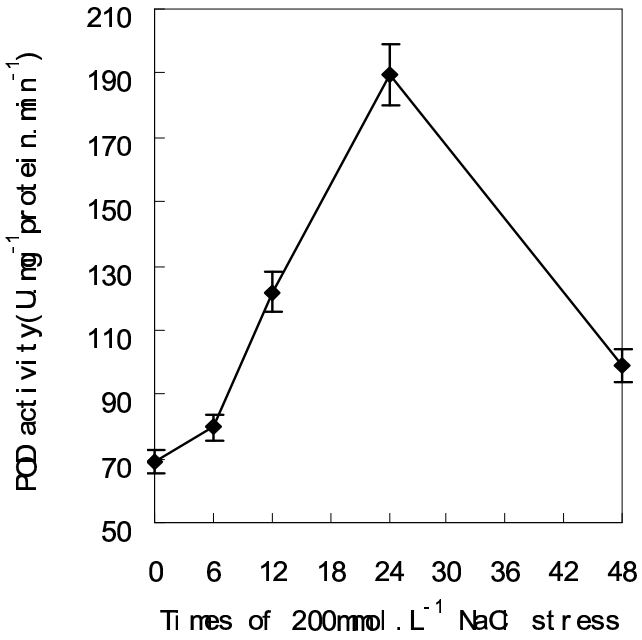

Figure 4. Changes of POD activity in *Brassica napus* at 200 mmol.l^{-1} for 0, 6, 12, 24 and 48 h. The data represents the means and standard errors of three replications.

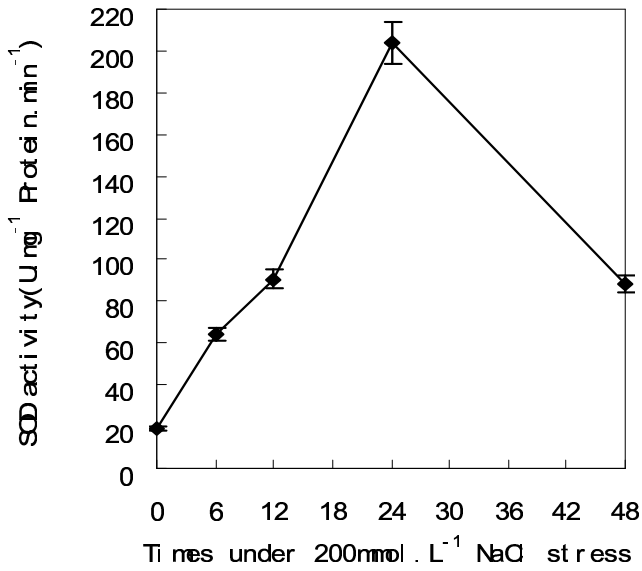

Figure 5. Changes of SOD activity in *Brassica napus* at 200 mmol.l^{-1} for 0, 6, 12, 24 and 48 h. The data represents the means and standard errors of three replications.

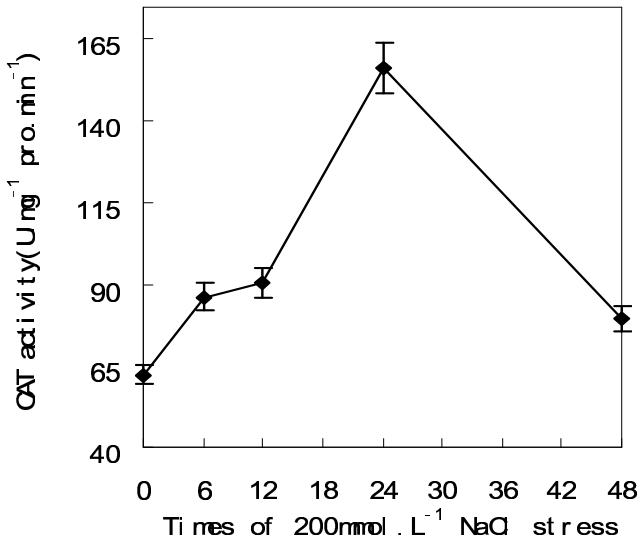

Figure 6. Changes of CAT activity in *Brassica napus* at 200 mmol.l^{-1} for 0, 6, 12, 24 and 48 h at 0, 100, 150, 200, 250 and 300 mmol.l^{-1} for 24 h. The data represents the means and standard errors of three replications.

activity at 0h, a little increasing at 6h, distinct increasing at 12 h, peaking at 24 h and declining at 48 h.

DISSCUSION

The most important forms of reactive oxygen species in plants mainly include O^{2-}, H_2O_2, $^{\cdot}OH$ and HO^{2-} (Kumaresan et al., 2008). Plant subjected to drought, low temperature and salt stress underwent impairment of electron transport systems of membranes that caused increase of ROS (ROS, including O_2^{-}, H_2O_2, $^{\cdot}OH$ and HO^{2-}) production (Smirnoff 1993; Navari-Izzo and Rascio, 1999). The assimilation of CO_2 in chloroplast was limited, which caused energy consumption reducing, the ratio of photosynthetic electron transported to O_2 correspondingly

increasing and the content of reactive oxygen species *in vivo* also largely increasing (Asada, 1999). In addition, stress can also affect the transport of the respiratory electron in plant mitochondrial respiratory chain which leak two single electrons to reduce molecular oxygen into O^{2-} and H_2O_2 (Yu et al., 1998). Once excessive reactive oxygen species in plant were not removed in time, plants would be subjected to seriously oxidative damage. Therefore, enzymatic and non-enzymatic antioxidant defense system can protect plant cells from injury. Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are the most important protective enzymes to remove reactive oxygen species. SOD may catalyze the dismutation of superoxide radical to generate O_2 and H_2O_2, the produced H_2O_2 removed by POD and CAT (Asada, 1999).

In this study, the results showed that the activity of three antioxidant enzymes in *B. napus* seedlings under 200 mmol.l^{-1} NaCl stress was associated with the ability of anti-oxidative stress. From Figures 4, 5 and 6, the activity of SOD, POD and CAT gradually increased from 0-24 h under 200 mmol.l^{-1} NaCl stress. The maximal activity of POD and SOD in *B. napus* leaves came to 189.22 and 204.21 U.mg^{-1}.protein.min^{-1} and that of CAT was 156.44 mg H_2O_2.g^{-1} protein.min^{-1} separately, which had separately a higher increase by 1.7, 10 and 1.5 times compared to the untreated control. The activity of antioxidant enzymes in leaves of oilseed rape could be induced by a certain degree of NaCl stress. The increased enzyme activity coincided with enhanced H_2O_2 in *Cassia angustifolia* (Agarwal1 and Pandey, 2004) and *Afzal* plants (Khosravinejad et al., 2008) in response to increased salt concentration. Among the antioxidant enzymes, CAT activity was increased the most drastically (Khosravinejad et al., 2008). POD activity was found to increase along with activities of other antioxidant enzymes like CAT, SOD and GSH reductase in response to various environmental stress factors, suggesting that the components of ROS-scavenging systems were co-regulated (Shigeoka et al., 2002). These enzymes have a possible synergy to commonly resist oxidative damage caused by salt stress. We presume irreversible damage in plants was caused by severe stress, but its regulative mechanism has yet to be confirmed by further studies.

Expression of three antioxidant enzymes genes in leaves of rape seedlings under salt stress was analyzed by quantitative real-time PCR (Figure 3). It showed that change of POD, SOD and CAT genes expression was the same as that of their enzymatic activity which was positively correlated with expression of their genes.

Despite the activity of SOD, POD and CAT having increased under NaCl stress, cell membrane was oxidative status at all times and membrane damage had not been ruled out and then the damaged degree of the plasma membrane gradually aggravated with the extension. The increasing electrolyte exuded from leave tissue cells and the electrical conductivity continually rose. The relative electrical conductivity rate reached 47.35 at 24 h under 200 mmol.l^{-1} NaCl stress, which was more than 1.6 times higher than that under no NaCl treatment (Figure 2). At the same time, ion concentration around plant rhizospheres under salt stress was higher than other areas and thereby it was difficult for plant roots to take up water. In addition to plant water loss by transpiration which lowered leaf surface temperature, the water content in plants decreased. The relative water content in leaves of oilseed rapes was 69.05% after 48 h under 200 mmol.l^{-1} NaCl stress, which was a lower decrease by 25.4% than that with no NaCl stress (Figure 1).

In a conclusion, the expression of POD, SOD and CAT genes in leaves of was induced by salt (such as NaCl) and their activities were positively correlated with expression of their genes.

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