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ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT RESPONSES OF ALFALFA LEAVES AND ROOTS UNDER DIFFERENT SALINITY LEVELS

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The effect of increasing NaCl concentrations on biomass, hydrogen peroxide (H_2O_2), ascorbic acid (ASC), proline and total thiol, and the activity of some antioxidant enzymes in alfalfa (*Medicago sativa* L. cv. *Gara-Yonjeh*) were investigated. The dry weights of roots and shoots with increasing NaCl concentrations decreased progressively, and the strongest toxicity was detected at NaCl treatment of 200 mM. Superoxide dismutase (SOD) activity in the leaves increased gradually up to NaCl concentrations of 100, while the higher concentration of NaCl reduced SOD activity in both leaves and roots. The maximum levels of ascorbate peroxidase (APX) activity were increased at 150 mM and 100 mM NaCl in leaves and roots of *Gara-Yonjeh,* respectively. Peroxidase (POD) activity in roots of *Gara-Yonjeh* increased (82% at 200 mM) by salinity, while it decreased (43% at 200 mM) in leaves. In contrast, catalase (CAT) activitiy increased (84% at 200 mM) in leaves, and decreased (57% at 200 mM) in the roots of *Gara-Yonjeh.* Electrophoresis analysis suggested that different patterns in SOD, CAT and POD isoenzymes depend on NaCl concentrations, and the staining intensities of these isoforms are supported the results obtained from the spectrophotometric determinations. In POD and CAT, activity of isoform III was detected at all concentrations, by a "low–high–low" pattern, with the maximum activity at 50 mM of NaCl. Results imply that the function of antioxidant systems in higher NaCl concentration is responsible for the salt tolerance observed in *Gara-Yonjeh.*

Keywords: Medicago sativa L. – *Gara-Yonjeh* – antioxidant system – salt stress – isoenzyme pattern

INTRODUCTION

Salinity is considered to be a primary environmental factor limiting plant development and productivity, mainly of important crops. Salinization is rapidly increasing on a global scale and currently affects more than 10% of arable land, which results in a decline of the average yields of major crops greater than 50%. Therefore, understanding the mechanisms of plant tolerance high salinity is a crucial environmental research topic [2].

Generally, exposure to salt stress triggers many common reactions in plants that lead to ion imbalance and hyperosmotic stresses. The consequence of exposure to

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these stresses is the generation of reactive oxygen species (ROS). In the lack of effective protective mechanism, ROS can seriously damage plants by lipid peroxidation, protein degradation, breakage of DNA and programmed cell death [27]. To overcome with the increased ROS level, plants possess developed antioxidative systems which are composed of non-enzymatic mechanism such as ascorbate, glutathione, phenolic compounds and proline and enzymatic mechanism such as superoxide dimutases (SOD), peroxidases (POD), ascorbate peroxidases (APX) and catalases (CAT) [13].

SOD converts superoxide radicals ($O_2^{\cdot -}$) into hydrogen peroxide (H_2O_2), POD reduces H_2O_2 to water using various substrates as electron donors, APX uses ascorbate as an electron donor to reduce H_2O_2 to water, and CAT dismutates H_2O_2 into water and oxygen. Some evidence suggests that resistance to oxidative stress may, at least in part, be involved in salt stress tolerance [1].

Legumes are the main single source of vegetable protein in human diets and livestock feeds. These plants have ability of symbiotic nitrogen fixation; and improve soil organic fertility [11]. Alfalfa (*M. sativa* L.) is one of the most important forage legumes with more than 10 types of vitamins and a main component of many crop rotation systems worldwide. In addition, alfalfa's deep-root system can help prevent from soil and water loss in semi-dry lands [20]. As a glycophyte, alfalfa exhibits significantly reduced biomass under severe salt stress with varying responses among different cultivars. *M. sativa* L. cv. *Gara-Yonjeh* is one of important crop alfalfa cultivars that is cultivated in north-west of Iran.

The antioxidative system-based response of alfalfa plants has been studied to abiotic stresses including petroleum sludge [19], drought [21], heavy metals toxicity [35] and selenium toxicity and sulfur deficiency [10]. Furthermore, the responses of antioxidative systems towards salt stress condition have been studied in leaves of some alfalfa cultivars mainly *Zhongmu 1, Defi, Xinmu* and *Northstar* [28, 32].

Therefore the aims of present study were to determine the adaptation strategies of *Gara-Yonjeh* cultivar towards salt stress conditions for the first time, and to evaluate if there is any difference between roots and leaves regarding the mechanism of salt tolerance.

MATERIALS AND METHODS

Plant materials and NaCl treatment

One of the local cultivars of alfalfa (*M. sativa* L. cv. *Gara-Yonjeh*) was used for this study. Seeds were provided from Azarbaijan Agricultural Research Center and Natural Resources of Tabriz.

To obtain adult *Gara-Yonjeh* plants, seeds were surface-sterilized using sodium-hypochlorite 5% (v/v). Twelve seeds cultured in plastic 2 L pots containing perlite under controlled environmental conditions with a temperature regime of 25/18 °C day/night, 14/10 h light/dark period, a relative humidity of 70/80% and at a photon flux density of about 400 μmol/m^2/s. The plants were watered in field capacity limit

every 2 days with 50% Hoagland nutrient solution for 1 week, with full strength Hoagland nutrient solution for 2 weeks and then the nutrient solution was supplemented with NaCl. Plants were thinned to six plants per pot. The treatments comprised 5 levels of NaCl (0, 50, 100, 150 and 200 mM) in Hoagland's nutrient solutions were applied to three-week-old plants. The NaCl concentration was increased stepwise in aliquots of 50 mM every day until the appropriate concentration was attained. After salt treatment for 2 weeks, the plants were used for further physiological and biochemical analyses. For biochemical analyzes, leaf and root samples were harvested, quickly frozen and stored at liquid nitrogen.

Physiological analyses

Leaf area was measured using a planometer. Each plant was divided into shoot and root. Shoot and roots were washed with double-distilled water for 5 min and after blotting dry, fresh weight (FW) and root length were determined [29]. After drying at 70 °C for 1 day, dry weight (DW) was determined.

Biochemical analyses

Hydrogen peroxide levels were determined according to Velikova et al. [31]. The ascorbic acid content were determined by indophenol method as described by Okeri et al. [24]. Free proline content in the leaves and roots was determined following the method of Bates et al. [3]. Total thiol concentration or sulfhydryl groups (–SH) were measured by the methods originally described by Ellman [8] and modified by Hu [12].

Enzyme activity and isoform analyses

Leaves and roots were homogenized in phosphate buffer (0.01 M, pH 7.0), containing polyvinylpyrrolidone (0.2% w/v) for stabilizing the extract. The homogenate was centrifuged at 14,000 *g* at 4 °C for 20 min and the supernatant was used as the crude extract for the following assay and electrophoresis. Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Winterbourn et al. [33]. One unit of SOD was defined as the amount of enzyme required to induce a 50% inhibition of NBT (*p*-nitro blue tetrazolium chloride) reduction as measured at 560 nm, compared with control samples without enzyme aliquot. Catalase (CAT, EC 1.11.1.6) activity was assayed by monitoring the decrease in absorbance of H_2O_2 at 240 nm [23]. Unit activity was taken as the amount of enzyme that decomposes 1 μmol of H_2O_2 in one minute. Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test [9]. The enzyme unit was calculated as enzyme protein required for the formation of 1 μmol tetraguaiacol for 1 min. The activity of ascorbate

peroxidase (APX, EC 1.11.1.11) was measured using a procedure modified from that described in Boominathan et al. [5]. One unit of APX oxidizes ascorbic acid at the rate of 1 μmol/min at 25 °C. Protein content was determined according to the method of Bradford with BSA as a standard [6].

The isoenzymes of SOD, CAT and POD were separated on discontinuous polyacrylamide gels (PAGE) under the non-denaturing conditions. The stacking and separating gels contained 2.5% and 7.5% polyacrylamide, respectively. Proteins were electrophoretically separated at 4 °C and 20–25 mA in maximum voltage.

SOD activity was determined on the gel as described by Beauchamp et al. [4]. The gels were soaked in 100 ml of K-phosphate buffer (50 mM, pH 7.8) containing EDTA (1 mM), NBT (0.1 mM), riboflavin (0.05 mM) and EDTA (3% v/v). After 30 min incubation in the dark, the gel was rinsed in distilled water and exposed to a UV light for 15 min in order to visualize the enzyme bands.

Separation of POD isoenzymes and detection the bands were carried out on the gel by submerging the gel in a staining solution containing sodium citrate buffer (50 mM, pH 4.5), H_2O_2 (2 mM) and guaiacol (20 mM). Bands corresponding to POD activity emerged after 40–60 min [15].

For the detection of CAT isoenzymes activity, gels were incubated in 0.003% (v/v) H_2O_2 for 10 min and developed in a 1% (w/v) $FeCl_3$ and 1% $K_3[Fe(CN)_6]$ (w/v) solution for 10 min [34]. We originally performed four independent replicates of SOD, CAT and POD gels.

Statistical analysis

Data are presented as the mean ± SD for each treatment ($n = 4$). Significant differences among treatments were analyzed by one-way ANOVA followed by Tukey (HSD) test at the $P<0.05$ probability level.

RESULTS

A significant growth improvement of 48% in shoot DW and 22% in leaf area of *Gara-Yonjeh* was observed at NaCl concentration of 50 mM. In contrast, at the same NaCl concentration, strong toxic effect was observed in root length of *Gara-Yonjeh,* which decreased up to 23%. The effect of NaCl treatment on DW of root and shoot was not significant at 100 mM compared with control plants. With the increase of NaCl concentration, the DW of root and shoot toxically affected and the strongest toxicity was detected at NaCl treatment of 200 mM, decreasing up to 24% of root and up to 46% shoot DW (Table 1).

Concentration of H_2O_2 in root and leaves of *Gara-Yonjeh* increased significantly with increased NaCl concentrations. The level of H_2O_2 in root of NaCl-treated plants quickly increased from 6% (at 50 mM) to 321% (at 200 mM). Similar trend also

Table 1
Effect of various concentration of NaCl on shoot and root dry weight, leaf area and root length in *Gara-Yonjeh*

NaCl (mM)	Leaf area (cm^2/plant)	Root length (cm/plant)	Shoot dry weight (mg/plant)	Root dry weight (mg/plant)
0	14.04±1.4^b	80.28±4.9^a	56.91±5.9^b	8.34±0.4^a
50	17.21±0.7^a	61.67±5.8^b	84.52±3.3^a	7.93±0.8^{ab}
100	15.7±0.4^{ab}	45.69±5.6^c	53.94±4.2^b	7.40±0.5^{ab}
150	9.35±0.6^c	30.38±0.9^d	30.67±1.8^c	6.35±0.4^b
200	4.96±0.7^d	29.33±1.6^d	28.67±1.0^c	6.29±0.3^b

Each value indicated is the means of four replicates recorded ± S.E. Means followed by the same letters are not significantly different at $P < 0.05$.

occurred in leaves with a significantly higher increase that ranged from 79% (at 50 mM) to 1066% (at 200 mM) (Table 2).

The reduced ascorbate content in leaves of *Gara-Yonjeh* significantly decreases with NaCl concentrations at 100 to 200 mM, which decreased up to 40% at 200 mM. On the other hand, exposure to NaCl induced an increase up to 157% in ascorbate levels in root of *Gara-Yonjeh* at 200 mM (Table 2).

The level of total thiol in roots of *Gara-Yonjeh* quickly increased from 85% (at 100 mM) to 286% (at 200 mM). However, only a slight and significant increase in total thiol levels was observed within the 100 mM of NaCl in leaves of *Gara-Yonjeh* that followed with significant decrease during exposure to increased NaCl concentration (Table 2).

Addition of varying levels of NaCl to the growth medium caused a consistent increase in the accumulation of proline in both leaves and roots of *Gara-Yonjeh*. The level of proline in leaves of NaCl-treated plants quickly increased from 42% (at 50 mM) to 326% (at 200 mM). At similar trend also happened in roots that ranged from 53% (at 50 mM) to 174% (at 150 mM).

We detected at least three isoforms of SOD in leave of *Gara-Yonjeh* (Fig. 1A). The total SOD activity in the leaves of *Gara-Yonjeh* increased significantly with increasing NaCl concentrations compared to the control, and the maximal levels increased by 29% at 100 mM, while the higher concentration of NaCl reduced SOD activity. In roots of *Gara-Yonjeh* the total SOD activity generally reduced at exposure to NaCl and the maximal levels decreased by 29% at 150 mM (Table 3).

Only one isoenzyme of CAT in the leaves of *Gara-Yonjeh* was detected (Fig. 1B). Single isoform of CAT had a higher activity in expose with NaCl (403% at 200 mM). In contrast, the total activities of CAT in roots of *Gara-Yonjeh* were severely decreased by 57% for the NaCl concentration at 150 mM (Table 3).

Examination of POD isoenzyme profiles in leaves of *Gara-Yonjeh* by non-denaturing PAGE revealed three isoenzyme of POD. Isoform I was found at 50, 100 and 150 mM NaCl. Isoform II was found at all treatment of NaCl except for 200 mM. The

Table 2

Effect of various concentration of NaCl on the content of ascorbic acid, proline, total thiol and H_2O_2 in leave and root of *Gara-Yonjeh*

NaCl (mM)	Ascorbic acid (mg/g FW)		Total proline (µmol/g FW)		Total thiol (mM/g FW)		H_2O_2 (µmol/g FW)	
	Leave	Root	Leave	Root	Leave	Root	Leave	Root
0	9.99±0.1^{a}	3.4±0.1^{d}	5.11±1.1^{c}	13.77±1.4^{d}	1.38±0.2^{a}	1.88±0.3^{c}	21.43±1.4^{d}	339.61±7.2^{c}
50	9.74±1.1^{a}	4.07±0.3^{cd}	7.29±0.4^{c}	21.07±0.9^{c}	1.33±0.3^{a}	1.84±0.2^{c}	38.5±5.2^{cd}	360.7±24.67^{c}
100	7.94±0.1^{b}	4.69±0.4^{c}	11.88±1.0^{b}	27.03±2.6^{bc}	1.69±0.2^{a}	3.48±0.4^{b}	57.42±6.7^{bc}	494.35±26.3^{bc}
150	5.15±0.4^{c}	6.39±0.3^{b}	21.02±3.1^{a}	37.82±2.8^{a}	0.69±0.04^{b}	5.32±1.1^{a}	74.89±12.86^{b}	776.22±97.9^{b}
200	5.98±0.1^{c}	8.76±0.4^{a}	21.77±0.9^{a}	28.08±2.2^{b}	0.66±0.1^{b}	6.5±0.5^{a}	249.9±4.96^{a}	1429.74±287.7^{a}

Each value indicated is the means of four replicates recorded ± S.E. Means followed by the same letters are not significantly different at $P < 0.05$ within the same tissue.

Table 3

Effect of various concentration of NaCl on the activities of SOD, CAT, POD and APX in leave and root of *Gara-Yonjeh*

NaCl (mM)	SOD (Unit/mg Pro/min)		CAT (Unit/mg Pro/min)		POD (Unit/mg Pro/min)		APX (Unit/mg Pro/min)	
	Leave	Root	Leave	Root	Leave	Root	Leave	Root
0	6.96±0.9^{b}	5.12±0.3^{a}	23.89±3.7^{c}	1.64±0.13^{a}	1.46±0.05^{b}	4.94±0.1^{c}	20.69±0.2^{c}	17.67±2.8^{c}
50	6.93±0.2^{b}	4.41±0.3^{ab}	50.85±2.2^{b}	0.99±0.01^{b}	2.10±0.23^{a}	5.07±0.1^{c}	33.65±5.6^{c}	32.11±2.6^{a}
100	9.02±0.1^{a}	4.29±0.3^{ab}	52.78±1.7^{b}	1.01±0.09^{b}	1.41±0.01^{bc}	6.76±0.2^{b}	84.24±8.7^{b}	33.11±0.3^{a}
150	5.36±0.2^{c}	3.60±0.1^{b}	102.7±8.3^{a}	0.69±0.09^{c}	1.13±0.06^{c}	8.40±0.7^{a}	101.96±3.5^{a}	25.15±0.9^{b}
200	5.06±0.3^{c}	3.61±0.1^{b}	120.2±21^{a}	0.72±0.03^{c}	0.82±0.01^{d}	9.04±0.1^{a}	90.25±3.4^{ab}	21.98±1.7^{bc}

Each value indicated is the means of four replicates recorded ± S.E. Means followed by the same letters are not significantly different at $P < 0.05$ within the same tissue.

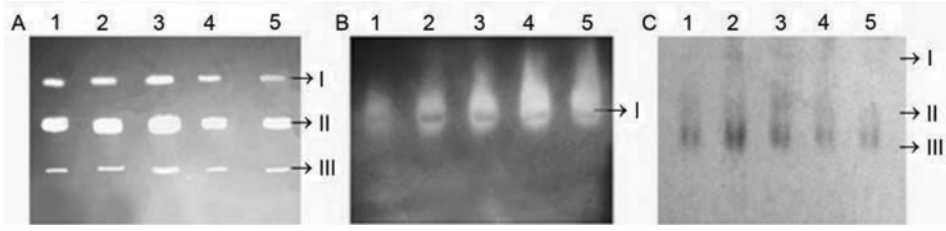

Fig. 1. Effect of various concentration of NaCl on the activities of SOD, CAT and POD in shoot of *Gara-Yonjeh*. For determination of SOD, CAT and POD isoform activities, the shoot extracts containing 60 µg proteins were loaded into the native PAGE. The activities of SOD (A), CAT (B) and POD (C) on gels were visualized by staining. The arrows point to the bands corresponding to the isoforms. Lanes from 1 to 5 were 0, 50, 100, 150 and 200 mM, respectively

activity of isoform III was detected at all concentration of NaCl. The pattern of isoform III activity Similar to SOD showed a "low–high–low" pattern, with the maximum activity at 50 mM of NaCl (Fig. 1C). The gel results also supported the results obtained from the spectrophotometric determination. The activities of POD in roots of *Gara-Yonjeh* show a general increase with the concentrations of NaCl applied. The activities of this enzyme were greatly stimulated by 83% after the exposure to NaCl at 200 mM, respectively (Table 3).

The assessment of antioxidant enzyme activity showed that APX activity in both roots and leaves of *Gara-Yonjeh* with increased salinity. The maximal levels of APX activity were increased by 392% at 150 mM and 87% at 100 mM NaCl in leaves and roots of *Gara-Yonjeh,* respectively (Table 3).

DISCUSSION

Sodium addition exerted a dual effect on the growth of *Gara-Yonjeh* depending on its concentration. In plants supplied with trifle sodium (50 mM), a growth-promoting effect was exerted on the shoot with up to 48% increase in DW compared with control plants but with the increase of NaCl concentration the growth parameters (e.g. dry weight) toxically affected. The beneficial effects of sodium on the growth of nonhalophytes (glycophytes) are well known in agriculture and horticulture. Thus, growth stimulation by sodium is caused mainly by its effect on cell expansion and on the water balance of plants. Not only can sodium replace potassium in its contribution to the solute potential in the vacuoles and consequently in the generation of turgor and cell expansion, it may surpass potassium in this respect since it accumulates preferentially in the vacuoles [18]. Studies have been revealed that toxic effects of salinity on plant growth may be due to ion cytotoxicity (mainly due to Na^+, Cl^-) and osmotic stress [36].

In the results presented here, H_2O_2 was rapidly accumulated in both root and leaves of *Gara-Yonjeh* during salt treatments. H_2O_2 has many essential roles in plant metab-

olism but at the same time, accumulation related to practically any environmental stress is potentially damaging. It is also well known that oxidative metabolism, and particularly H_2O_2, is involved in a wide variety of reactions and signaling cascades necessary for all aspects of plant growth and the integration of activity. Thus, while the involvement of H_2O_2 in stress responses is of particular interest, it really must be considered in the context of, and even as a special case of, H_2O_2 involvement in "normal" growth and metabolism. Accordingly, the plant antioxidant defense network is important in controlling the life-time of the ROS signals and in preventing uncontrolled oxidation [19, 36]. However, some authors reported no changes [16] or a significant reduction of the H_2O_2 concentration during salt treatments [14].

Our results demonstrate that the reduced ascorbate content in leaves of *Gara-Yonjeh* significantly decrease with the NaCl concentrations at 100 to 200 mM. Decrease in the level of accumulation of ascorbate was in correlation with the increase of activity of APX in leaves of *Gara-Yonjeh* in increased NaCl concentrations. Ascorbate is one of the nonenzymatic antioxidants for plant cells which control the ROS levels. Therefore, the decrease of ascorbate level in leaves of *Gara-Yonjeh* may be due to the pronounced consumption of ascorbate for scavenging H_2O_2 in the water-water cycle (Table 2). On the other hand, the increase of accumulation of ascorbate was more than the increase of activity of APX in salt-treated roots, suggesting that the rapid synthesis of ascorbate might occur. Therefore, the need for ascorbate through the ascorbate–glutathione cycle was reduced in salt-treated roots and possibly ascorbate is directly scavenging H_2O_2 [22].

Our data indicated that the NaCl salinity induces an increase in the content of non-protein thiol compounds in roots of *Gara-Yonjeh*. However, in leaves of *Gara-Yonjeh* a slight and significant increase was observed that was followed with a significant decrease during exposure to increased NaCl concentration (Table 2). High accumulation of antioxidant thiol compounds, which is possibly through the up-regulation of glutathione production, can be associated with tolerance of *Gara-Yonjeh* to increasing NaCl concentrations [7].

Treatment with NaCl also affected the accumulation of proline in leaves and roots of *Gara-Yonjeh*. Roots accumulated significantly higher levels of proline in responses to NaCl exposure (Table 2). Several physiological studies demonstrated that non-toxic compatible solutes, such as proline, glycinebetaine and sugars, can accumulate under salt stress conditions without any negative influence on the cell physiology. It is suggested that proline regulates the accumulation of useable N, contributes to osmotic adjustment and mitigates the damaging effects of NaCl that lead to cell membrane disruption [26]. Furthermore, under NaCl treatment an effect of proline accumulation on the inhibition of root growth has been established [17]. Accordingly, the root-length of *Gara-Yonjeh* decreased parallel to accumulation of proline in the roots.

The control of ROS levels can also be obtained by enzymatic antioxidant system (SOD, CAT, POD and APX). The total SOD activity in the leaves of *Gara-Yonjeh* increased significantly with increasing NaCl concentrations compared to the control but the higher concentration of NaCl reduced SOD activity. In roots of *Gara-Yonjeh* the total SOD activity generally reduced during exposure to NaCl. SOD is one of the

ubiquitous enzymes in aerobic organisms and plays a key role in cellular defense mechanisms against ROS. Its activity modulates the relative amounts of $O_2^{\cdot -}$ and H_2O_2 and decreases the risk of –OH radical formation. Whereas increased ascorbate accumulation indicated in root of *Gara-Yonjeh* in high concentration of NaCl, the reduction of SOD activity in roots can be interpreted by the employment of non-enzymatic routes for conversion of $O_2^{\cdot -}$ to H_2O_2 using antioxidants like GSH and ascorbate [30].

CAT activities increased by salinity in leaves of *Gara-Yonjeh*, and decreased in the roots of *Gara-Yonjeh*. In contrast to CAT, POD activity increased in roots of *Gara-Yonjeh* but it decreased in leaves of *Gara-Yonjeh* during exposure to salinity. The elevated POD activity in roots of *Gara-Yonjeh* treated with NaCl may reflect an increased ROS scavenging capacity and decreased damage of the plasma membrane lipids under stress conditions. PODs are involved not only in scavenging H_2O_2 but also in plant growth, development, lignification, suberization, and cross-linking of cell wall compounds [25].

APX is one of the major involving enzymes in the water-water cycle and the ascorbate-glutathione cycle which reduces H_2O_2 using ascorbate as an electron donor. Therefore, our results show that leaves and roots of *Gara-Yonjeh* responded to NaCl toxicity by improving the capacity of antioxidative enzymes, particularly APX, involved in the water-water cycle and the ascorbate-glutathione cycle, respectively [22].

The present results confirm that both non-enzymatic and enzymatic antioxidant systems are responsible for the salt tolerance observed in *M. sativa* L. cv. *Gara-Yonjeh*. The mechanism of tolerance in both roots and leaves was similar with some differences regarding the antioxidant enzyme activity.

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