WFL Publisher
Science and Technology
Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.10 (2): 371-374. 2012 *www.world-food.net*

Leaf water status, proline content, lipid peroxidation and accumulation of hydrogen peroxide in salinized Chinese kale (*Brassica alboglabra*)

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Received 16 January 2012, accepted 6 May 2012.

Abstract

In responding to stress, plant cells may secrete compatible compounds and at the time demonstrate an increase in others which would reflect the status of reactive oxygen species (ROS) scavenging capacity of plants under oxidative stress. In this study the ability of four cultivars of Chinese kale (*Brassica alboglabra*) to tolerate salinity stress was evaluated. Four diverse cultivars of Chinese kale (cv. 'Standard kailan', 'Hong Kong kailan', 'Kale Curly Leaf' and Hong Kong stem flower') were subjected for 14 days to varying levels of NaCl, i.e. 0, 25, 50 and 75 mM in Hoagland's nutrient solution in a static aerated hydroponic system. Salinity induced changes in all assayed parameters. Leaf relative water content (RWC) was reduced by 7.6, 13.3 and 15.5% relative to the control as NaCl concentration increased at 25, 50 and 75 mM, respectively. In addition, accumulation of proline in leaf tissue was induced significantly at high NaCl concentration. However, 'Standard kailan' contained the lowest concentration of proline among cultivars. Salinity stress noticeably raised the concentration of H_2O_2 in leaves with their respective values of 47.5, 56.0 and 56.2% for 25, 50 and 75 mM compared to control. Almost similar response was also recorded for accumulation of malondialdehyde (MDA). The level of MDA in leaves, which represents the rate of lipid peroxidation process, was considerably increased under saline condition. Lower concentrations of H_2O_2 were found in leaf tissues of 'Standard kailan' and 'Hong Kong kailan' compared to those of 'Curly leaf' and 'Hong Kong stem flower'. The lowest amount of MDA in leaf tissues of cv. Standard kailan in comparison with others, suggests that the cv. Standard kailan is better protected from oxidative damage under salinity stress and being more tolerant.

Key words: Salt stress, relative water content, oxidative stress, proline, hydrogen peroxide, lipid peroxidation, Chinese kale.

Introduction

Salinity is one of the most serious stress factors that limit crop production. It can disrupt the plants' metabolic functions and can be easily noticed on the entire plant subsequently leading to decrease in productivity or even plant death. Osmotic stress and ionic imbalance derived from salinity can affect major plant physiological and biochemical processes such as photosynthesis, protein synthesis and lipid metabolism [1]. Increasing osmotic stress in plants leads to stomatal closure, resulting in reduction of CO_2 availability and photosynthesis, thus increasing the possibility of reactive oxygen species formation [2]. The immediate response of Calvin cycle to such condition leads to decrease in carbon reduction and oxidized $NADP^+$.

Lack of $NADP^+$ which serves as an electron acceptor in photosynthesis is the underlying cause of electron donation from over-reduced ferredoxin to oxygen to form superoxide radicals by Mehler reaction, which initiates chain reaction that generates more noxious oxygen radicals, referred to as reactive oxygen species (ROS). ROS are highly active and can disrupt normal cellular functions. Lipid peroxidation, protein denaturation and DNA mutation are the results of the oxidative damage to membranes, proteins and nucleic acids [3, 4]. In mediating salt stress effect, vast array of regulators might be used by plants. Among them include synthesis and accumulation of low molecular weight water-soluble metabolites such as proline as observed in most plants growing in saline environments [5]. Formation of ROS in salt-stressed plants triggers antioxidative defense mechanisms, resulting in the accumulation of a wide range of enzymatic and non-enzymatic antioxidants which can quench the ROS [6]. Changes in hydrogen peroxide content and rate of lipid peroxidation (as shown by changes in MDA content) are good indicators of the status of ROS scavenging capacity of plants under oxidative stress [7]. The variations in some biochemical indicators, i.e. proline, H_2O_2 and MDA in relation to leaf water status of four Chinese kale (*Brassica alboglabra*) cultivars in response to salinity treatment are reported in this paper.

Material and Methods

The seeds of four Chinese kale (*Brassica alboglabra*) cultivars ('Standard kailan', 'Hong Kong kailan', 'Kale curly leaf'' and 'Hong Kong stem flower') obtained from commercial seeds supplier were used in the study. The seeds were sown in trays and kept under water spray until emergence. Young seedlings in the seed trays were put in a plastic film pool and irrigated with a nutrient solution containing (mg L^{-1}) 250 N, 67 P, 239 K, 160 Ca, 30 Mg, 80 S, 3 Fe, 0.62 Mn, 0.44 B, 0.02 Cu, 0.11 Zn and 0.048 Mo.

The seedlings were allowed to grow until 15th day after

germination, and then transplanted and installed on the static aerated hydroponic system using plastic trays as containers (twelve plants per container). The salt stress treatment began when the seedling were at 21 days after germination by adding 0, 25, 50 and 75 mM NaCl in the solution. The salt was added gradually to avoid osmotic shock. Two weeks after the treatment, new fully expanded leaves were harvested for further physiological and biochemical measurements.

Determination of relative water content: Five leaf discs fresh weights (FW) were determined and then floated on deionized water for 4 hours under low irradiance condition. Wet surface of turgid tissues were then quickly dried prior to measuring turgid weight. Samples were dried at 70°C for 72 hours in oven and DW was determined. The RWC was finally calculated using the following formula [8]:

$$RWC = (FW - DW / \text{turgid weight} - DW) \times 100$$

Determination of proline content: Content of proline was measured according to the procedure of Bates *et al.* [9]. Leaf samples (0.2 g) were homogenized with 3 mL sulphosalicylic acid (3% w/v) and centrifuged for 15 min. Then 2 mL of glacial acetic acid and ninhydrin solution were added to 2 mL of the supernatant. Tubes were incubated in boiling water for 60 min and placed on ice to stop the reaction. The supernatants were mixed with 4 mL of toluene. The test tubes were incubated to separate toluene and water phases. Upper phase's absorbance was read at 520 nm using spectrophotometer (Varian Cary 50 UV-Vis spectrophotometer, USA). The content of proline was calculated using a standard curve.

Determination of hydrogen peroxide: Content of hydrogen peroxide was measured according to the procedure of Velikova *et al.* [10]. Fresh leaf samples (0.5 g) were homogenized with 5 mL 0.1% (w:v) trichloroacetic acid (TCA) in ice bath and the homogenate was centrifuged at 12,000 g for 15 min. Then 0.5 mL of 10 mM phosphate buffer and 1 mL of 1 M KI were added to 0.5 mL of the supernatant. The absorbance of supernatant was read at 390 nm. The content of H_2O_2 was estimated using a standard curve.

Determination of lipid peroxidation: Lipid peroxidation was estimated by the level of malondialdehyde (MDA) production based on the thiobarbituric acid (TBA) method described by Heath and Packer [11]. Fresh leaf (250 mg) samples were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 15,000 g for 15 min. Of the supernatant 1.0 mL was added to 4 mL of 0.5% (w/v) TBA in 20% TCA (w/v), heated in a boiling water bath for half an hour and immediately cooled in an ice bath to stop reaction. The absorbance of the resulting supernatant was recorded at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. MDA concentrations were calculated by means of an extinction coefficient of 155 mM^{-1} cm^{-1} adapting the formula used by Du and Bramlage [12]:

$$MDA\ (\mu mol/g\ fresh\ wt.) = [(A_{532} - A_{600})/155] \times 10^3 \times dilution\ factor$$

Statistical analysis: The study was conducted using a split plot design with four replications. The data were analysed by using SAS 9.1 software (SAS Institute, Inc., Cary NC, USA). Mean comparison was conducted using LSD test at 5% level of probability.

Results and Discussion

Salinity treatment significantly reduced relative water content in all cultivars. The RWC was highest in the control plants but did not differ between plants treated with 50 mM and 75 mM NaCl (Fig. 1 A). Overall, leaf relative water content decreased by 7.6%, 12.3% and 15.5% relative to the control as salt concentration increased to 25, 50 and 75 mM, respectively. Salt stress induces both osmotic stress and ion toxicity and results in a clear decline in biochemical activity and cell turgor pressure [13]. Hence reduction in leaf water status (RWC and osmotic potential) can be explained using selected biochemical indicators in response to salt stress [14, 15].

It is apparent that salt stress results in marked change in proline accumulation. Proline content of all cultivars rose significantly with increase in salinity. The proline contents also differed among cultivars with cv. 'Standard kailan' containing the lowest concentration at all salinity levels (Fig. 1 B).

Biosynthesis and accumulation of compatible solutes such as proline is a distinctive phenomenon that possibly plays a role in facilitating the retention of water in the cytoplasm of plants and maintain leaf RWC under salinity stress [5]. It is understood, but not yet demonstrated that there might be a relationship between salt tolerance and the role of proline and other metabolites for osmotic adjustment in plants. However, some studies suggested that the amplification in proline concentration may not be associated with salinity tolerance [16, 17]. It has been previously reported that high proline concentration may also have contributed in additional regulatory or as an osmoprotective factor in salt

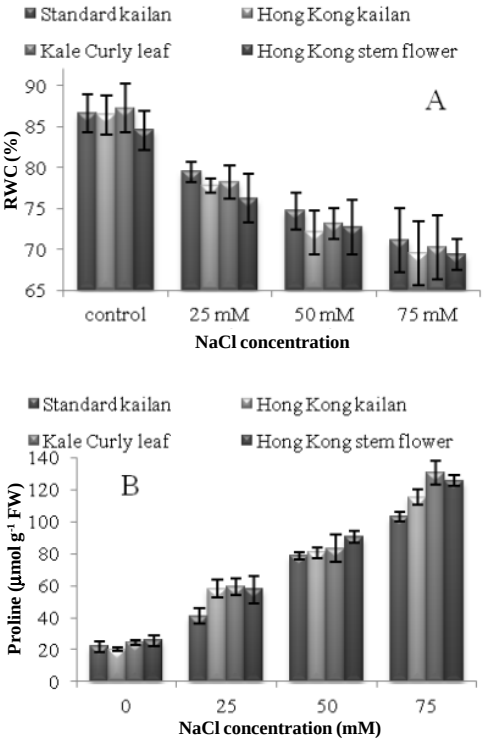

Figure 1. Effect of salinity levels on (A) RWC and (B) proline contents in Chinese kale cultivars. Vertical bars indicate ±S.E. of means (n = 4). Data is significant at p = 0.05 for treatments and cultivars.

stress, such as its role in cell osmotic adjustment in barley roots, which may control the activity of plasma membrane transporters [18]. Proline biosynthesis is a highly energy-demanding process and only small amount of that may be required to control plasma membrane transporters [18]. Excessive production of proline may not be defined by these processes, and it could be a reflection of poor performance and greater damage in response to salt stress. Nevertheless our results showed accumulation of proline was amplified with the increase in salt stress. Furthermore, the elevation of proline content was followed by a reduction in relative water content as shown by the negative correlation between the two parameters (r = -0.735) (Fig. 2 A).

Salt stress may cause molecular damage to plant cell either directly or indirectly through the formation of ROS. According to Foyer *et al.* [19], hydrogen peroxide, as a strong oxidant causes oxidative damage and disturbs metabolic functions at site where it accumulates. Also hydrogen peroxide and other active oxygen species like $OH^{\cdot}$, $^1O_2$ and $O_2^{\cdot-}$ were noted to be responsible for the lipid peroxidation [20, 21].

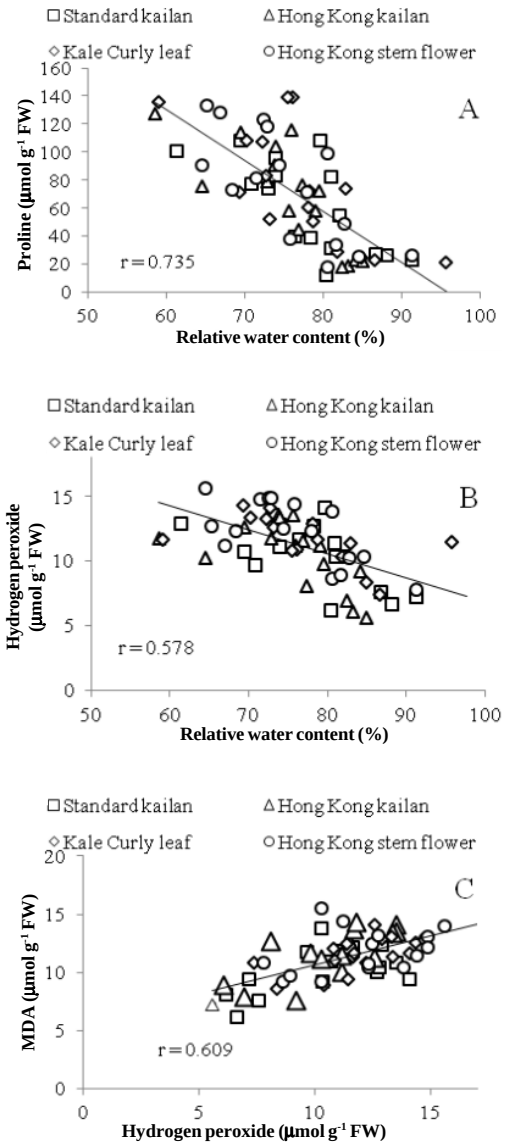

Figure 2. Relationship between (A) proline and RWC, (B) H_2O_2 and RWC, and between (C) H_2O_2 and MDA.

In this study, hydrogen peroxide content in the leaves of all cultivars increased significantly with increase in salinity. Among the cultivars, 'Standard kailan' and 'Hong Kong kailan' accumulated lower amount of H_2O_2 in their leaf tissues compared to the other cultivars. Increases in salinity induced the concentration of H_2O_2 with respective values of 47.5%. 56.0% and 56.2% for 25, 50 and 75 mM compared to the control (Fig. 3 A), clearly suggest that plant tissue could be destroyed more rapidly at high salinity levels not only due to osmotic stress as indicated by reduction in RWC, but also due to excessive accumulation of toxic materials such as H_2O_2. Results in Fig. 2 B depicted that the concentration of H_2O_2 was negatively correlated (r = -0.578) with leaf relative water content (RWC).

The level of MDA in leaves, which represents the rate of lipid peroxidation process, varied by the salinity treatment. The contents of MDA were noticeably induced by salt treatments; hence the average highest content of MDA was formed in the most stressed plants. The MDA contents also differed among cultivars. The lowest amount of MDA was accumulated in leaf tissues of cv. 'Standard kailan' in comparison with the other three cultivars (Fig. 3 B).

One of the issues that emerge from the findings of the present study is that stress induced a progressive increase in H_2O_2 content and MDA accumulation in the leaves indirectly due to oxidative stress. The observed significant difference in the hydrogen peroxide production levels in the leaves of all cultivars with cvs. 'Standard kailan' and 'Hong Kong kailan' besides, the lowest amount of MDA in leaf tissues of 'Standard kailan' in comparison with others implied that the 'Standard kailan' is better protected from oxidative damage under salt stress. On the other hand, it appears that higher oxidative stress in other cultivars probably

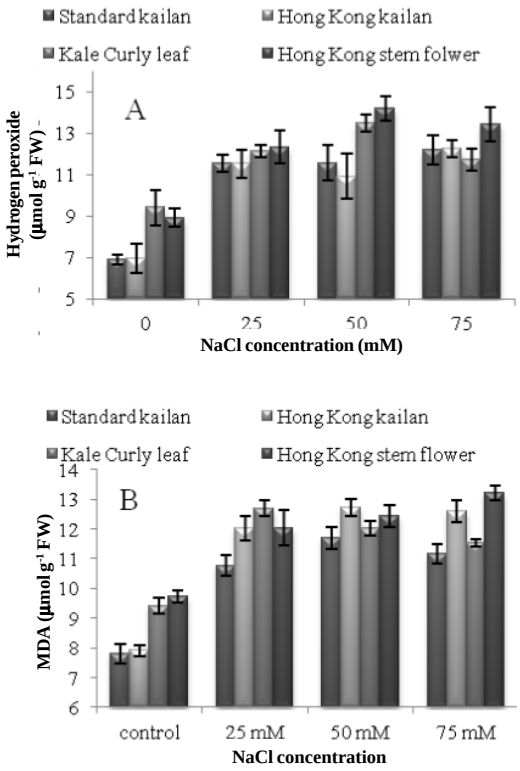

Figure 3. Effect of salinity levels on (A) H_2O_2 and (B) lipid peroxidation (MDA content) in Chinese kale cultivars. Vertical bars indicate ±S.E. of means (n = 4). Data is significant at p = 0.05 for treatments and cultivars.

results from the lack of or insufficient up-regulation in their antioxidative system.

Changes in lipid peroxidation as an index of membrane stability have been shown to be correlated with oxidative damage in the presence of various abiotic stresses [22-25]. In this study a positive correlation between contents of hydrogen peroxide and MDA with the *r* value 0.609 was observed (Fig. 2 C).

In summary, leaf water content of cv. 'Standard kailan' provided with lower proline concentration compared to other cultivars under salinity. Lower signs of oxidative stress shown in this cultivar suggest that cv. 'Standard kailan' is comparatively more tolerant to salt stress that may be linked to the controlled synthesis of proline, a high energy demanding process.

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