Nutrio-physiological study of salinity tolerance mechanism in rosemary (*Rosmarinus officinalis* L.)

(ローズマリー(*Rosmarinus officinalis* L.)における耐塩性機構に関する栄養生理学的研究)

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CHAPTER I

Introduction

Water scarcity and salinity in water used for irrigation are critical problems in the arid and semi-arid regions of the world. Irrigated agriculture provides approximately 40% of the world's food, although it corresponds to only 17% of cultivated land and it is estimated that the irrigated area is reduced by 1%–2% each year because of salinization (FAO, 2002). In addition, an increase of 2 billion people is predicted in the next 25 to 30 years in the tropics and subtropics, so it will be necessary to increase yield on irrigated land to avoid malnutrition and famine (Pitman and Läuchli, 2002).

In Mexico, approximately 50% of the land surface is located in arid and semi-arid regions, and the main agricultural region has suffered from the adverse effects of droughts frequently and persistently throughout history (Ortega-Gaucin, 2018). Understanding how plants respond to drought, salinity, and coexisting stresses could be crucial to improve crop yield under these conditions (Larcher, 2003).

Salinity on cultivated land is a major problem affecting food security because it reduces crop growth and productivity (Butcher et al., 2016). The stress imposed by salinity arises because water is osmotically retained in the saline solution and becomes less accessible to the plant. Subsequently, an excess of Na^+ ions in the protoplasm disturbs the balance of ions, for example, K^+, Ca^{2+}, Mg^{2+}, which in turn affects proteins including enzymes, and membranes. If the osmotic and ionic adverse effects of salt absorption exceed the tolerance level of the plant, oxidative stress occurs (Larcher, 2003).

Currently, three main mechanisms by which plants tolerate salinity stress are recognized: osmotic tolerance, ion exclusion, and tissue tolerance (Munns and Tester, 2008; Rajendran et al., 2009; Roy et al., 2014). Osmotic tolerance is regulated by longdistance signals that reduce shoot growth and is triggered before shoot Na accumulation. Ion exclusion is related to Na and Cl transport processes in the root, which reduce the concentration of these ions within the leaf. Tissue tolerance involves the removal of Na from the cytosol and compartmentalization in the vacuole before it causes detrimental effects in cellular processes.

It is accepted that Na^+ is the most abundant ion that competes with nutrients for the absorption by plants, mainly with K^+, an essential nutrient that is crucial for cell osmo-regulation, stomatal function, and activation of large number of enzymes involved in photosynthesis, protein synthesis, and oxidative metabolism (Grattan and Grieve, 1999; Shabala, 2003). In glycophytes, salinity tolerance has been related to the maintenance of low Na concentrations in the whole plant (Horie et al., 2012) and acquisition and maintenance of K (Wakeel, 2013); therefore, the Na/K ratio has been considered as an accurate indicator of plant performance under salt stress (Kronzucker et al., 2008). Based on the selective absorption of K over Na by the root, and the selective transport of K over Na from root to stem, and stem to leaf, salt-tolerant plants can be classified as salt-excluding plants, salt-secreting plants, and salt-diluting plants (Wang et al., 2002).

Photosynthesis is considered to be an important indicator of the whole-plant performance, because it is one of the primary processes affected by water or salt stress (Chaves et al., 2009; Ashraf and Harris, 2013). Photosynthesis involves photochemical processes driven by light; enzymatic processes that do not require light; and diffusion processes, by which CO_2 and O_2 are exchanged between chloroplasts and external air. Stress-induced damage to any of these processes can reduce the overall photosynthetic capacity of the plant (Ashraf and Harris, 2013; Larcher, 2003).

On the other hand, the antioxidative enzyme system contributes to the ability to adapt to different types of environmental adversities that generally induce oxidative stress

(Caverzan, 2016). Oxidizing agents, called reactive oxygen species (ROS), are generated during normal physiological processes such as growth, photosynthesis, and respiration, but increase with abiotic stresses such as heat, salinity, and drought (Mittler, 2002). ROS are a group of free radicals, reactive molecules, and ions derived from oxygen. The most common ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot$). ROS are found in different cellular compartments (Mittler, 2002; Caverzan, 2016).

The mechanism of defense against ROS is composed of enzymatic and non-enzymatic components. Non-enzymatic antioxidants are found in all cellular compartments and may act directly in the detoxification of ROS and other radicals (Mittler, 2002); they are: ascorbate (AsA) and glutathione (GSH) as well as tocopherol, carotenoids, and phenolic compounds. Enzymatic components of the antioxidative defense system include (a) superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), which catalyze ROS degradation; and (b) enzymes of the ascorbate-glutathione cycle (AsA-GSH), such as ascorbate peroxidase (APX) and glutathione reductase (GR), which regenerate soluble antioxidants (Mittler, 2002, Gill and Tuteja, 2010). During stress, defense mechanisms against oxidative damage are activated to regulate toxic levels of ROS; however, when ROS production exceeds the capabilities of the defense mechanism, the cell is in a state of oxidative stress (Gill and Tuteja, 2010).

The present study deals with the nutritional and physiological characteristics of rosemary (*Rosmarinus officinalis L.*) and the mechanisms of salinity tolerance in this species. In a first approach, the absorption and selective transport characteristics of K over Na were analyzed to determine if rosemary excludes, excretes, or dilutes Na (Wang et al., 2002); the results were compared between rosemary and common thyme (*Thymus vulgaris* L.; hereafter 'thyme'), which are two species of aromatic herbs from the same taxonomic family, Labiatae (Lamiaceae) (Raja 2012). Subsequently, the nutritional imbalance caused by the competition of Na with essential nutrients such as K, Ca, and Mg in eight varieties of rosemary and its relationship with tissue tolerance was determined. Finally, the characteristics of gas exchange and the enzymatic components of the antioxidant system were evaluated in the same eight rosemary varieties; chlorophyll (*a*+*b*), malondialdehyde (MDA), and H_2O_2 contents were evaluated as indicators of protein oxidation, lipid peroxidation, and oxidative stress respectively.

Rosemary has been used since ancient times for food and medicinal purposes. It is currently used as a functional food and a material for the medicinal, cosmetic, and food-additive industries and is one of the major species in the market of aromatic herbs with organic management in Mexico (Juárez-Rosete et al., 2013). Several publications deal with the effect of saline stress on rosemary. Salinity exceeding 25 mM NaCl reduces growth, increases Na concentration and reduces K in the root, stem, and leaves, although a greater K/Na ratio is maintained in the shoot than in the roots (Tounekti et al., 2008). Stress induced by NaCl causes an accumulation of proline, total phenols, and antioxidants (Singh et al., 2010). Salinity tolerance has been associated with reduced leaf area and stomatal conductance, and with Na uptake for osmotic adjustment (Alarcon et al., 2006).

In addition, salinity affects the composition of rosemary essential oil, mainly by decreasing 1,8-cineole, possibly due to a decrease in cell hydration (Tounekti et al., 2008). These studies have been conducted in a single variety or not specify the studied variety, and the mechanisms of tolerance underlying these characteristics have not been addressed. In a study of other Labiatae herbs, Tanaka et al. (2018) reported that the growth of basil (*Ocimum basilicum* L.) and sage (*Salvia officinalis* L.) was not affected by cultivation in a salinized nutrient solution containing 50 mM NaCl, but that of thyme (*Thymus vulgaris*

L.) and oregano (*Origanum vulgare* L.) was reduced by approximately 60% and 50%, respectively.

CHAPTER II

Selective absorption and transport of K over Na as a salinity tolerance mechanism in rosemary and thyme plants

Sodium is one of the most abundant cations in soil salts; it competitively inhibits the absorption of other elements by plants, especially K, which is an essential multi-functional element influencing several processes (Shabala, 2003). Therefore, characterizing the processes that control the absorption and transport of Na and K in plants exposed to salinity stress is crucial for understanding the mechanisms of salinity tolerance and for increasing agricultural productivity.

Wang et al. (2002) classified the responses of salt-tolerant plants to salinity into three main types based on two physiological indexes: the selective absorption capacity of the root system (SA) and the selective transport of K over Na in root and stem (ST). The classifications were salt-excluding plants, salt-secreting plants, and salt-diluting plants, in decreasing order of SA capacity. Higher values of SA or ST mean higher Na control and absorption of K.

In this study, the salinity tolerance mechanisms of rosemary and thyme were clarified by analyzing the selective absorption of K over Na by the roots and the selective transport of K over Na among organs.

Materials and methods

Plant materials and cultivation

Rosemary and thyme seeds were purchased from a Japanese seed company (Fujita Seed Co., Ltd., Hyogo, Japan). On 17 April 2015, the seeds were sown in a plastic tray (50 cm × 36 cm × 8 cm) filled with moistened vermiculite. The tray was incubated in a glasshouse at the Faculty of Agriculture, Tottori University, Japan (35°51′52″N, 134°17′36″E). On 19 July 2015, three rosemary seedlings and eight thyme seedlings were transplanted to single plastic pots (3.5 L) filled with a soil mixture comprising non-salinized (i.e., control) or a salinized mixture of sand-dune soil and clay loam soil (1:1 v/v). The soil salinization was achieved according to the saturation extraction method (Rhoades et al., 1999), in which NaCl solution was used to adjust the soil electrical conductivity (ECe) to 3 dS m^{-1} (ECe 3; low-salt treatment) or 6 dS m^{-1} (ECe 6; high-salt treatment). The control, ECe 3, and ECe 6 soils underwent chemical analysis before plants were cultivated. The cultivation involved four replicates, with pots for two species and three treatments (i.e., control and two salinity treatments) arranged in a complete randomized design. A basal fertilizer consisting of NO_3, P_2O_5, and K_2O was applied to final concentrations of 20.0, 8.56, and 17.22 kg 1000 m^{-2}, respectively. We also applied magnesium lime as a soil acidity corrector. The amount of evaporated water was measured daily, and soil was irrigated with tap water to maintain 80% (w/w) field capacity. To stabilize the glasshouse temperature at 25–35 °C, the windows along the sides and on the roof were manually opened or closed as necessary. Rosemary and thyme plants were harvested on 20 September 2015.

Biomass and water content

The harvested rosemary and thyme plants were separated into leaf blades, stems with petioles, and roots, after which they were immediately weighed to determine the fresh weight of each organ. Samples were then dried in a forced-air oven at 70 °C for 72 h and weighed to measure dry weight (g $plant^{-1}$). The water content of each organ was calculated according to the following equation:

Water content (g g^{-1}) = (Fresh weight − Dry weight) / Dry weight.

Mineral concentration in plants and soils

Dried leaf blades, stems, and roots were each ground to a fine powder in a blender. Approximately 0.2 g of each powdered sample was wet digested with 15 ml of acid mixture (sulfuric acid - nitric acid - perchloric acid, 1:10:4 vol.) at 350°C for subsequent determination of Na and K concentrations with an AA-660 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan). Additionally, we prepared aqueous extracts from saturated soil pastes by using 300 g soil samples of the control and each salinity treatment at the initial conditions (Rhoades et al., 1999), after which Na and K concentrations were determined by atomic absorption spectrophotometry.

Selective absorption and transport

The selective absorption of K over Na by the roots ($SA_{K.Na}$) was calculated according to the following equation (Wang et al., 2002): $SA_{K.Na}$ = (Na/K in the root zone soil)/(Na/K in the whole plant). In addition, the selective transport of K over Na from the roots to the stems [$ST_{K.Na(roots/stems)}$] and from the stems to the leaf blades [$ST_{K.Na(stems/leaf\ blades)}$] was calculated according to the following equation (Wang et al., 2002): $ST_{K.Na(A/B)}$ = (Na/K in part A)/(Na/K in part B). Increasing $ST_{K.Na(A/B)}$ values corresponded to an increasing ability of plants to selectively transport K over Na from part A to part B.

Statistical analysis

Data were analyzed with SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, NY, USA), with ANOVA and the means among treatments were compared using the Tukey test. *P*-values less than 0.05 were classed as statistically significant.

Results

Dry weight and water content

In rosemary, the dry weights of the whole plant and individual organs did not differ significantly between the control and ECe 3 treatments; in contrast, the dry weights of the whole plant, leaf blades, and stems were significantly lower for the ECe 6 treatment than for the control treatment (Fig. 1). In thyme, among the analyzed organs, only the stem dry weight was significantly lower for the ECe 3 treatment than for the control treatment; however, the dry weights of the whole plant, leaf blades, and stems were significantly lower for the ECe 6 treatment than for the control treatment. The decreases (relative to the controls) in whole-plant dry weights when plants were subjected to ECe 6 treatment were similar for rosemary (37%) and thyme (38%).

Under control conditions, the water contents of the roots, stems, and leaf blades were 7.03, 2.27, and 3.21 g g^{-1} dry weight, respectively, for rosemary; and 4.92, 1.63, and 2.68 g g^{-1} dry weight, respectively, for thyme. For both rosemary and thyme, the water contents of the whole plant and individual organs were not significantly affected by the soil salinity.

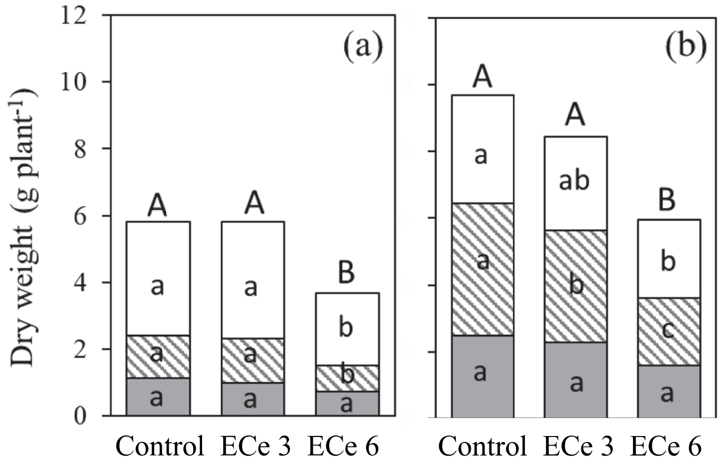


Fig. 1. Dry weights of the whole plant and each organ in rosemary (a) and thyme (b) under different soil salinity conditions. Leaf blades ☐, stems ▨, roots ■ Control, non-salinized; ECe 3, salinized at 3 dS m^{-1}; ECe 6, salinized at 6 dS m^{-1}. Different letters (lowercase, organ; uppercase, whole plant) indicate significant differences among plots, according to a Tukey test ($P < 0.05$, n = 4).

Na and K concentrations and Na/K ratio

In the control rosemary and thyme plants, the Na concentration was highest in the roots (Table 1). The Na concentrations in the leaf blades and stems were 18%–19%, 22%–24%, and 35%–40% of those in the roots in the control, ECe 3, and ECe 6 treatments, respectively.

The Na concentration in all rosemary organs increased significantly with increasing soil salinity; in contrast, the K concentration in the rosemary organs was unaffected by soil salinity, with the exception of a significant decrease in the stems in response to the ECe 6 treatment. Additionally, in rosemary organs, the ECe 3 treatment did not significantly affect the Na/K ratio, except for a significant increase in the roots; however, the ECe 6 treatment resulted in a significant increase in Na/K ratios in all organs.

In thyme, the ECe 3 treatment significantly increased the Na concentration compared with the control only in the roots, and the ECe 6 treatment significantly increased the Na concentration from that induced by ECe 3 only in the stems. In comparison with the control, the K concentration significantly decreased in thyme stems and roots in response to the ECe 3 treatment, and in all organs following the ECe 6 treatment. The ECe 3 treatment increased the Na/K ratio only in the roots, while the ECe 6 treatment increased it in the roots and stems compared with the control.

At the whole-plant level, the Na/K ratios in rosemary and thyme were 0.07 and 0.06 (control), 0.13 and 0.13 (ECe 3 treatment), and 0.30 and 0.22 (ECe 6 treatment), respectively.

Table 1. Na and K concentrations and Na/K ratios in each organ of rosemary and thyme under control and salinity conditions.

	Na (mg g^{-1} DW)			K (mg g^{-1} DW)			Na/K ratio		
	leaf blades	stems	roots	leaf blades	stems	roots	leaf blades	stems	roots
Rosemary									
Control	0.84c	0.90c	4.63b	20.75a	18.57a	20.05a	0.04b	0.05b	0.23c
ECe 3	1.71b	1.57b	6.85ab	21.33a	18.55a	16.30a	0.08b	0.08b	0.42b
ECe 6	4.29a	3.79a	10.84a	19.36a	16.10b	15.83b	0.22a	0.24a	0.68a
Thyme									
Control	0.31a	0.68b	3.06b	21.09a	20.01a	14.02a	0.01a	0.03b	0.22b
ECe 3	0.29a	0.63b	6.64a	20.58a	18.52b	10.90b	0.01a	0.03b	0.61a
ECe 6	1.66a	2.15a	7.24a	17.77b	16.04c	8.96c	0.09a	0.13a	0.81a

Control, non-salinized; ECe 3, salinized at 3 dS m^{-1}; ECe 6, salinized at 6 dS m^{-1}. Different letters indicate significant differences among plots according to Tukey test ($P < 0.05$, $n = 4$).

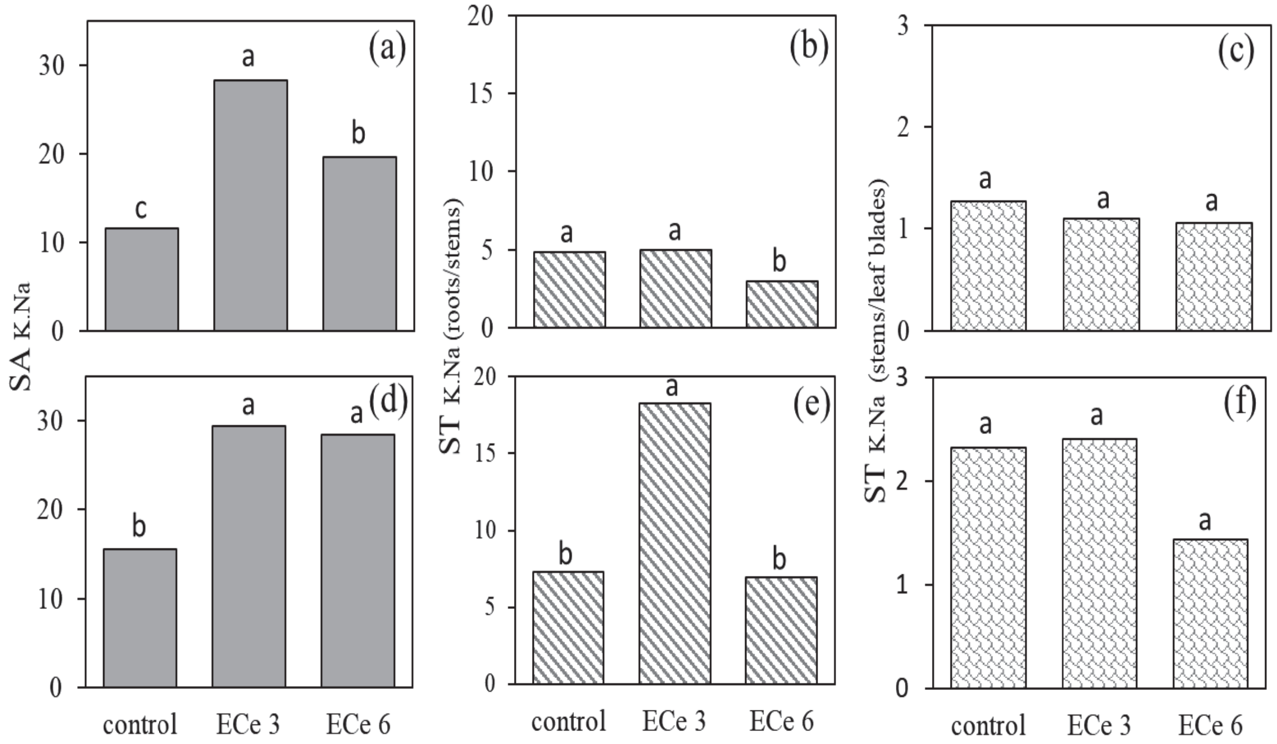
Selective absorption of K over Na by the roots

The $SA_{K.Na}$ values were similar in the control rosemary and thyme plants, and increased significantly in response to ECe 3 treatment (Fig. 2a and d). In rosemary, the $SA_{K.Na}$ value induced by the ECe 6 treatment was significantly lower than that due to the ECe 3 treatment, but was still significantly higher than that in the control treatment. In thyme, there was no significant difference between the $SA_{K.Na}$ values for the ECe 3 and ECe 6 treatments, but both were significantly higher than that in the control.

Selective transport of K over Na among organs

In rosemary, $ST_{K.Na(roots/stems)}$ did not significantly differ between the ECe 3 and control treatments, but the value decreased significantly in response to the ECe 6 treatment (by 38% relative to the control; Fig. 2b). $ST_{K.Na(stems/leaf\ blades)}$ did not differ significantly among treatments. In thyme, $ST_{K.Na(roots/stems)}$ did not differ significantly between the control and ECe 6 treatments, but increased significantly in response to the ECe 3 treatment. Although $ST_{K.Na(stems/leaf\ blades)}$ appeared to decrease following the ECe 6 treatment, the change was not significant.

Fig. 2. Selective absorption of K over Na from soils to roots ($SA_{K.Na}$), Selective transport of K over Na from roots to stems ($ST_{K.Na\ roots/stems}$) and from stems to leaf blades ($ST_{K.Na\ stems/leaf\ blades}$) of rosemary (a - c) and thyme (d – f) under control and different salinity conditions.



Different alphabetic letters indicate significant differences of dry weight in each organ and in whole plant among plots according to Tukey test ($P < 0.05$, $n = 4$).

Discussion

The growth of whole rosemary and thyme plants was unaffected by ECe 3, but decreased by approximately 40% following the ECe 6 treatment compared with the control (Fig. 1), suggesting that the salinity tolerance of these two plant species is similar. The salinity treatments in this study did not significantly affect the water contents of specific organs or the whole plant in either species.

Similar to most glycophyte species (Maathuis et al., 2014), rosemary and thyme accumulate Na mainly in the roots (Table 1), which protects the photosynthetic tissues from salinity stress. Previous studies revealed that salinity tolerance is associated with Na exclusion and the maintenance of the Na/K ratio (Apse and Blumwald, 2007; Horie et al., 2012; Kronzucker et al., 2008; Shabala, 2003). The higher whole-plant Na/K ratios in the ECe 6 treatment compared with the control and ECe 3 treatment is likely the result of an ionic imbalance that is inhibitory to growth

For both rosemary and thyme, the ECe 3 treatment did not significantly affect the whole-plant dry weight (Fig. 1), and it significantly increased the Na/K ratio of roots only (Table 1); however, Na and K concentrations in organs differed depending on the species. In rosemary, the ECe 3 treatment significantly increased the Na concentration in all the organs, but the K concentration was not significantly affected. In thyme, the Na concentration was not significantly changed in the stems or leaf blades, but the K concentration decreased significantly in the roots and stems. In thyme stems, the observed decrease in dry weight may have been partly due to K deficiency.

The ECe 3 treatment of rosemary and thyme also influenced the selective absorption capacity of the root system, with $SA_{K.Na}$ values that were approximately double those of the control treatments (Fig. 2a and d). These results suggest that the capacities of the rosemary and thyme root systems to take up or select K over Na from the medium were similar. In contrast, a comparison of rosemary and thyme plants that underwent the ECe 3 treatment revealed obvious differences in $ST_{K.Na(roots/stems)}$ and $ST_{K.Na(stems/leaf\ blades)}$. In rosemary, $ST_{K.Na(roots/stems)}$ and $ST_{K.Na(stems/leaf\ blades)}$ were similar to those of the control treatment (Fig. 2b and c), possibly because of the moderate tolerance of the stems and leaf blades to Na, which enables the K concentration to remain stable in the whole plant. Thus, to some extent rosemary might be a salt-diluting plant. In contrast, in thyme plants, the ECe 3 treatment induced a considerable increase in $ST_{K.Na(roots/stems)}$, while $ST_{K.Na(stems/leaf\ blades)}$ was unaffected (Fig. 2e and f). The observed increase in $ST_{K.Na(roots/stems)}$ reflects the ability of the roots to enhance the transport of K to the stems (Wang et al., 2002), which may be important for preventing an increase in the Na content of photosynthetic tissues (Table 1).

In rosemary, $SA_{K.Na}$ and $ST_{K.Na(roots/stems)}$ were significantly decreased in ECe 6 treatment relative to ECe 3 treatment. This decrease may be indicative of a disturbance in K absorption by the roots and the subsequent transport to the stems, and may ultimately contribute to the higher inhibition of growth under the ECe 6 condition than the ECe 3 condition (Fig. 1). Therefore, rosemary roots may be able to retain Na and selectively transport K to the stem under low-saline but not under high-saline conditions. In contrast, in thyme plants that underwent the ECe 6 treatment, $SA_{K.Na}$ was similar to that induced by the ECe 3 treatment, but $ST_{K.Na(roots/stems)}$ decreased to the control level. Although $ST_{K.Na(stems/leaf\ blades)}$ appeared lower in ECe 6 than in ECe 3, this change was not significant. Taken together, the results suggest that the reduction in growth in thyme plants treated under the ECe 6 condition compared with the ECe 3 condition (Fig. 1) is related to the decrease in the concentration of K in the stems and leaf blades (Table 1), which leads to an ionic imbalance in the leaves, but there is no conclusive evidence that this is the result of a decrease in selective transport of K over Na from the stems to the leaf blad-es. The precise Na and K transport system remains uncharacterized, although an earlier investigation found that Na and K compete for the same transport proteins (Hasegawa et al., 2000).

Summary

The salinity tolerance mechanisms of rosemary and thyme were compared in terms of the selective absorption of K over Na by the roots ($SA_{K.Na}$) and the selective transport of K over Na from the roots to the stems [$ST_{K.Na(roots/stems)}$] and from the stems to the leaf blades [$ST_{K.Na(stems/leaf\ blades)}$]. Plants were cultivated in non-saline (control), low-saline (ECe = 3 dS m^{-1}), or high-saline (ECe = 6 dS m^{-1}) soil. In both species, whole-plant dry weight decreased only following the ECe 6 treatment. In rosemary, $SA_{K.Na}$ increased after both salinity treatments, but more so after the ECe 3 treatment. $ST_{K.Na(roots/stems)}$ was unaffected by ECe 3, but decreased in response to ECe 6, while $ST_{K.Na(stems/leaf\ blades)}$ was unchanged by salinity stress. In rosemary, the suppressed selective absorption of K over Na by the roots under high-salt conditions may have inhibited the selective transport from the roots to the stems, ultimately decreasing dry matter. In thyme, $SA_{K.Na}$ increased in response to the ECe 3 and ECe 6 treatments. $ST_{K.Na(roots/stems)}$ increased only for the ECe 3 treatment, while $ST_{K.Na(stems/leaf\ blades)}$ decreased only for the ECe 6 treatment, although not significantly. In thyme, the increased selective absorption of K over Na by the roots may stabilize the selective transport from the roots to the stems at the control level; however, the decrease of about 40% in $ST_{K.Na\ (stem/leaves)}$ was not statistically significant, and a larger study is required to confirm their contribution to the decrease in dry matter.

CHAPTER III

Salinity tolerance mechanism and its difference among varieties in rosemary (*Rosmarinus officinalis* L.) - nutritional status of eight varieties under salt conditions

In the previous chapter, we found that rosemary plants showed increased selective absorption of K over Na by their root system in salinized soil of ECe 3 dS m^{-1}, but that the increase in this capacity was partially suppressed in rosemary grown in salinized soil of ECe 6 dS m^{-1}; this is the likely cause of the observed resistance to salinity stress at ECe 3 dS m^{-1} but dramatic growth inhibition at ECe 6 dS m^{--1}. Na alters the ionic homeostasis and disturbs the absorption of essential nutrients (Larcher, 2003). Therefore, the effect of moderate or severe salinity (50 and 100 mM NaCl) on the absorption of three important nutrients, K, Ca, and Mg, in a hydroponic system was investigated next. In addition, using this system, the relationship between nutrient absorption and salinity tolerance in eight rosemary varieties was analyzed to elucidate salinity tolerance mechanism in this species.

Materials and methods

Plant materials and cultivation

Eight varieties of rosemary were purchased from a Japanese seedling company (Shirokiya Shubyo Co. Ltd., Tottori, Japan): i.e., Arp, Benenden Blue, Lockwood de Forest, Primley Blue, Prostratus, Salem, Tuscan Blue and Officinalis. Seedlings were transplanted into plastic pots (width, 20 cm; length, 20 cm; depth, 26 cm) filled with an organic substrate (Metro-Mix 360, Sungro Horticulture, Agawam MA, USA) and raised using common practices to obtain cuttings.

The plants were grown in a greenhouse of the Faculty of Agriculture, Tottori University, Japan (35°51′52″N, 134°17′36″E), from 6 March to 9 July 2017. The average daytime (0700 to 1900) and nighttime (1900 to 0700) temperatures were 30.6 ± 5.2 °C and 21.4 ± 4.1 °C, respectively, and the relative humidity was 50% ± 16% and 81% ± 8%, respectively.

Cuttings were grown in Metro-Mix 360 in 200-cavity trays. At 43 days, when roots became longer than 1 cm, they were transplanted into hydroponic beds (40 L) and supplied with a sodium-free solution consisting of the following nutrients (mM): N (3.55), P (0.32), K (1.32), Ca (0.64), Mg (0.85), Fe (0.03597), Cu (0.00016), Mn (0.01120), Zn (0.00174), and B (0.01617). After a week for adaptation to the hydroponic system, homogeneous 2-month-old plants were selected and transplanted into 3-L pots, 3 plants per pot, to begin salinity treatments.

The treatments for the pots comprised two levels of salinity (50 and 100 mM NaCl) and control (3 mM NaCl). The salinity of 50 and 100 mM was achieved by daily increments of 25 mM NaCl. All salinity levels were reached at the same time, at which point the experiment was started. The above nutritive solution with the corresponding level of salinity was renewed weekly. The pots were randomly arranged, with 4 replicate pots per treatment. The plants were harvested after 4 weeks of salinity treatment.

Measurements

Growth

Immediately after harvest, each plant was separated into root, stem, and leaf blades, and the fresh weight of each organ was recorded. The fresh leaves of each plant were spread flat in transparent folders to measure the leaf area (cm^2) (Li-3100C, Li-Cor, Lincoln, NE, USA). Fresh samples were stored in paper bags and dried in a convection oven at 70 °C for 72 h. The dry weights (g plant^{-1}) were recorded as leaf dry mass (LDM), stem dry mass (SDM), root dry mass (RDM), and total dry mass (TDM). The biomass allocation to leaf, stem, and root was calculated according to Poorter et al. (2012). The water content was calculated as:

Water content (g g^{-1}) = (Fresh weight − Dry weight) / Fresh weight

Mineral concentrations

The dried samples of leaf, stem, and root of each plant were pulverized (Wonder Blender, Chemical Co., Ltd., Osaka, Japan), and approximately 0.2 g of sample was acid-digested at 350 °C. The contents of Na, K, Ca, and Mg were determined by atomic absorption spectrophotometry (mg) (Z-2310, Hitachi, Tokyo, Japan).

Salinity tolerance

Relative Tolerance Index (RTI) (Udovenko, 1976) was used to evaluate salinity tolerance: for this index, TDM at each salt condition was calculated relative to that in the control. The tissue tolerance to Na was estimated from the relation between the percentage of necrotic leaf tissue (ratio of necrotic leaf dry weight to total leaf dry weight at harvest) and the leaf Na concentration (Rajendran et al., 2009).

Statistical analyses

Means of different parameters for each variety were compared between control and salt treatments by Tukey's test ($P < 0.05$), and the correlations between parameters were determined according to Pearson's test, in SPSS v. 25 software (IBM Corp., Armonk, NY, USA).

Results

Growth

TDM (g plant^{-1}) of the control plants differed markedly among varieties (Fig. 3). TDM was reduced in all varieties as salinity increased; this result was significant for all varieties and conditions except Primley Blue at 50 mM NaCl. Compared with the control, the average reduction in TDM among varieties was approximately 30% at 50 mM NaCl and 47% at 100 mM NaCl; at 50 mM NaCl, the reduction in TDM in Primley Blue was the least (4.8%) and that in Officinalis was the greatest (46%). Leaf area (cm^2 plant^{-1}) significantly decreased as salinity increased in all varieties (Fig. 3). At 50 mM NaCl, the average leaf area reduction among varieties was 50%, but in Officinalis it reached 74%. At 100 mM NaCl, it was >70% in Salem, Arp, Tuscan Blue, and Officinalis.

In the control condition, the average biomass allocation among varieties was approximately 3:1:1 leaf:stem:root (Table 2); Tuscan Blue had the highest allocation to leaf and the lowest to root, and Benenden Blue had the lowest to leaf and the highest to root. At 50 mM NaCl, Benenden Blue and Officinalis showed significantly increased biomass allocation to root, and Primley Blue and Lockwood de Forest maintained biomass allocation to stem compared with the control. Salinity of 50 mM did not significantly alter biomass allocation to the leaf in any variety. At 50 mM NaCl, the water content (g g^{-1} fresh weight) increased significantly in roots in all varieties except Salem (Fig. 4) compared with the control. In leaves, the water content was not significantly affected at either salinity level, except in Benenden Blue (where it increased at both 50 mM and 100 mM NaCl) and Officinalis (where it was significantly lower at 100 mM than 50 mM but neither of the salinity treatments was significantly different from the control.).

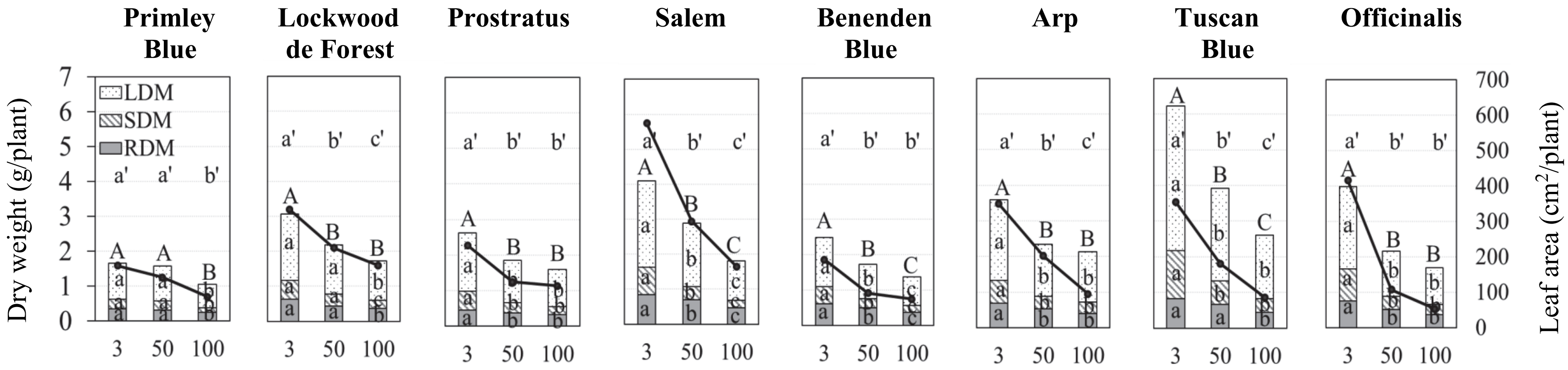


Fig. 3. **Effect of salinity on dry mass and leaf area in eight rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl .** Varieties are ordered by relative tolerance index (RTI) (highest to lowest, left to right). Total bar height indicates total dry mass. LDM, leaf dry mass; SDM, stem dry mass; RDM, root dry mass. Different letters indicate significant differences among treatments, according to Tukey test ($P < 0.05$, n = 4). Lowercase letters (a, b, c) relate to dry weight in the indicated organ and uppercase letters relate to total dry mass. Lowercase letters with a prime symbol (a′, b′, c′) relate to leaf area.

Table 2. Biomass allocation in eight rosemary varieties exposed to 3 (control), 50, or 100 mM NaCl.

Variety	NaCl (mM)	LMF	SMF	RMF
Primley Blue	**3**	0.624a	0.170a	0.204a
	50	0.637a	0.164a	0.198a
	100	0.637a	0.121b	0.240a
Lockwood de Forest	**3**	0.615b	0.179a	0.205a
	50	0.641ab	0.158a	0.200a
	100	0.657a	0.127b	0.214a
Prostratus	**3**	0.624b	0.200a	0.174a
	50	0.641ab	0.152b	0.205a
	100	0.657a	0.138b	0.204a
Salem	**3**	0.603a	0.189a	0.207b
	50	0.630a	0.127b	0.242ab
	100	0.626a	0.114b	0.258a
Benenden Blue	**3**	0.560a	0.194a	0.245b
	50	0.561a	0.152b	0.285a
	100	0.588a	0.144b	0.266ab
Arp	**3**	0.624a	0.179a	0.196a
	50	0.622a	0.153b	0.223a
	100	0.660a	0.153b	0.186a
Tuscan Blue	**3**	0.649a	0.218a	0.131a
	50	0.659a	0.163b	0.177a
	100	0.679a	0.148b	0.172a
Officinalis	**3**	0.584a	0.226a	0.189b
	50	0.589a	0.172b	0.237a
	100	0.602a	0.179b	0.218ab

LMF, leaf mass fraction; SMF, stem mass fraction; RMF, root mass fraction. For each column, in each variety, different letters indicate significant differences among treatments according to Tukey test ($P < 0.05$, n = 4).

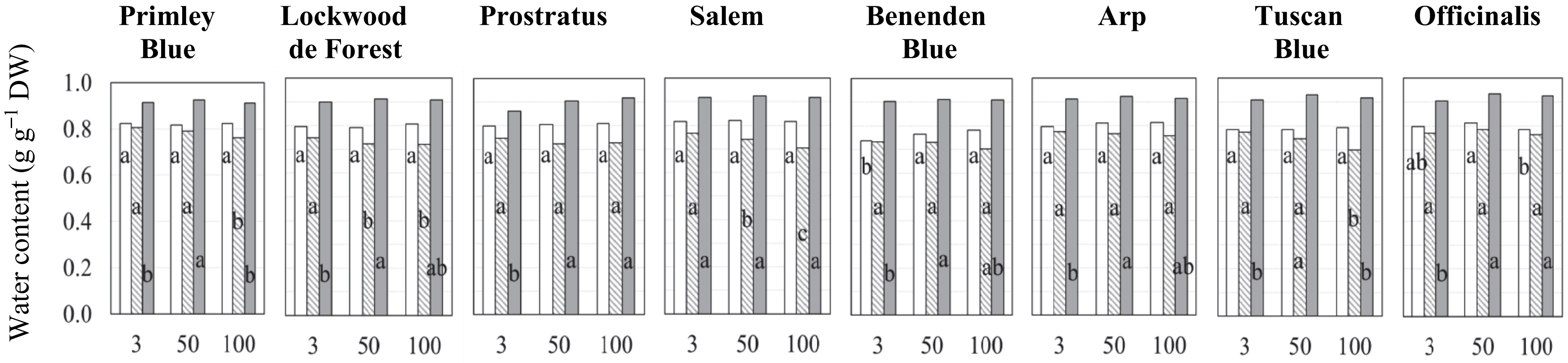


Fig. 4. Effect of salinity on water content in leaf □, stem ▨, and root ■, of eight rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl..Different letters indicate significant differences among treatments for each organ, according to Tukey test (*P* < 0.05, n = 4).

Mineral concentrations

In the control condition, the highest Na concentration was in the roots in all varieties; although there was a slight varation between stem and leaf, (Fig. 5). In plants treated with 50 mM NaCl. Leaf Na concentrations in Primley Blue and Benenden Blue were similar to those of stem, whereas those in Lockwood de Forest, Arp, and Officinalis matched those in root. At 100 mM NaCl, Na concentrations in root and leaf were similar in all varieties.

In the control condition, K was primarily concentrated in the leaf (Fig. 5), but in Salem, Benenden Blue or Tuscan Blue, it was equally high in root. In the salinity treatments, K was significantly reduced in all organs, particularly root. At 50 mM NaCl, leaf K was reduced by 40% on average but by almost 50% in Officinalis.

The Ca concentration tended to be similar in stem and leaf in most varieties in the control condition (Fig. 5). As salinity increased, Ca decreased in all organs except in the leaf and root of Prostratus and the leaf of Officinalis.

In the control condition, the Mg concentration was highest in leaf (Fig. 5). In the salinity treatments, it decreased by approximately 25% in all organs, with small differences among varieties.

At 50 mM NaCl, the leaf Na/K ratio ranged widely among varieties from 0.626 (Primley Blue) to 1.733 (Officinalis) (Table 3). At 100 mM NaCl, the ratios and the range among varieties were larger than those at 50 mM.

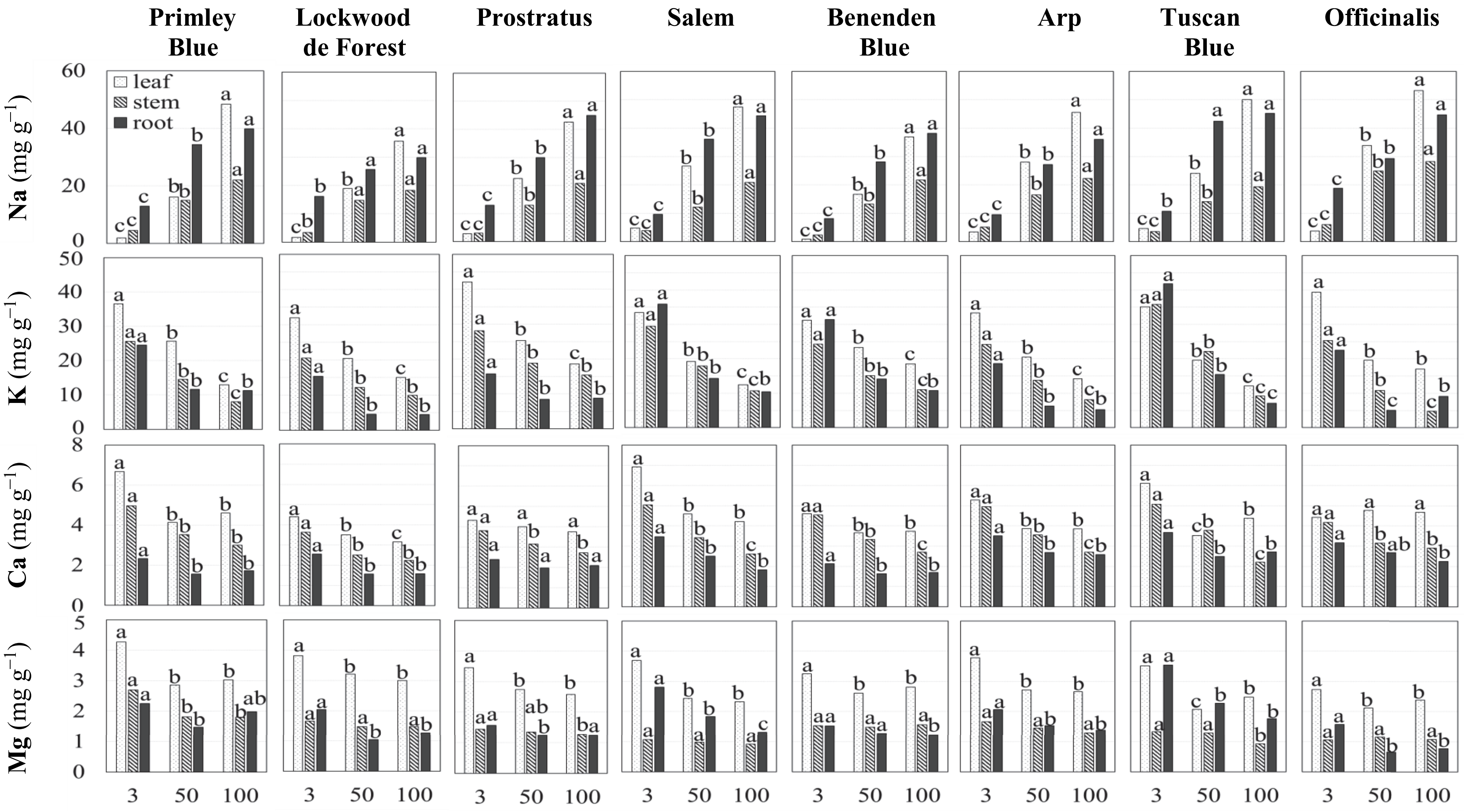


Fig. 5. Effect of salinity on concentrations of Na, K, Ca, and Mg (mg g^{-1} DW) in leaf, stem, and root of eight rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl. Different letters indicate significant differences among treatments in each organ, according to Tukey test ($P < 0.05$, n = 4).

Salinity tolerance

According to the RTI, Primley Blue was the most salt-tolerant variety, because it maintained 95.8% of TDM at 50 mM NaCl and 63.8% at 100 mM NaCl (Table 3). Officinalis was the most sensitive, because it maintained only 56% of TDM at 50 mM NaCl and 43% at 100 mM NaCl.

Under control conditions no leaf necrosis occurred. In salinity, all varieties showed leaf tissue damage by necrosis (Table 3), which began at the tip of the youngest leaves of the apex and then progressed to the branches. At 50 mM NaCl, the percentage of leaf necrosis was lowest in Primley Blue (10.8%) and Lockwood de Forest (13.4%); moderate in Prostratus, Salem, Benenden Blue, Arp, and Tuscan Blue (~20%–27%); and highest in Officinalis (43%). At 100 mM NaCl, the percentage of leaf necrosis doubled in Primley Blue and Tuscan Blue compared with that at 50 mM NaCl, but was similar in the other varieties to that at 50 mM NaCl.

To characterize tissue tolerance, a scatter graph of leaf Na concentration versus percentage of leaf necrosis in all varieties was prepared (Fig. 6). At 50 mM NaCl, in Officinalis, a higher percentage of necrosis was associated with a higher leaf Na concentration. In Primley Blue and Lockwood de Forest, a lower percentage of leaf necrosis was associated with a lower leaf Na concentration. Benenden Blue had a low leaf Na concentration but a moderate level of necrosis, whereas Salem, Arp, and Tuscan Blue had high leaf Na concentrations but a low level of necrosis, therefore, they may have some tissue tolerance. In 100 mM most varieties overlap.

Table 3. Relative Tolerance Index (RTI), leaf Na/K ratio, and leaf necrosis (%) in eight rosemary varieties exposed to 50 or 100 mM of NaCl.

Variety	NaCl (mM)	RTI	Leaf Na/K	Necrosis (%)
Primley Blue	**50**	95.8a	0.626c	10.8c
	100	63.8a	3.737ab	22.0cd
Lockwood de Forest	**50**	71.4b	0.926bc	13.4c
	100	56.9ab	2.371ab	13.9d
Prostratus	**50**	71.2b	0.888bc	27.0b
	100	60.9ab	2.286ab	26.0bcd
Salem	**50**	70.9b	1.380ab	20.6bc
	100	44.3ab	3.809ab	21.0cd
Benenden Blue	**50**	69.8b	0.717c	26.0b
	100	55.1ab	1.991b	34.2abc
Arp	**50**	65.9b	1.367ab	19.7bc
	100	60.8ab	3.202ab	22.9cd
Tuscan Blue	**50**	63.1b	1.228abc	19.3bc
	100	41.9b	4.122a	40.8ab
Officinalis	**50**	55.6b	1.733a	43.0a
	100	42.7b	3.127ab	46.4a

Different letters indicate significant differences among varieties for each salinity level, according to Tukey test ($P < 0.05$, n = 4).

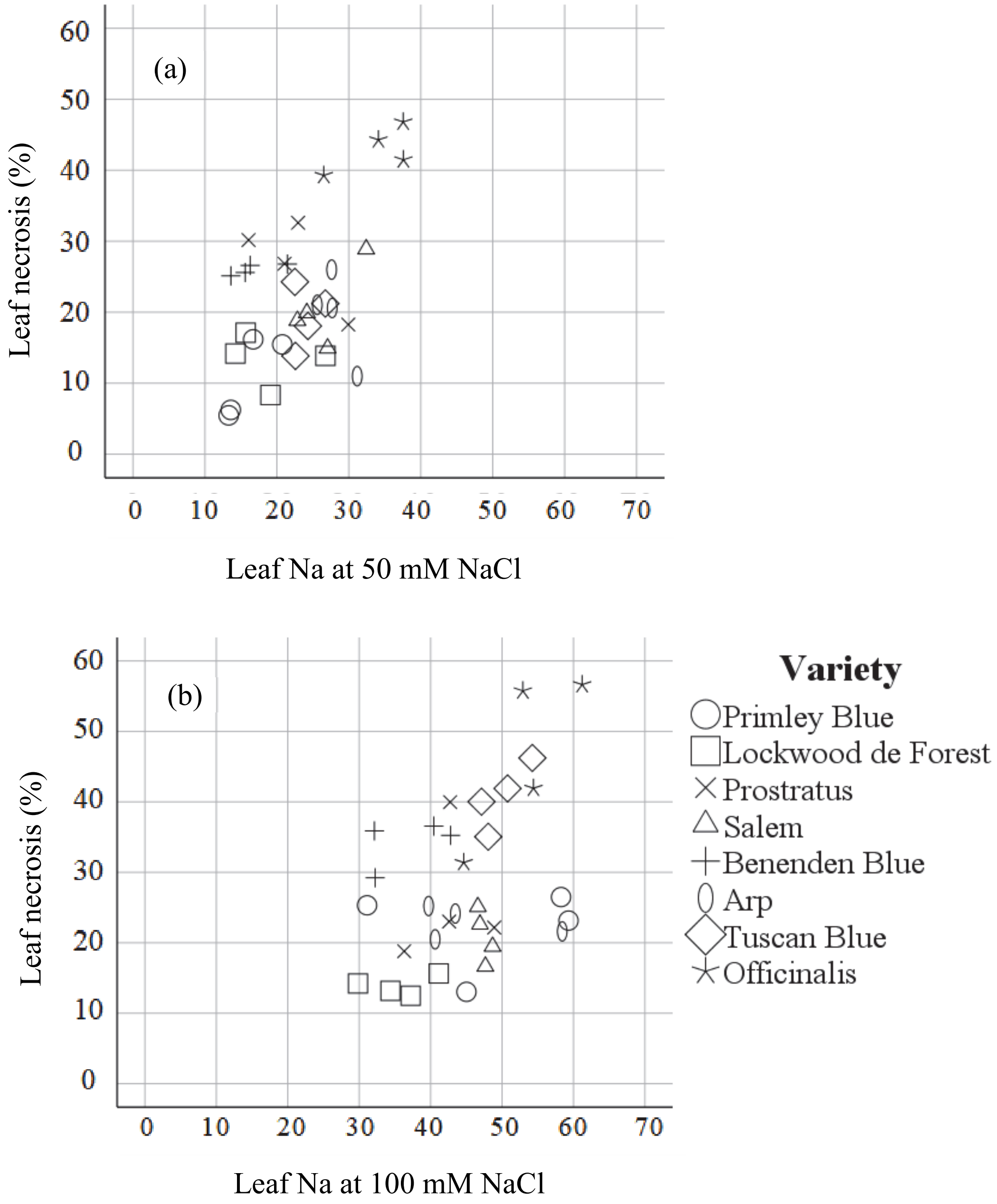


Fig. 6. Relationships between leaf sodium concentration (mg g^{-1} DW) and leaf necrosis (%) in eight rosemary varieties at (a) 50 or (b) 100 mM NaCl. A unique symbol represents each variety (4 replicates for variety).

Correlation analysis

When the data for all varieties were combined, we identified significant correlations between variables (Table 4). All variables, except RTI were determined in the leaf blades. At 50 mM NaCl, the RTI was correlated positively with K concentration ($r = 0.494$, $P < 0.01$) and Mg concentration ($r = 0.366$, $P < 0.05$), and negatively with Na concentration ($r = -0.623$) and the Na/K ratio ($r = -0.619$) (both $P < 0.01$). At 100 mM NaCl, it was correlated positively with Mg concentration ($r = 0.521$, $P < 0.01$). At both levels of salinity, it was correlated negatively with the percentage of leaf necrosis (50 mM, $r = -0.441$, $P < 0.05$; 100 mM, $r = -0.464$, $P < 0.01$).

At both levels of salinity, the percentage of leaf necrosis correlated positively with Na concentration (50 mM, $r = 0.539$, $P < 0.01$; 100 mM, $r = 0.430$, $P < 0.05$), and at 50 mM NaCl it correlated positively with the Na/K ratio ($r = 0.490$, $P < 0.01$).

Ca concentration was positively correlated with the Na/K ratio (50 mM, $r = 0.479$; 100 mM, $r = 0.490$) and the percentage of leaf necrosis (50 mM, $r = 0.513$; 100 mM, $r = 0.541$) ($P < 0.01$). Na and K concentrations were negatively correlated at 50 mM NaCl ($r = -0.652$) and 100 mM NaCl ($r = -0.483$) (both $P < 0.01$).

Table 4. Pearson correlation coefficients between mineral concentrations (Na, K, Ca, and Mg), Na/K ratio, Relative Tolerance Index (RTI), percentage of necrosis, and water content (WC), in eight rosemary varieties subjected to 50 or 100 mM NaCl.

	Na 50	Na 100	K 50	K 100	Ca 50	Ca 100	Mg 50	Mg 100	Na/K 50	Na/K 100	RTI 50	RTI 100	Necrosis 50	Necrosis 100	WC 50	WC 100
Na 50			−0.652**		0.508**		−0.443*		0.971**		−0.623**		0.539**		0.517**	
Na 100				−0.483**		0.552**		−0.469**		0.817**		−0.337		0.430*		0.019
K 50					−0.091		0.356*		−0.783**		0.494**		−0.202		−0.090	
K 100						−0.316		0.113		−0.864**		0.220		0.101		−0.476**
Ca 50							−0.283		0.479**		−0.104		0.513**		0.616**	
Ca 100								−0.244		0.490**		−0.278		0.541**		−0.048
Mg 50									−0.451**		0.366*		−0.521**		−0.044	
Mg 100										−0.274		0.521**		−0.397*		0.237
Na/K 50											−0.619**		0.490**		0.447*	
Na/K 100												−0.345		0.180		0.320
RTI 50													−0.441*		−0.005	
RTI 100														−0.464**		0.245
Necrosis 50															−0.024	
Necrosis100																−0.720**
WC 50																
WC 100																

All parameters except RTI were measured in the leaf. Mineral concentrations (mg g^{-1} DW), Necrosis, Leaf necrosis (%); RTI, Relative Tolerance Index; WC, water content (g/g^{-1} DW). Data for eight varieties of rosemary (n = 4 plants per variety) were combined for the analysis. *$P < 0.05$; **$P < 0.01$

Discussion

The rosemary varieties differed in salinity tolerance under moderate salinity (50 mM NaCl): the RTI decreased in the order of Primley Blue >> Lockwood de Forest ≈ Prostratus ≈ Salem ≈ Benenden Blue > Arp ≈ Tuscan Blue > Officinalis (Table 3). The remarkable difference in the TDM between Tuscan Blue and Primley Blue (Fig. 3) can be related to survival strategies in their natural environments: whereas Tuscan Blue grows rapidly under favorable environmental and abiotic conditions (a competitive strategy), Primley Blue grows slowly, maintaining a balance in the expenditure of carbon, minerals, and water, even under stress (a stress strategy) (Larcher, 2003). In general, the varieties with smaller mass showed higher salinity tolerance (Fig. 3, Table 3). The results suggest that Officinalis follows a competitive strategy, whereas Lockwood de Forest, Prostratus, Salem, Benenden Blue, and Arp are intermediate between a competitive and stress strategy.

All the varieties absorbed Na, and it was stored mainly in the root, although a considerable amount was transported to the stem, and particularly to the leaf (Fig. 5), where it may function as an osmolyte to avoid dehydration (Alarcón et al., 2006). The accumulation of sodium in the leaf plays a role in salinity tolerance of rosemary, because it helps to maintain turgor; however, if it is not properly regulated by the processes that control the net delivery of sodium into the root xylem, it might cause ionic toxicity (Munns and Tester, 2008).

Salinity in the solution around the root system disturbs ionic homeostasis, primarily that of K and Ca, altering the electrochemical potential gradient of the plasma membrane and possibly also of the tonoplast (Hasegawa et al., 2000; Munns and Tester, 2008). Na attenuates the influx of K, and the difference in the uptake of these two ions is manifested in the leaf Na/K ratio, which is used as an indicator of salinity tolerance in different aromatic herb species (Tanaka et al., 2018). Here, the Na/K ratio showed a negative correlation with the RTI at 50 mM NaCl ($r = -0.619$, $P < 0.01$), which suggests the importance of K in the salinity tolerance of rosemary (Grattan and Grieve, 1999). Therefore, leaf Na/K ratio could be a reliable indicator of salinity tolerance in rosemary under moderate salinity conditions.

Ca is an essential nutrient involved in the structure of the cell wall and membranes, and particular Ca sensors transduce abiotic and biotic stimuli (Grattan and Grieve, 1999). Notably, Ca concentration was positively correlated with Na concentration, Na/K ratio, and percentage of leaf necrosis at both salinity levels (Table 4). Under saline conditions, Ca might be taken up to play a physiological role, such as in the transport of ions (White and Broadley, 2003), but the metabolic disturbance induced by ionic stress might increase the necrotic damage.

Mg in plants is found interacting with enzymes and residing in the chlorophyll molecule. Under salinity, Mg competes strongly with Ca at the binding sites of cell membranes of the root (Grattan and Grieve, 1999). In all the varieties of rosemary examined here, salinity significantly reduced the concentration of Mg, primarily in the leaf (Fig. 5). Reduction in Mg under salinity could reduce the chlorophyll content of aromatic herbs (Tanaka et al., 2018).

In the salinity treatments, necrosis began at the tip of the younger leaves, changing the color to dark brown, and then appeared in the fully developed leaves. Necrosis is the cell death that occurs in response to pathogens or abiotic stress and is due to mitochondrial dysfunction and the generation of ROS (Minina et al., 2013). The high percentage of necrotic leaf tissue in Officinalis could be due to the imbalance in the supply of nutrients, mainly K, and high Na/K ratio, that reduced the production of biomass, reducing the relative ability of leaves to store Na in the vacuoles (Munns and Tester, 2008), and ROS could increase. We also determined the allocation of biomass to leaf, stem, and root under salinity (Table 2). Notably, Primley Blue and Lockwood de Forest maintained the allocation of biomass to stem under moderate salinity, suggesting an important role of this trait in salinity tolerance. Benenden Blue and Officinalis increased the allocation of biomass to root, which would increase the surface area for the uptake not only of more water but also of more salts. Therefore, this root trait of Benenden Blue and Officinalis could have positive or negative effects, depending on the ability of roots to selectively take up or exclude ions.

In conclusion, under moderate salinity, the Na/K ratio in leaf was significantly positively correlated with the RTI of rosemary; however, under high salinity, the correlation between the RTI and the Na/K ratio in leaf was inconsistent. By contrast, leaf water content was not significantly affected at either level of salinity, except for the significant increase at 50 mM and 100 mM NaCl in Benenden Blue. On the basis of the scatter graph of leaf Na concentration versus percentage of leaf necrosis, it was found a relatively high tissue tolerance to Na in a few varieties (ie: Salem, Arp and Tuscan Blue), but this tissue tolerance was not an indicator of salinity tolerance that could account for total growth. The excessive amount of Na and/or the imbalance between Na and K in leaf could have induced ionic toxicity, which then directly reduced growth.

Summary

To investigate the nutritional status of rosemary subjected to salinity stress, and its relation with salinity tolerance mechanisms, we grew eight varieties under moderate or severe salinity (50 or 100 mM NaCl) or control conditions (3 mM NaCl) in hydroponic culture and measured nutrient and water contents. At 50 mM NaCl, (1) the Relative Tolerance Index (RTI), which is a measure of growth relative to that in the non-saline control condition, decreased in the order of Primley Blue >> Lockwood de Forest ≈ Prostratus ≈ Salem ≈ Benenden Blue > Arp ≈ Tuscan Blue > Officinalis; (2) the leaf Na/K ratio and leaf necrosis percentage were significantly and negatively correlated with the RTI in all eight varieties; and (3) leaf tissue tolerance, indicated by a low percentage of necrosis with high Na concentration, differed only slightly among varieties, but Salem, Arp, and Tuscan Blue showed the highest tolerance. At both concentrations, NaCl had little effect on leaf water content. At 100 mM NaCl, the correlation between the RTI and the leaf Na/K ratio or percentage leaf necrosis became inconsistent. Therefore, rosemary may tolerate salinity by maintaining appropriate levels of nutrients with a low Na/K ratio in leaves before water content is affected. Although the transport of some Na to a leaf might create osmotic pressure, the excessive transport of Na to leaves creates ionic stress that reduces growth.

CHAPTER IV

Salinity tolerance mechanism and its difference among varieties in rosemary (*Rosmarinus officinalis* L.) - Physiological response of eight varieties under salt conditions

In the Chapter III was revealed that leaf Na/K ratio could be used as a reliable indicator to screen for salinity tolerance in rosemary plants, and Ca might play an important role in salinity tolerance, because it was strongly correlated with Na. Also, it was found that tissue tolerance could contribute slightly to salinity tolerance in some rosemary varierties.

In this chapter, physiological aspects, specifically characteristics of gas exchange and response of the antioxidative enzymatic system, were investigated to understand the mechanisms or adjustments that this species triggers to increase its resistance or tolerance to salinity stress.

Materials and methods

Plant materials and cultivation

Plant materials and cultivation were as described in the previous chapter. Eight rosemary varieties were grown hydroponically under moderate or high salinity (50 mM or 100 mM NaCl, respectively) or under control conditions (3 mM NaCl).

Measurements

Gas exchange

After the plants were grown for 3 weeks under saline or control conditions, gas exchange traits were evaluated in the eight rosemary varieties. Photosynthetic rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (Ci, μmol CO_2 mol^{-1}), and electron transport rate (flux of photons driving photosystem II [PS II]; ETR, μmol m^{-2} s^{-1}) were measured with a Li 6400 XT portable photosynthesis system and leaf chamber fluorometer LCF 6400-40 (both from LI-COR), with reference CO_2 set at 400 μmol CO_2 mol^{-1}, flow rate to the sample set at 500 μmol s^{-1}, and photosynthetically active radiation set at 500 μmol m^{-2} s^{-1}; ETR was inferred from chlorophyll fluorescence measurements.

Four consecutive sunny days were selected and two varieties were evaluated per day: Prostratus and Lockwood de Forest were evaluated on the first day, followed by Salem and Primley Blue, then Arp and Officinalis, and finally Benenden Blue and Tuscan Blue. After each photosynthesis measurement, the area of the rosemary leaf inside the chamber was measured. Data are the means of four replicates.

Chlorophyll content

Total chlorophyll (chlorophyll *a*+*b*) was determined in 99.5% ethanol extracts as described by Tanaka et al. (2018).

Leaf hydrogen peroxide content

The hydrogen peroxide (H_2O_2) was determined according to the method of Ferguson et al. (1983), as modified by Tanaka et al. (2018).

Leaf antioxidative enzymes activities

The activities of the leaf antioxidative enzymes, catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and superoxide dismutase (SOD) were evaluated. The measurements were made according to the method of Tanaka (1982), as modified by Tanaka et al. (2018).

Leaf malondialdehyde content

The leaf malondialdehyde (MDA) content was determined according to Ghanem (2008), as modified by Tanaka et al. (2018).

Results

Gas exchange

A was decreased by salinity in most varieties to a similar extent at 50 and 100 mM NaCl compared with the control (Fig. 7); however, it was significantly reduced by 100 mM NaCl, but not 50 mM NaCl, in Benenden Blue and Officinalis.

g_s tended to decrease gradually as salinity increased: at 50 mM NaCl, it was significantly reduced in Lockwood de Forest, Salem, and Arp only compared with the control (Fig. 7), but at 100 mM NaCl, all varieties, except Prostratus, showed significant reduction.

Ci showed slight variation with salinity, but it was stable at both NaCl levels in most varieties (Fig. 7); it was significantly reduced only in Primley Blue at 100 mM NaCl.

At 50 mM NaCl, ETR remained at a level similar to the control for all varieties except Tuscan Blue, which showed a reduction (Fig. 7). At 100 mM NaCl, ETR was significantly reduced in Lockwood de Forest, Salem, Benenden Blue, Tuscan Blue, and Officinalis compared with the control.

Chlorophyll content

At 50 mM NaCl, the content of chlorophyll (*a*+*b*) decreased significantly in Salem, Tuscan Blue, and Officinalis (Fig. 8) compared with the control. At 100 mM NaCl, chlorophyll content decreased significantly in all varieties.

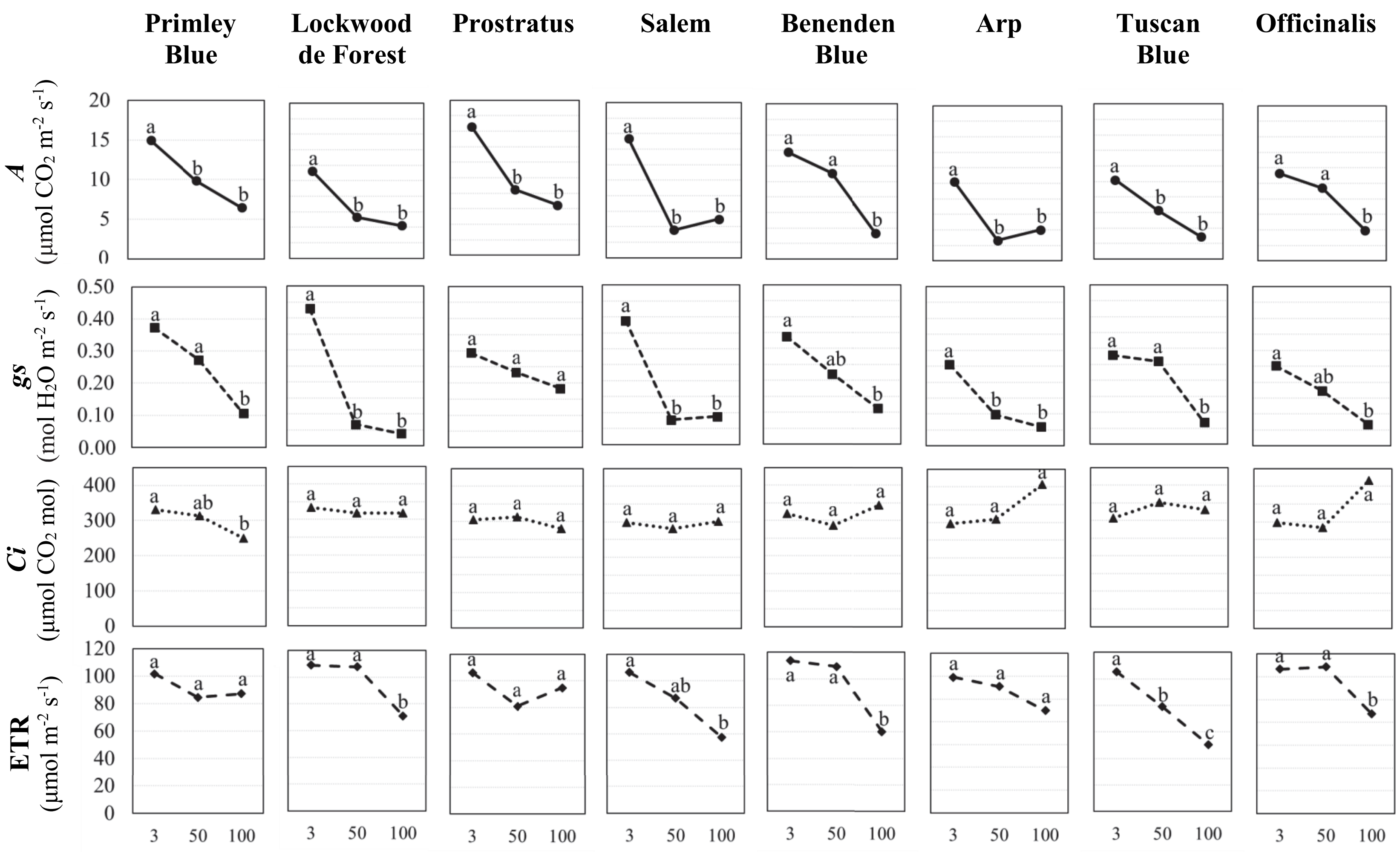


Fig. 7. Effect of salinity on photosynthetic rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (Ci) and electron transport rate (ETR) of rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl. Different letters indicate significant differences among treatments according to Tukey test ($P < 0.05$, n = 4).

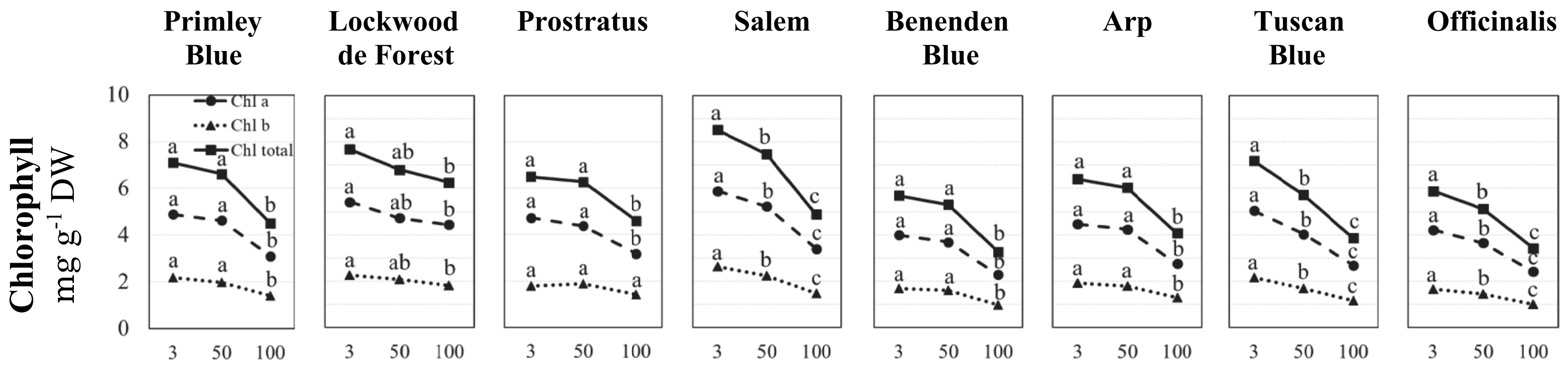


Fig. 8. Effect of salinity on chlorophyll *a*, chlorophyll *b*, and total chlorophyll content of rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl. Different letters indicate significant differences among treatments according to Tukey test ($P < 0.05$, n = 4).

Leaf hydrogen peroxide content

At 50 mM NaCl, leaf H_2O_2 content was slightly increased in Benenden Blue compared with the control, but this increase was not statistically significant (Fig. 9). At 100 mM NaCl, leaf H_2O_2 content was significantly increased in Benenden Blue and Tuscan Blue compared with the control. Conversely, leaf H_2O_2 content tended to reduce in some other varieties under saline conditions: in Lockwood de Forest it showed a non-significant reduction at 50 mM NaCl and a significant reduction at 100 mM NaCl; in Arp, it showed a significant reduction at 50 mM NaCl and a non-significant reduction at 100 mM NaCl, and in Prostratus and Officinalis it showed a significant reduction at both salinities compared with the control.

Leaf antioxidative enzymes activities

Marked differences in the activity of antioxidative enzymes were observed among rosemary varieties in response to saline treatments (Fig. 10). At 50 mM NaCl, SOD activity (units mg^{-1} protein min^{-1}) increased significantly in Tuscan Blue compared with the control; it also increased in Officinalis but this change was not significant. At 100 mM NaCl, SOD activity increased significantly in Prostratus and Officinalis. In contrast, it was significantly reduced in Salem at both salinities to a similar extent, and in Lockwood de Forest at 100 mM NaCl compared with the control.

CAT activity (mmol H_2O_2 mg^{-1} protein min^{-1}) showed a non-significant increase in Prostratus and Tuscan Blue, and a significant increase in Officinalis at 50 mM NaCl (Fig. 10), but in these varieties, it decreased to become similar to the control level at 100 mM NaCl. In contrast, CAT activity was significantly decreased in Salem at 50 mM NaCl and Arp at 100 mM NaCl. CAT activity was not significantly affected by salinity in Primley Blue, Lockwood de Forest, or Benenden Blue.

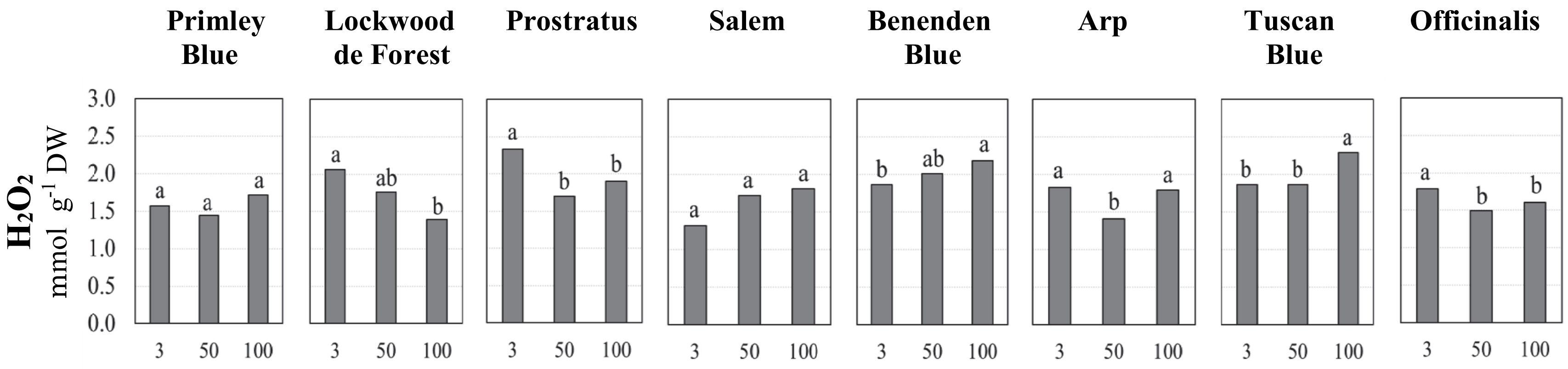


Fig. 9. Effect of salinity on leaf H_2O_2 content of rosemary varieties subjected to 3 (control), 50 and 100 mM NaCl. Different letters indicate significant differences among treatments, according to Tukey test ($P < 0.05$, n = 4).

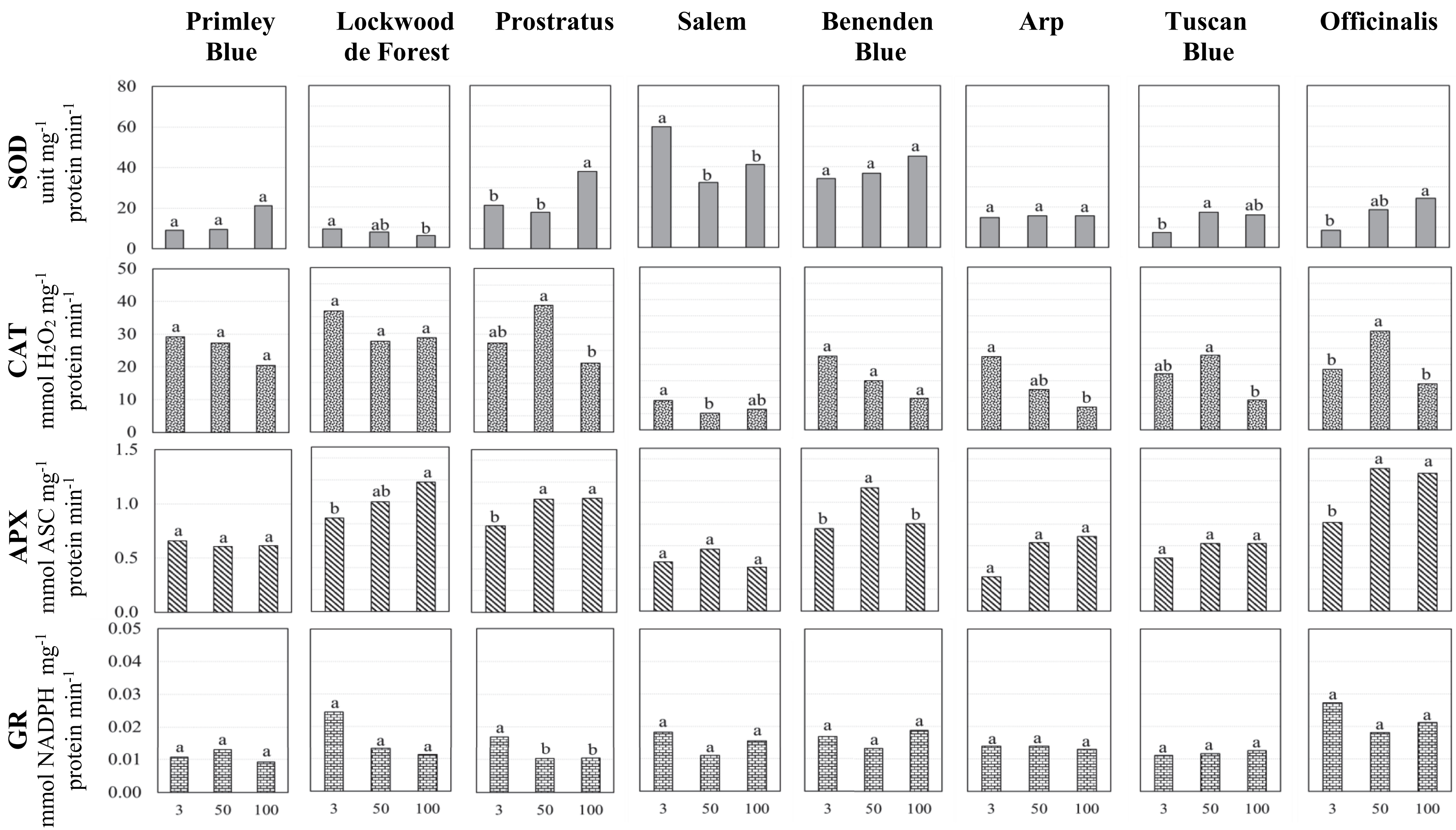


Fig. 10. Effect of salinity on the activities of leaf antioxidant enzymes in rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl. SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase. Different letters indicate significant differences among treatments, according to Tukey test (*P* < 0.05, n = 4)

APX activity (mmol AsA mg^{-1} protein min^{-1}) increased significantly in Prostratus, Benenden Blue, and Officinalis and showed a non-significant increase in Lockwood de Forest at 50 mM NaCl compared with the control. At 100 mM NaCl, this increase was maintained for Prostratus and Officinalis, and it increased further to become significant in Lockwood de Forest; however, in Benenden Blue, the APX activity showed a significant decline to the control level. Saline stress tended to reduce the GR activity in some varieties, although this reduction was only significant for Prostratus (50 mM and 100 mM NaCl).

Leaf malondialdehyde content

MDA is an indicator of damage in the cell membrane and organelles due to ROS. At 50 mM NaCl, leaf MDA content increased significantly only in Benenden Blue (Fig. 11). At 100 mM NaCl, leaf MDA tended to increase in Benenden Blue, Arp and Tuscan Blue, but this was not statistically significant.

Correlation analysis

Correlations between the physiological traits described above in combined data for the eight varieties are shown in Table 5. All parameters were measured in leaf. At 50 mM NaCl, A was positively correlated with g_s ($r = 0.531$) and CAT activity ($r = 0.386$) (both $P < 0.01$) and APX activity ($r = 0.410$, $P < 0.05$), and was negatively correlated with chlorophyll content ($r = -0.377$, $P < 0.01$). SOD activity was positively correlated with H_2O_2 content ($r = 0.413$) and negatively with CAT activity ($r = -0.478$) (both $P < 0.01$). APX activity was correlated with ETR ($r = 0.433$) and CAT activity ($r = 0.450$) (both $P < 0.01$) and also with percentage of necrosis ($r = 0.610$, $P < 0.01$), and was negatively correlated with chlorophyll content ($r = -0.458$, $P < 0.01$). Chlorophyll content was also negatively correlated with g_s ($r = -0.365$) and percentage of necrosis ($r = -0.467$) (both $P < 0.05$). Na/K ratio was positively correlated with ETR ($r = 0.361$, $P < 0.01$).

At 100 mM NaCl, RTI was positively correlated with ETR ($r = 0.364$, $P < 0.05$) and CAT activity ($r = 0.433$, $P < 0.01$) and negatively with GR activity ($r = -0.392$, $P < 0.01$). Conversely, percentage of necrosis was positively correlated with GR activity ($r = 0.516$, $P < 0.05$) but was negatively correlated with chlorophyll content ($r = -0.706$, $P < 0.05$) and MDA ($r = -0.350$, $P < 0.01$). GR activity was also negatively correlated with A ($r = -0.354$) and chlorophyll content ($r = -0.440$) (both $P < 0.01$). Chlorophyll content was positively correlated with MDA ($r = 0.457$) and CAT activity ($r = 0.495$) (both $P < 0.05$), but negatively with SOD activity ($r = -0.374$) and leaf H_2O_2 content ($r = -0.392$) (both $P < 0.01$). H_2O_2 was negatively correlated with the CAT activity ($r = -0.545$, $P < 0.05$) and APX activity ($r = -0.352$, $P < 0.01$). CAT and APX activities were positively correlated with each other ($r = 0.476$, $P < 0.05$). Leaf MDA was negatively correlated with SOD activity ($r = -0.488$, $P < 0.05$). Na/K ratio was negatively correlated with APX activity ($r = -0.371$, $P < 0.01$).

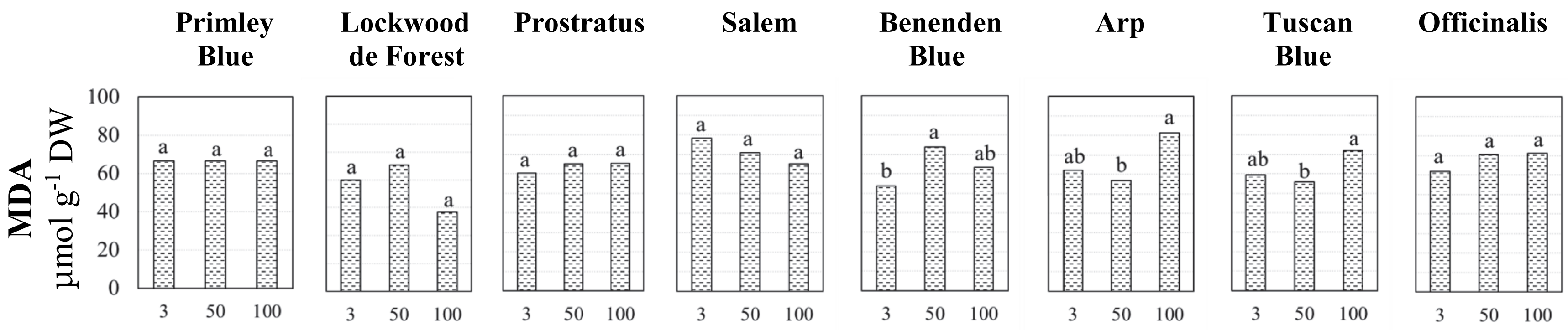


Fig. 11. Effect of salinity on leaf MDA content of rosemary varieties subjected to 3 mM (control), 50 mM, or 100 mM NaCl. Different letters indicate significant differences among treatments, according to Tukey test ($P < 0.05$, n = 4)

Table 5. Pearson correlation coefficients between the Relative Tolerance Index (RTI), percentage of leaf necrosis (NEC), Na/K ratio, gas exchange parameters, and activities of antioxidative enzymes in eight rosemary varieties subjected to 50 or 100 mM NaCl.

	Necrosis 50	Necrosis 100	Na/K 50	Na/Ka 100	A 50	A 100	gs 50	gs 100	ETR 50	ETR 100	ChlT 50	ChlT 100	H_2O_2 50	H_2O_2 100	MDA 50	MDA 100	CAT 50	CAT 100	APX 50	APX 100	GR 50	GR 100	SOD 50	SOD 100
RTI 50	-0.441*		-0.619**		0.203		0.151		-0.279		0.163		-0.063		0.145		0.051		-0.279		-0.101		-0.217	
RTI 100		-0.464**		-0.345		0.228		0.146		0.364*		0.158		-0.083		0.011		0.433*		0.055		-0.392*		-0.041
Necrosis 50			0.490**		0.284		0.047		0.277		-0.467**		-0.002		-0.037		0.123		0.610**		0.210		0.250	
Necrosis 100				0.180		-0.302		0.115		-0.084		-0.706**		0.328		-0.350*		-0.314		0.177		0.516**		0.195
Na/K 50					-0.306		-0.274		0.361*		-0.040		-0.181		-0.205		-0.200		0.050		0.262		0.135	
Na/K 100						0.003		-0.152		-0.101		-0.096		0.097		0.069		-0.346		-0.371*		0.082		-0.092
A 50							0.531**		0.198		-0.377*		-0.116		0.122		0.386*		0.410*		0.229		0.207	
A 100								0.230		0.225		0.110		-0.269		0.033		0.151		-0.105		-0.354*		0.204
gs 50									-0.098		-0.365*		-0.020		-0.256		0.317		0.009		-0.137		0.124	
gs 100										0.237		-0.077		0.102		-0.296		0.042		-0.122		-0.029		0.441*
ETR 50											-0.327		-0.231		0.125		-0.049		0.433*		0.281		0.131	
ETR 100												0.032		-0.214		-0.014		0.107		0.271		-0.004		-0.057
ChlT 50													0.017		0.129		-0.148		-0.458**		-0.250		-0.122	
ChlT 100														-0.392*		0.457**		0.495**		0.117		-0.440*		-0.374*
H_2O_2 50															0.130		-0.284		-0.019		-0.293		0.413*	
H_2O_2 100																-0.347		-0.545**		-0.352*		0.040		0.287
MDA 50																	0.064		0.293		0.053		-0.083	
MDA 100																		0.197		0.196		-0.092		-0.488**
CAT 50																			0.450**		0.063		-0.478**	
CAT 100																				0.476**		-0.323		-0.283
APX 50																					0.298		0.085	
APX 100																						0.165		-0.232
GR 50																							0.059	
GR 100																								0.322

All parameters except RTI were measured in the leaf. Abbreviations: photosynthetic rate (*A*), stomatal conductance (g_s), electron transport rate (ETR), total chlorophyll content (ChlT), hydrogen peroxide (H_2O_2), malondialdehyde concentration (MDA); catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR). Data for eight varieties of rosemary (n = 4 plants per variety) were combined for the analysis. * $P < 0.05$; ** $P < 0.01$.

Discussion

Different gas exchange characteristics and responses of the antioxidative enzyme system were triggered in rosemary plants treated with moderate (50 mM) and high (100 mM) salinity. When the data for the eight rosemary varieties were combined, the tolerance to salinity (RTI) did not directly correlate with A. Contrasting associations have been reported between growth and A under stress, and A has been considered an important indicator of the health status of the plant (Ashraf and Harris, 2013, Larcher, 2003). At 50 mM NaCl, A was reduced by salinity in most rosemary varieties (Fig. 7), but interestingly two varieties, a moderately salt-tolerant one (Benenden Blue) and the most salt-sensitive one (Officinalis), did not show reduced A. When A is reduced, excess excitation energy can be dissipated by so-called non-photochemical quenching; otherwise, it could lead to oxidative damage of photosynthetic machinery. Rosemary can safely remove excess energy through the xanthophyll cycle (Munné-Bosch and Alegre, 2000), but the non-reduction of A could reflect a delay in the response to stress, which could favor the loss of water.

In the combined data for the eight rosemary varieties, A was positively correlated with g_s ($r = 0.531$, $P < 0.01$). This relationship has been reported frequently in plant responses to salinity and other environmental stresses including drought. It is recognized that reduction in g_s is induced by the osmotic effect of salt outside the root (Munns and Tester, 2008). Reduced g_s lessens transpiration, thereby avoiding dehydration in the mesophyll cells, rather than reducing Ci (Chaves et al., 2009; Ashraf and Harris, 2013), and is considered an immediate response to salinity to mitigate ion influx to the shoot (Hasegawa et al., 2000). In addition, A was correlated with the antioxidative activity of CAT ($r = 0.386$, $P < 0.05$) and APX ($r = 0.410$, $P < 0.05$) in leaf; APX activity was also positively correlated with percentage of leaf necrosis ($r = 0.610$, $P < 0.05$). These results suggest that antioxidative enzyme activity was increased and maintain physiological functions such as A, as in Benenden Blue and Officinalis, but that necrosis still occurred probably due to the high Na influx.

Under saline conditions, the leaf content of the most stable ROS in plants, H_2O_2, and SOD activity were both similar to the levels in the control in most varieties; at 50 mM NaCl, H_2O_2 content was positively correlated with SOD activity ($r = 0.413$, $P < 0.05$). H_2O_2 increased only in Tuscan Blue, and tend to increase in Officinalis. This result could indicate the efficient elimination of the superoxide radical by SOD, which does not require an increase in its activity.

At 50 mM NaCl, total chlorophyll content was negatively correlated with percentage of necrosis ($r = -0.467$, $P < 0.01$), g_s ($r = -0.365$, $P < 0.05$), A ($r = -0.377$, $P < 0.05$), and APX activity ($r = -0.458$, $P < 0.01$). This result suggests that reduction in the chlorophyll content could be a symptom of increased dehydration or related to oxidative stress induced by salt (Tanaka, et al., 2018). Generally, the content of chlorophylls is reduced by salt stress, due to alterations in biosynthesis or their rapid degradation, which could affect the efficiency of the capture of light energy that activates photosystems I and II (Ashraf and Harris, 2013; Larcher, 2003). Here, total chlorophyll content was not affected by salinity in Primley Blue, the most salt-tolerant variety, but was decreased in Officinalis, the most salt-sensitive variety; the moderately tolerant varieties presented variable responses. The loss of chlorophyll has been considered an efficient mechanism to modulate the amount of light intercepted by leaves in rosemary under severe drought stress (Munné-Boch and Alegre, 2000). Previous researchers have detected reduction of chlorophyll content in salt-sensitive species; hence, chlorophyll content has been proposed as an indicator of salinity tolerance in various crops such as wheat, melon, and sunflower, but not in others, such as tomato (Ashraf and Harris, 2013). Here, total chlorophyll content in rosemary was not correlated with salinity tolerance (RTI) at 50 mM NaCl, hence it would not be a reliable index of salinity tolerance in rosemary under moderate salinity stress.

Ci remained at control levels even in saline conditions, probably due to the equilibrium between the consumption of CO_2 by carboxylation and the re-supply by photorespiration and the ambient air (Larcher, 2003). However, CO_2 could play a part in photosynthesis reduction due to limitations beyond the intercellular space of mesophyll, such as resistance to dissolving and transport of CO_2 into the liquid phase of the cell wall and the protoplasm, or resistance to carboxylation (Chaves et al., 2009; Larcher, 2003).

A different physiological response to salinity stress was triggered at 100 mM NaCl when compared with 50 mM NaCl. In the combined data, leaf Na/K ratio was negatively correlated with the antioxidative activity of APX ($r = -0.371$, $P < 0.05$) in leaf. It should be mentioned that Na/K ratio increased remarkably in the leaf at 100 mM NaCl compared with 50 mM NaCl; therefore, the activity of CAT and APX might be limited due to the toxicity of Na.

Leaf H_2O_2 content showed a heterogeneous response among varieties (Fig. 9); however, under the 100 mM NaCl condition, it was negatively correlated with the activities of CAT ($r = -0.545$, $P < 0.01$) and APX ($r = -0.352$, $P < 0.05$) in leaf. In plant cells, H_2O_2 is produced predominantly during photosynthesis and photorespiration, and its production is controlled by the enzymatic and non-enzymatic antioxidant system; hence, the antioxidative activities of CAT and APX are indispensable for the detoxification of H_2O_2 (Gill and Tuteja, 2010; Mathuis, 2014). At 100 mM NaCl, CAT activity tended to decrease and APX activity was maintained similar to that at 50 mM NaCl in most varieties; despite this, the effective action of antioxidative enzymes in the removal of H_2O_2 can be seen in Lockwood de Forest, Prostratus, and Officinalis, although weak activity only was seen in Benenden Blue and Tuscan Blue. Perhaps other antioxidative enzymes are involved.

The remarkable negative correlations of chlorophyll content with the percentage of leaf necrosis, and leaf GR and CAT activities (Table 5), suggest that the synthesis of chlorophyll could be affected by oxidative stress. On the other hand, its positive correlation with leaf MDA, which results from oxidative stress, might be because of the relatively high MDA in the varieties that maintained high chlorophyll content. At 100 mM NaCl, ETR was diminished in most varieties, and its reduction was correlated with RTI ($r = 0.364$, $P < 0.05$).

The antioxidative activity of GR in leaf was significantly correlated with several physiological parameters at 100 mM NaCl, although it was not affected by salinity, except in Lockwood de Forest. GR activity was negatively correlated with RTI ($r = -0.392$, $P < 0.05$), A ($r = -0.354$, $P < 0.05$), and chlorophyll content ($r = -0.440$, $P < 0.05$), but positively with percentage of leaf necrosis ($r = 0.516$, $P < 0.01$). GR is the enzyme responsible for maintaining the reduced form of glutathione (GSH), an essential metabolite in plants for the activation of genes that lead to acclimation, stress tolerance, and other defense responses (Foyer et al., 1997, Gill and Tuteja, 2010). The activity of this enzyme did not increase during salt stress; therefore, its activity might be an intrinsic trait of each variety, and the positive correlation with percentage of leaf necrosis could be related with greater acclimation requirement against environmental conditions.

In conclusion, the characteristics of gas exchange and antioxidative enzyme activity responded to salt stress differently in different rosemary varieties. The features associated with greater tolerance in moderate salinity were reduction in A and g_s, which might lessen transpiration and dehydration in the mesophyll cells and mitigate ion influx. In addition, increases in CAT and APX activities kept an appropriate level of H_2O_2 in leaf. In high salinity, RTI was associated with leaf CAT activity, but its activity was limited by ionic stress. In addition, RTI was negatively correlated with the intrinsic concentration of GR in the eight varieties combined, probably as a requirement for adaptation to stressful conditions. Interestingly, Primley Blue, the most salt-tolerant variety did not require increases in its antioxidative enzyme system to minimize leaf H_2O_2 accumulation under salt stress. Possibly Primley Blue has some other strategy to avoid the stress. Mittler (2002) proposed different mechanisms or changes in plant metabolism to avoid the effect of stress, and therefore, ROS production. Among these mechanisms, there are anatomical adaptations of the leaves, molecular mechanisms of reorganization in the photosynthetic apparatus, and reduction of photosynthetic rate In addition, in rosemary, differences in oxidative stress induced by salt in the leaf could be related to the concentration of carnosic acid, which acts as an antioxidant in rosemary chloroplasts, eliminating free radicals that are formed as a result of photosynthetic activity (Munné-Bosch and Alegre, 2001).

Summary

Physiological aspects, specifically characteristics of gas exchange and response of the antioxidative enzymatic system, were investigated to understand the mechanisms that rosemary triggers to increase its resistance to salinity stress.

Under hydroponic culture in moderate salt (50 mM NaCl) most of the eight rosemary varieties examined decreased photosynthetic rate (A) and stomatal conductance (g_s), possibly to prevent damage to the photosystems and adjust the osmotic pressure to make the use of water more efficient. In all varieties, the intercellular concentration of CO_2 (Ci) did not change significantly, and in almost all varieties, the electron transport rate (ETR) did not change significantly. When the data for all eight rosemary varieties were combined, A was correlated with the antioxidative enzyme activities of CAT ($r = 0.386$, $P < 0.05$) and APX ($r = 0.410$, $P < 0.05$) in leaf, but APX activity was positively correlated with percentage of leaf necrosis ($r = 0.610$, $P < 0.01$). This suggests that the increased antioxidative enzyme activity was triggered to eliminate the increase of reactive oxygen species (ROS), but nevertheless necrosis occurred. Leaf SOD activity increased only in the most salt-sensitive varieties, Tuscan Blue and Officinalis, possibly for scavenging of the superoxide radical, suggesting that the more tolerant varieties do not require an increase in SOD activity. In the data combined across varieties, leaf SOD activity was positively correlated with leaf H_2O_2 ($r = 0.413$, $P < 0.05$). Total chlorophyll content was negatively correlated with percentage of leaf necrosis, g_s, A, and leaf APX activity (Table 5). This result suggests that reduction in the chlorophyll content could be a symptom of increased dehydration or related to oxidative stress induced by salt.

At high salt (100 mM NaCl), A, g_s, ETR, and chlorophyll content were impaired in almost all varieties, suggesting severe metabolic disturbance. In the data combined across varieties, A was negatively correlated with leaf GR activity, which in turn was positively

correlated with percentage of leaf necrosis but negatively with salinity tolerance (RTI). These results suggest that in rosemary stressful conditions might lead to an increase of ROS, and then detoxifying mechanisms would be triggered to protect the cell from oxidative stress.

General discussion

Sodium is the most abundant ion in the saline soil components that competes with nutrients for the absorption by plants; it competes mainly with K, which is an essential nutrient crucial for cell osmo-regulation, stomatal function, and as the activator for many enzymes, and is involved in photosynthesis, protein synthesis, and oxidative metabolism (Grattan and Grieve, 1999; Shabala, 2003). In glycophytes, salinity tolerance has been related to the maintenance of low Na concentration in the whole plant (Horie et al., 2012) and acquisition and maintenance of K (Wakeel, 2013). Here, to determine the ionic selectivity in rosemary, the selective absorption and selective transport of K over Na, according to Wang et al. (2002), were compared between rosemary and thyme, two Labiatae aromatic herbs.

Rosemary and thyme had similar tolerance to salinity: both species were unaffected by the low-salt treatment (soil electrical conductivity (ECe), 3 dS m^{-1}), but total dry mass (TDM) decreased by approximately 40% following the high-salt treatment (ECe, 6 dS m^{-1}) (Fig. 1). Rosemary in salinity conditions increased Na concentration in all the organs, but it maintained the leaf K concentration even in high salinity, whereas thyme did not alter the concentration of Na in the leaf but reduced K. Some plants may absorb Na to help maintain turgor (Munns and Tester, 2008), and so may rely on Na (Tounekti et al., 2011a); the results here suggest that rosemary can be included in this group; in contrast, thyme avoids transporting Na to the photosynthetic tissue, but decreases the absorption of K.

Here, study of selective absorption (SA) and selective transport (ST) of K over Na revealed that rosemary and thyme in the low-salinity condition increased their selective absorption of K over Na by the roots ($SA_{K.Na}$), but only thyme had a dramatic increase in the selective transport of K over Na from root to stem [$\mathrm{ST}_{\mathrm{K.Na\ (roots/stems)}}$], which allowed it to exclude Na from the photosynthetic tissue. When high salinity was compared with low salinity, the selective absorption of K over Na by the roots decreased in rosemary, which might indicate alterations in the absorption of K by the roots, and subsequent transport to the stem, which ultimately would reduce growth; in contrast, in thyme, a weak selective transport of K over Na from the stem to the leaves [$\mathrm{ST}_{\mathrm{K.Na\ (stem/leaves)}}$] could lead to ionic imbalance in the leaves. These differences in the absorption of nutrients result from the interaction between passive and active transport, as well as the plant's capacity for ionic selectivity and ion accumulation, particularly in vacuoles (Larcher, 2003). From the results obtained in the above experiments, rosemary could be classified as a salt-diluting plant.

Then was investigated the effect of Na on the status of the essential nutrients K, Ca, and Mg, and its relationship with salinity tolerance mechanism in this species. The remarkable difference in the TDM between Tuscan Blue (6.22 g plant^{-1}) and Primley Blue (1.64 g plant^{-1}) (Fig. 3) under the non-salinized control condition can be related to survival strategies in their natural environments (Larcher, 2003). The relative tolerance index (RTI) from highest to lowest was in the order of Primley Blue, Lockwood de Forest, Prostratus, Salem, Benenden Blue, Arp, Tuscan Blue, and Officinalis. Salinity decreases growth and crop yield because it causes changes at all functional levels of the organism, since it can limit the absorption of water by the roots, alter the absorption of nutrients, alter the essential metabolism such as photosynthesis, cause oxidative stress and, finally, overcome the adaptation or resistance capabilities of the plant and cause cell death (Grattan and Grieve, 1999; Munns and Tester, 2008; Chaves et al., 2009; Ashraf and Harris, 2013).

Here, the absorption of Na in the leaves of rosemary varieties altered the ionic homeostasis, mainly of K and Ca. Nutrient deficiencies related to Na uptake have been reported in rosemary (Tounekti et al., 2008 and 2011b; Singh et al., 2010), other Labiatae species (basil, sage, thyme, and oregano) (Tanaka et al., 2018), and in other aromatic plants (Said-Al Ahl and Omer, 2011). Disturbances in ionic homeostasis could relate to alterations in the gradient of electrochemical potential of the plasma membrane and possibly also of the tonoplast (Hasegawa, et al., 2000; Munns and Tester, 2008).

When plants were grown in hydroponic cultures under moderate-salt (50 mM NaCl) or high-salt (100 mM NaCl) conditions, salinity tolerance (measured as RTI) was negatively and significantly correlated with Na/K ratio at 50 mM NaCl, but at 100 mM NaCl this relation became inconsistent (Table 3). This result indicates that leaf Na/K ratio could be a reliable indicator of salinity tolerance in rosemary under moderate salinity conditions. Previous studies in different species have supported such a relationship (Kronzucker et al., 2008; Horie et al., 2012; Tanaka et al., 2018). This result is consistent with the previous finding that K homeostasis is crucial for optimal metabolic plant performance (Grattan and Grieve, 1999; Shabala, 2003). When the data for the eight varieties of rosemary were combined, leaf Ca concentration was positively correlated with leaf Na concentration, leaf Na/K ratio, and percentage of leaf necrosis at both salinity levels (Table 4). Under salinity conditions, Ca might be absorbed to play a physiological role such as transport of ions (White and Broadley, 2003) and perhaps the synthesis of carnosic acid (Tounekti et al., 2011b), but metabolic disturbances induced by ionic stress might increase necrotic damage. In addition, compared with the control condition (3 mM NaCl), biomass allocation to the leaf was not significantly altered by either salinity condition in most varieties. At 50 mM NaCl, biomass allocation to the stem was not significantly different in Primley Blue and Lockwood de Forest (the top two tolerant varieties) but was significantly decreased in all others. At 100 mM NaCl, all the varieties had a significant decrease in SMF, compared with the control. Prostratus and Salem showed a significant increase in biomass allocation to the root at 50 mM; but an increase in biomass allocation to the root could be dangerous if the plant does not have a good selectivity of ion uptake.

Tissue tolerance, one of the main protective mechanisms by which plants face salinity conditions, is related to the ability to regulate the salt content in the cytosol (Munns and Tester, 2008; Rajendran et al., 2009; Roy et al., 2014). Some rosemary varieties such as Salem, Arp and Tuscan Blue have relatively high tissue tolerance, as indicated by a low percentage of leaf necrosis with high leaf Na concentration (Fig. 6); however, the significant correlation of Na/K ratio with RTI suggests that tissue tolerance did not contribute substantially to salinity tolerance in rosemary—the excessive amount of Na and/or the imbalance between Na and K in the leaf could induce ionic toxicity, which directly reduced growth. The exact mechanism by which Na exerts its toxicity is not yet known; however, if Na that has entered the plant is not compartmentalized efficiently in the vacuoles, it could be stored in the apoplast and cause dehydration of the cell; if concentrated in the cytoplasm it could inhibit enzymes required for optimal growth; or if it accumulates in the chloroplasts it would directly affect photosynthesis (Munns and Tester, 2008).

Photosynthesis is one of the primary processes affected by drought or salt stress; the effects can be direct, due to limitations in the diffusion of CO_2 through the stomata or the mesophyll, or changes in photosynthetic metabolism; or indirect, such as the oxidative stress (Chaves et al., 2009; Ashraf and Harris, 2013). In the current study, 50 mM NaCl lowered the photosynthetic rate (A) in most rosemary varieties. In the combined data for the eight rosemary varieties, A was positively correlated with stomatal conductance (g_s)

and the antioxidative activities of CAT and APX in leaf, but was negatively correlated with chlorophyll content. This result suggests that A was affected by stomatal limitation of CO_2 diffusion and reduction of chlorophyll content; however, increased activity of the antioxidative enzymes might maintain A. In addition, reduction in g_s might be related to inhibition of transpiration to avoid dehydration of the mesophyll cells (Chaves et al., 2009; Ashraf and Harris, 2013). In a study of rosemary plants stressed by severe drought, the reduced chlorophyll content occurred in association with reduced water content, which contributed to reduction of the amount of photons absorbed by the leaves, increased the xanthophyll cycle, and prevented damage to cell membranes, making recovery possible after autumn rains (Munné-Bosch and Alegre, 2000). In other aromatic herbs, thyme and oregano, decreased chlorophyll content was associated with a decrease in Mg (Tanaka et al., 2018), and in other salt stressed plants, decreased chlorophyll content has been associated with oxidative stress (Said-Al Ahl and Omer, 2011).

At 50 mM NaCl, Primley Blue, the most salinity-tolerant variety, showed reduced A and g_s but stable chlorophyll content and no increased activity of antioxidative enzymes in leaf compared with the control; probably the control in *gs* increased efficiency in water usage might prevent chlorophyll degradation. On the other hand, Officinalis, the most sensitive variety, did not show reduced A, probably due to higher antioxidative activity of CAT and APX, but higher APX activity was associated with a higher percentage of necrosis.

In contrast, when the plants were grown in 100 mM NaCl, A, g_s, photon flux of photosystem II (ETR), and chlorophyll content were impaired in almost all varieties, suggesting severe metabolic disturbance. At this salinity level, A was negatively correlated with GR activity, and higher GR activity was correlated with greater percentage of leaf necrosis, leading to lower salinity tolerance. It is recognized that stressful conditions might increase ROS levels, and that detoxifying mechanisms would then be triggered to protect the cell from oxidative stress (Foyer et al., 1997; Gill and Tuteja, 2010; Caverzan, 2016). The most tolerant variety, Primley Blue, maintained unaltered ETR, H_2O_2, and MDA contents, and antioxidative enzyme activity in leaf; conversely, in Officinalis, the most sensitive variety, ETR was impaired, and SOD and leaf APX activities were increased and CAT activity was reduced in leaf. In the varieties with moderate salinity tolerance, various responses in the characteristics of gas exchange and activity of antioxidative enzymes were observed.

In previous studies, changes in the level of antioxidative response has been related with salinity tolerance. Munns and Tester (2008) suggest that differences between varieties in antioxidative activity might be due to differences in the processes of stomatal closure or CO_2 fixation that occur to avoid photoinhibition in stress conditions.

General summary

Salinity in cultivated land is one of the main problems affecting food security because it reduces crop growth and productivity (Butcher et al., 2016). The stress imposed by salinity on the plant arises because the water is osmotically retained in the saline solution surrounding the roots and becomes less accessible to the plant. Subsequently, an excessive absorption of salt could disturb the uptake of essential nutrients like K, Ca, or Mg, which harms biochemical and physiological processes in the plant. If the osmotic and ionic adverse effects of salt absorption exceed the tolerance level of the plant, oxidative stress occurs (Larcher, 2003). Rosemary (*Rosmarinus officinalis* L.) is a Labiatae aromatic herb widely cultivated in arid and semi-arid regions because of its medical and cosmetic benefits and moderate salinity tolerance. Various characteristics of rosemary have already been evaluated under salinity conditions (Alarcón et al., 2006; Tounekti et al., 2008, Singh et al., 2010; Tounekti et al., 2011; Juárez-Rosete et al., 2013) however, these evaluations were made in a single variety and is not specified, and the mechanisms of tolerance underlying such characteristics have not been investigated. In this study, we aimed to elucidate the salinity tolerance mechanisms in rosemary by comparing the selective absorption and transport of K over Na between rosemary and another Labiatae aromatic herb, thyme (*Thymus vulgaris* L.); and by comparing nutritional and physiological aspects of the tolerance mechanism among eight rosemary varieties under salt stress.

Sodium is the most abundant salt component that competes with nutrients in the absorption by plants; it mainly competes with K, an essential nutrient that is crucial for cell osmo-regulation, stomatal function, and as the activator for many enzymes, and is involved in photosynthesis, protein synthesis, and oxidative metabolism. First we measured the processes that regulate the salt content in the plant—selective absorption and the selective transport of K over Na—in rosemary and thyme plants, to elucidate the responses that can lead to acclimation or tolerance in these species. The results revealed that in low salinity (ECe 3 dS m^{-1}), rosemary and thyme doubled the selective absorption of K over Na by the roots ($SA_{K.Na}$) with respect to the control, but only thyme presented a remarkable increase in the selective transport of K over Na from the roots to the stems [$ST_{K.Na(roots/stems)}$] compared with the control. Under high-salt conditions (ECe 6 dS m^{-1}), rosemary partially suppressed the increase in $SA_{K.Na}$; this might inhibit the $ST_{K.Na\ (root/stem)}$ and $ST_{K.Na\ (stem/leaves)}$, leading to growth reduction. On the other hand, in thyme, the increased $SA_{K.Na}$ under high salinity compared with the non-saline control could help stabilize $ST_{K.Na\ (root/stem)}$ to levels similar to that in the control, but a decrease in $ST_{K.Na\ (stem/leaves)}$ may contribute to a decrease in dry matter due to an ionic imbalance.

Secondly, we evaluated the nutritional status of rosemary under salinity, to determine which nutrient levels became disturbed and could lead to functional damage and growth reduction. Eight rosemary varieties were hydroponically cultivated in moderate (50 mM NaCl) or high (100 mM NaCl) salinity conditions; the control condition was set as 3 mM NaCl. At 50 mM NaCl, the Relative Tolerance Index (RTI; defined as growth relative to the control condition), decreased in the order of Primley Blue >> Lockwood de Forest ≈ Prostratus ≈ Salem ≈ Benenden Blue > Arp ≈ Tuscan Blue > Officinalis. In both salt conditions, the Na/K ratio and occurrence percentage of leaf necrosis were significantly and negatively correlated with RTI in the data for all eight varieties combined. Leaf tissue tolerance, indicated by a low percentage of leaf necrosis with a high leaf Na concentration, differed slightly among varieties; Salem, Arp, and Tuscan Blue showed the highest tissue tolerance, but did not show high RTI, indicating that tissue tolerance did not contribute to the salinity tolerance in this species. At both 50 and 100 mM NaCl, most varieties displayed reduced leaf concentrations of K, Ca, and Mg, but leaf water content was hardly affected by salinity. At 100 mM NaCl, the correlation between the RTI and the Na/K ratio or percentage leaf necrosis became inconsistent. Therefore, rosemary might tolerate salinity by maintaining appropriate levels of nutrients with a low Na/K ratio in leaves prior to water status. Although the transport of some Na to a leaf might improve osmotic adjustment, the excessive transport of Na to leaves creates ionic stress that reduces growth.

Thirdly, physiological aspects, specifically characteristics of gas exchange and the antioxidative response system, were investigated in the same eight rosemary varieties under hydroponic culture to further understand the salinity tolerance mechanisms in rosemary. At 50 mM NaCl, most varieties showed reduced photosynthetic rate (A) and stomatal conductance (g_s); these parameters were positively correlated with each other in the combined data from the eight varieties ($r = 0.531$, $P < 0.01$). These changes might occur to prevent damage to photosystems, adjust osmotic pressure, and make the use of water more efficient. The intercellular concentration of CO_2 (Ci) and electron transport rate (ETR) were maintained stable compared with the control. In addition, in the combined data from the eight varieties, A was correlated with the antioxidative activity of catalase (CAT) ($r = 0.386$, $P < 0.05$) and ascorbate peroxidase (APX) ($r = 0.410$, $P < 0.05$) in leaf, but APX activity was positively correlated with percentage of leaf necrosis ($r = 0.610$, $P < 0.01$). This result might suggest that an increase of CAT and APX antioxidative activities could maintain A, but excessive reactive oxygen species (ROS) led to an increased percentage of leaf necrosis. Superoxide dismutase (SOD) activity in leaf increased in the most salt-sensitive varieties, Tuscan Blue and Officinalis, possibly to scavenge the superoxide radical, suggesting that the most tolerant varieties did not require an increase of SOD activity. In the combined data, total chlorophyll content was negatively correlated with percentage of leaf necrosis ($r = -0.467$, $P < 0.01$), g_s ($r = -0.365$, $P < 0.05$), A ($r = -0.377$, $P < 0.05$), and leaf APX activity ($r = -0.458$, $P < 0.01$).

This result suggests that reduction in the chlorophyll content could be a symptom of dehydration or related to oxidative stress induced by salt. Different physiological responses to salinity stress were triggered at high salinity compared with moderate salinity. At 100 mM NaCl, in the combined dataset, glutathione reductase (GR) activity in leaf was significantly correlated with several physiological parameters, although it was not affected by salinity in any variety except Prostratus; it was negatively correlated with RTI ($r = -0.392$, $P < 0.05$), A ($r = -0.354$, $P < 0.05$), and chlorophyll content ($r = -0.440$, $P < 0.05$), but was positively correlated with percentage of leaf necrosis ($r = 0.516$, $P < 0.01$).

Salinity tolerance mechanisms in rosemary could be explained from the viewpoint of changes in nutritional and physiological characteristics. Selective absorption of K over Na from soil to root is crucial to maintain proper leaf nutritional status. Rosemary absorbs K over Na and holds Na in the root, although some Na absorbed plays a role as an osmolyte in the leaf. The results here suggest that leaf Na/K ratio could be an accurate indicator of salinity tolerance in this species. Antioxidative enzymes such as CAT and APX possibly effectively scavenge reactive oxygen species generated by salinity.

Summary in Japanese

農耕地の塩性化は、作物の生産性を減少させるために、食料安全上大きな問題となっている(Butcherら, 2016)。作物が塩ストレスを受けると、はじめに、根圏の土壌溶液中の水が、高い浸透圧により吸収されにくくなる。そしてそれに引き続き、過剰な塩分が体内に侵入し、K、Ca、Mgのような必須元素の吸収が撹乱され、生理学的反応が悪影響を受ける。これら浸透圧ストレスやイオンストレスが、許容範囲を超えると酸化ストレスが引き起こされる（Larcher, 2013）。ローズマリーは、シソ科のハーブであり、医薬品や化粧品として用いられている。また耐塩性が中程度であることから、乾燥地や半乾燥地で、広く栽培されている。塩性条件下におけるローズマリーの特性は、今までに調査されてきているが、品種が限定されていたことから、耐塩性機構の解明に至っているとは言えない。本研究では、ローズマリーの耐塩性機構を栄養生理学的な観点から明らかにすることを目的とし、そのために同じシソ科のハーブ類の種やローズマリーの8品種間で比較検討を行なった。

はじめに、ローズマリーにおけるNaに対するKの選択吸収能および選択輸送能、および栄養生理学的特性を測定することにより、本種の塩に対する馴化や耐塩性機構を理解することとした。Naは植物が吸収する養分と競合する塩の中

で最も多量に存在する。また、養分の中でも主にKの吸収を阻害する。Kは、植物にとって必須養分であり、細胞の浸透圧調節、気孔の開閉、光合成、タンパク質合成および酸化代謝に関する多くの酵素の活性因子として重要である。そこで、我々は植物体内の塩含量を調節するプロセスに注目し、ローズマリーにおけるNaに対するKの選択吸収能および選択輸送能を調査した。なお、対照植物として同じシソ科に属するタイムを選んだ。低塩区（ECe 3 dS m^{-1}）において、両種のK対Na選択吸収能（SA$_{K.Na}$）は対照区の2倍程に上昇し、タイムのみで根から茎へのK対Na選択輸送能（ST $_{K.Na(roots/stems)}$）が著しく上昇した。高塩区（ECe 6 dS m^{-1}）において、ローズマリーでは、SA$_{K.Na}$が低塩区と比べて低下し、このことが（ST $_{K.Na(roots/stems)}$）の低下につながり、最終的には成長が減退したものと考えた。一方、高塩区のタイムでは、高いSA$_{K.Na}$によりST$_{K.Na\ (root/stem)}$は、対照区の値を維持することが可能であったが、ST$_{K.Na\ (stem/leaves)}$は低下し、イオンインバランスを引き起こし成長は減退したと考えた。

次に、機能障害や成長減退につながり得る塩性条件が、ローズマリーの養分状態に及ぼす影響を評価した。そのために、ローズマリー8品種を中塩区（50 mMNaCl）および高塩区（100 mMNaCl）で水耕栽培した。なお対照区は3mMNaClとした。50 mMNaClにおいて、相対成長（相対耐性指数：RTI）は、Primley Blue >> Lockwood de Forest ≈ Prostratus ≈ Salem ≈ Benenden Blue > Arp ≈

Tuscan Blue > Officinalis の順で減少した。葉の Na/K 比とネクロシス発生率は、8 品種すべてに渡って RTI と有意に負の相関を示した。葉の Na 含有率が高く、ネクロシス発生率が低いことは葉 Na 組織耐性が高いことを意味するが、それは、品種間でわずかに異なっており、 Salem、Arp および Tuscan Blue では高い葉Na組織耐性を有している可能性が示唆された。しかし、本耐性が耐塩性を説明する訳ではなかった。中塩区および高塩区において、ほとんどの品種で、葉の K、Ca および Mg 含有率が低下したが、葉の水分含有率は、塩による影響をほとんど示さなかった。100 mMNaCl では、RTI と葉の Na/K 比あるいはネクロシス発症率との間の相関は、はっきりしなかった。以上のことから、ローズマリーは、水分状態よりも、適切な養分状態と低い Na/K 比を維持することにより、耐塩性を発揮するものと考えた。葉に取り込まれた一部の Na は浸透圧を形成し得るが、 葉に過剰に侵入した Na は成長減退を引き起こすイオンストレスを発生させた。

最後に、ローズマリーの耐塩性機構を解明するために、生理学的な面としてガス交換および抗酸化応答システムを調査した。50mMNaCl では、ほとんどの品種で、光合成速度（A）および気孔コンダクタンスが低下し、両者の間には、正の相関関係（r=0.531, $P < 0.01$）が認められた。このことは、光化学システム

への悪影響を防ぎ、浸透圧を調節し、また水利用効率を高めるためであった可能性がある。50mMNaCl では、葉内 CO_2 濃度（*Ci*）および電子伝達速度（ETR）は、対照区の値を維持した。さらに、*A* は抗酸化酵素 CAT（catalase）および APX（ascorbate peroxidase）の活性と正の相関関係（順に r=0.386*、(*r*=0.410, $P < 0.05$）を示したが、APX は葉のネクロシス発症率とも正の相関関係を示した（*r*=0.610, $P < 0.01$）。このことから、抗酸化酵素活性の上昇により A を維持することができたが、過剰な活性酸素種（ROS）によりネクロシス発症率が高まった可能性が考えられた。また、SOD 活性は最も塩感受性品種である Tuscan Blue および Officinalis で、上昇した。これは、おそらくスーパーオキシド・ラジカルの消去のためであったと考えられる。このことから、耐塩性品種では SOD 活性の上昇は、必要がなかったものと思われた。さらに全クロロフィル含有率は、葉のクロロシス発症率、*gs*、*A* そして APX 活性との間に負の相関関係（順に *r*=-0.467, $P < 0.01$、*r*=-0.365, $P < 0.05$、*r*=-0.377, $P < 0.05$、*r*=-0.458, $P < 0.01$）を示した。このことから、クロロフィル含有率の低下は、塩による脱水現象や酸化ストレスの兆候である可能性がある。100mMNaCl では、50mMNaCl とは異なる生理応答が示された。すなわち、全ての品種において、GR 活性はいくつかの生理的パラメーターと有意な相関関係を示すものの、Lockwood de

Forest を除いては、塩ストレスの影響を受けなかった。すなわち GR 活性は、RTI、A およびクロロフィル含有率との間に負の相関関係（それぞれ順に r=-0.392, $P < 0.05$、r=-0.354, $P < 0.05$、r=-0.440, $P < 0.05$）を示したが、クロロシス発症率との間には、正の相関関係（r=0.516, $P < 0.01$）を示した。

ローズマリーの耐塩性機構を栄養生理学的な面から明らかにした。土壌から根への K 対 Na 選択吸収能は、葉の適切な養分状態を保つために、極めて重要である。ローズマリーは、Na よりも K を多量に吸収し、Na を根に集中的に蓄積する。なお葉に取り込まれたNaの一部は、浸透圧調節の役割をもつと考えられる。葉における Na/K 比は、正確な耐塩性の指標になり得るといえる。CAT や APX のような抗酸化酵素は、活性酸素種を効率的に消去し得る。

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