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BASIL IONIC RESPONSES TO SEAWATER STRESS AND THE IDENTIFICATION OF GLAND SALT SECRETION

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ABSTRACT

Basil (*Ocimum Basilicum*) seedlings were subjected to 0,5%,10%,20% and 40% seawater for 10 days under shade house conditions. Increasing seawater concentration led to considerable reduction in plant dry matter accumulation. However, root growth was not affected till treated with 40% seawater. Accumulations of Na^+ and Cl^- were correlated with decline of Ca^{2+} and K^+ in stems, roots or both. Contents of Ca^{2+} and K^+ in leaves increased or remained relatively unchanged with increasing salt levels. In salinity-stressed plants, Na^+ were partitioned mainly in roots and more than 50% of the K^+ and Ca^{2+} were stored in leaves. Higher K^+/Na^+ and Ca^{2+}/Na^+ ratios in leaves rather than in roots and stems were observed in Basil plants. Scanning electron microscopy coupled with X-ray microanalysis showed that Basil leaf gland secretions contained Na^+ and Ca^{2+}. The Na^+ secretion increased while Ca^{2+} secretion decreased with the increasing seawater concentration. These results suggested that Basil salt tolerance is associated with the compartmentation of Na^+ in roots and the balance of K^+, Na^+ and Ca^{2+} in leaves as well as the salt secretion by the leaf glands.

Key words: *Ocimumbasilicum* L.; Basil; Ion balance; Salt secretion; Seawater stress; X-ray microanalysis

INTRODUCTION

Salinity toxicity is a worldwide agricultural and eco-environmentalproblem. The negative effects of salinity on plant growth and metabolic processes are mainly associated with ion toxicity and nutrition deficiency (Seday *et al*., 2014), which could be caused bya competition of Na^+ and $Cl^-$with inorganic ions such as K^+, Ca^{2+}, Mg^{2+},$NO_3^-$and$H_2PO_4^-$(Parida and Das, 2005).To adapt to saline conditions, plants reduce the accumulation of Na^+ and Cl^- through varying root permeability to different ions and enhance the selectivity of salt ion absorption (Neocleous and Vasilakakis, 2007). The maintenance of K^+ and Na^+ homeostasis in plant tissues is also involved, which plays a key role in the intracellular metabolism processes (Zheng *et al*., 2009). Additionally, some salt-tolerant species such as *zoysiagrass* (Marcum *et al*., 1998), *Atriplex* spp (Khan *et al*., 2000) and *Limonium bicolo*r (Ding *et al*., 2010) have been shown to acclimate the saline conditions by secreting excessive salt ions through salt-secreting structures, e.g. salt glands or salt bladders. Salt glands excrete salts directly through pores in the surface of cuticle (Oi*etal*., 2014). Whereas the salt bladders accumulate salt ions in their vacuoles and eventually the cells rupture releasing the salts to the surrounding environment (Fahn, 1988).

In fact, the secretory behaviour is not only found in halophytes, but also in many flowering glycophytes, which bear various types of glandular trichomes to secrete specific substances, such as polysaccharides, organic acids, terpenes, nectar, essential oils or nitrogenous compounds (McCaskill and Croteau, 1999). Basil (*Ocimum Basilicum* L.), belonging to the Lamiaceae, is a major essential oil producing glycophyte widely cultivated around the world for its important commercial and medicinal purposes (Attia *et al*., 2009). As in most Lamiaceae species, two type of glandular trichome, peltate and capitate, are distributed on surface of the Basil leaves and stems to secrete essential oils and other flavor compounds (Klimánková *et al.*, 2008). In peltate glandular trichomes, the compounds to be secreted are generally accumulated in a capacious subcuticular space and then they are released as the cutile is physically ruptured (Siebert, 2004), a similar secreting fashion to that of the salt bladders in some halophytes (Fahn, 1988). In contrast, the capitate glandular trichomes secrete compounds apparently through pores in the cuticle of the head cell(s) (Siebert, 2004), which is also observed in the salt secreting process of salt glands (Oi *et al*., 2014). In the last few decades, much research has been carried out on Basil with its capacity to produce flavors and essential oils and chemical compositions of the secreted compounds (Klimánková *et al*., 2008). Recently, several studies with this species showed that the production of essential oils was stimulated under moderate salinity, which could be due to a higher oil gland density and an increase in the absolute number of glands produced (Bernstein *et al.*, 2010; Tarchoune *et al*., 2013). Till now, studies in relation to Basil salt tolerance are relatively scarce. Especially, the capability of gland salt secretion and the impact of this secretory behavior on Basil salt adaptation has not yet been reported.

It is hypothesized that Basil will secrete a certain amount of salt ions mixed with organic substances through the glandular trichomes in a similar fashion to other halophytes possessing excretory structures. Thus, the aim of this study was to investigate the response of growth and ion uptake and distribution of Basil under seawater stress, to determine the elemental composition of gland secretions by means of scanning electron microscopy X-ray microanalysis and to ascertain if the Basil glands are able to demonstrate some salt secretion.

MATERIALS AND METHODS

Plant material and growth conditions: Basil (*Ocimumbasilicum* L.) seeds were sown into plastic pots containing 2.5 kg washed sand. The experiment was conducted in the shade house supplied with natural sunlight in the Institute of Agricultural Resources and Environment, Guangdong Academy of Agricultural Sciences(23°08′46″ N and 113°20′49″ E). After emergence, the seedlings were watered daily with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Seawater treatments began when Basil plants were 35-day old. Seawater was prepared by dissolving 33.33 g of sea salt(Multispecies Salt company, Guangzhou, China) in 1L deionized water, according to the manufacturer's instructions. The major ions of sea water were: 525.3 mM Na^+, 0.21 M K^+, 0.83 mM Mg^{2+}, 1.33 mMCa^{2+}, 560.4 mMCl^-, 0.51 mM SO_4^{2-} plus other less concentrated macro- and micro-nutrients. Four seawater treatments (5%, 10%, 20% and 40% seawater) were obtained by dissolving the corresponding sea salt in half-strength Hoagland's solution. The half-strength Hoagland's solution without additional seawater served as control. Basil plants were subjected to different seawater treatments for 10 days. During the plant culture and irrigation, care was taken to avoid accidental nutrient solution or salt projection and deposition. The five treatments were arranged in individual pots with three plants per treatment and twelve replicates each. The experiment was repeated three times under the same conditions.

Growth parameters and ions analysis: After Basil harvest, nine plants from each treatment were divided into separate root, stem and leaf fractions and weighed. The samples were oven-dried in paper bags at 70° to constant weight to determine dry weights. For inorganic-ion analysis, a 50 mg sample of root, stem or shoot was ashed in a muffle furnace. The ash was dissolvedin concentrated nitric acid and diluted to 100mL with distilled water (Zheng *et al*., 2009). The concentrations of K^+ and Na^+ were determined using a digital flame photometer (Cole-Parmer Instrument Company Model 2655-00, Chicago), while Ca^{2+} concentration was measured using an atomic-absorption spectrometer (HitachiZ-5000, Japan).The content of $Cl^-$was assayed using potentio metric titration with 0.01 mM $AgNO_3$.

Scanning electron microscopy observation and X-ray microanalysis on Basil leaf glands secretions: Fully expanded leaves (the third pair of leaves on the main stem from top) were collected for samples of scanning electron microscopy (SEM) observation and X-ray microanalysis. Six leaves consisted of three replications in each treatment were prepared according to the method described by Lu *et al*. (1995). Basil fresh leaves were cut into 1.0×0.5 cm pieces, air dried and fixed for 24 h with 2.5% glutaraldehyde at room temperature. The materials were then washed with 0.1 M phosphate buffer solution, fixed 1.5 h with 1% OsO_4 and washed again with distilled water, before being dehydrated in alcohol series concentrations (30, 50, 70, 80, 90% alcohol for 15 minutes and in 100% alcohol for 30 minutes). After infiltration for 30 minutes with tert-Butyl alcohol, leaf samples were subsequently freeze-dried in a JFD-310 freeze-drier (JEOL Ltd., Japan). Thereafter the samples were attached to SEM mounts, sputter coated with gold. Three SEM samples selected from three different replications in each treatment were performed X-ray microanalysis by using Hitachi S-3700N SEM equipped with a Brucker EDS X-ray detector (accelerating voltage, 20 kV). Seventeen elements including C, N, O, Na, Mg, Al, Si, P, S, Cl, K, Ca, Mn, Fe, Cu, Zn and Rb were determined and the results were expressed as the relative weight percentage.

Statistical analysis: A one-way analysis of variance (ANOVA) was applied to examine the seawater effects on each parameter using the SAS9.2 statistical software package. Means were compared by Fisher's least-significant difference test (LSD) at $P<0.05$.

RESULTS

Growth of Basil under seawater stress: All the plants remained alive at the end of seawater treatments, although shoot growth of Basil was strongly reduced. In plant treated with 40% seawater, shoot growth reduction was about 67%compared to the control (Table 1). Incontrast, root growth was not affected until40% seawater level (62% of control). Shoot growth was inhibited more significantly by seawater than that of roots. This conclusion was also supported by the change in root/shoot ratio, which increased with the increasing seawater stress, although there were no statistically significant differences among the salt treatments (Table 1).

Ion distribution and balance: Both Na^+ and Cl^- increased significantly in roots, stems and leaves with increasing seawater level (Fig.1). The Cl^- content in roots, stems and leaves treated by 40% seawater was 19.1, 24.5 and 25.2 times higher than that of the control plants. The highest seawater level (40% seawater) led to a 10-fold increase in Na^+ concentration in both roots and stems while a lower increase (2.6-fold) was observed in the leaves. In salinity-stressed plants, the Na^+ accumulated mainly in roots which accounted for 57-65% of the total, whereas only 8-11% of Na^+ was partitioned in leaves. Seawater stress decreased the K^+ content significantly both in roots and stems, whereas K^+ content in leaves increased markedly or remained unchanged. The Ca^{2+} content in stems decreased while it remained stable in roots and leaves under salinity. The higher K^+ and Ca^{2+} accumulations were observed in leaves than those in roots and stems as treated by seawater, i.e., 51-58% of the K^+ content, 56-62% of the Ca^{2+} content in leaves, respectively.

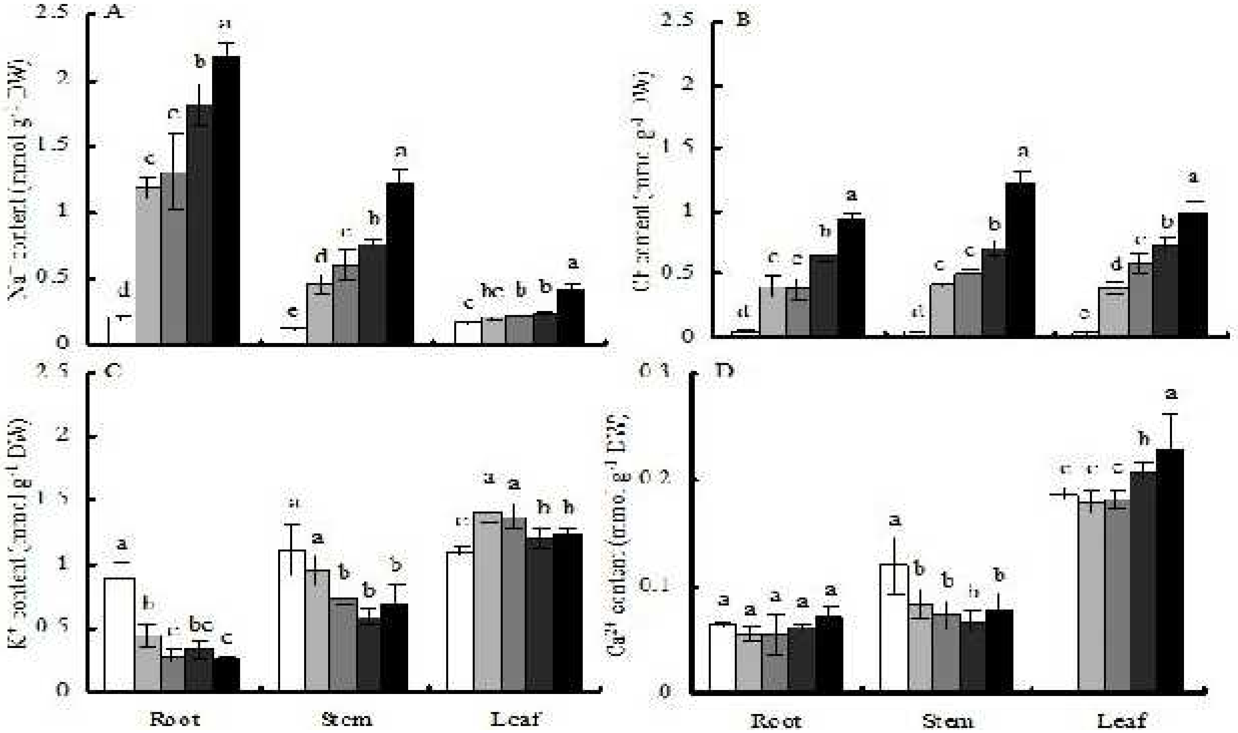
The ratio of K^+/Na^+ and Ca^{2+}/Na^+ in Basil roots, stems and leaves all decreased significantly under seawater treatments (Fig.2). However, Basil plants maintained considerably higher K^+/Na^+ and Ca^{2+}/Na^+ ratios in leaves than those in roots and stems under salinity. Particularly, the K^+/Na^+ ratios in leaves of stressed-plants ranged from 3.3 to 6.9.

Leaf gland secretions: A typical EDS X-ray spectrum of the capitate glandsecretions revealed prominent characteristic C, N, O, Na, P, and Ca X-ray emission peaks, with the corresponding weight percentage of 13.3%, 1.4%, 55.0%, 4.8%, 10.2% and 12.6%, respectively (Fig. 3). The weight percentages of other elements such as Mg, Al, Si, S, Cl, K, Mn, Fe, Cu, Zn, Rb were all less than 1% (Fig. 3). Predominant elements in the secretions were C and O, which accounted for about 70% of the total relative weight (Table 2). Two anionic elements P and N were present in the secretions, ranging from 1.5% to 3.9% and 6.9% to 10.7%, respectively (Table 2). Two cationic elements Na^+ and Ca^{2+} were detected in the secretions (Table 2). The relative weight of Na^+ increased with increasing salinity and the significant differences were also observed as seawater concentration exceeded 10%. As expected, the Na^+ content increased by 80% and 94% as treated with 20% and 40% seawater. Conversely, the Ca^{2+} content in gland secretions showed a decreasing trend as the salinity level increased and the reduction ranged from 20% to 58% under salinity.

Table 1. Effects of seawater stress on dry matter and root/shoot ratio of Basilplant

Seawater concentration (%)	Dry weight (gplant^{-1})		
	Root	Shoot	Root/shoot
Control	0.34 ±0.05 a	2.85 ±0.58 a	0.13 ± 0.04 b
5	0.37 ±0.11 a	1.84 ± 0.43 b	0.21 ±0.08 a
10	0.33 ±0.09 a	1.71 ± 0.38 b	0.20 ±0.06 a
20	0.36 ±0.12 a	1.70 ± 0.69 b	0.22 ±0.04 a
40	0.21 ± 0.06 b	0.94 ±0.20 c	0.23 ±0.09 a

[a]The data in the table represent means ± SD of three experiments.Data followed by different letters in the same column are statistically differentaccording to Fisher's least-significant difference test ($P<0.05$). The same as follows.



Bars with different letters are significantly different at $P<0.05$. The same as follows.

Fig. 1.Effects of seawater stress on Na^+ (A), Cl^- (B), K^+ (C) and Ca^{2+} (D) in roots, stems and leaves of Basil plants

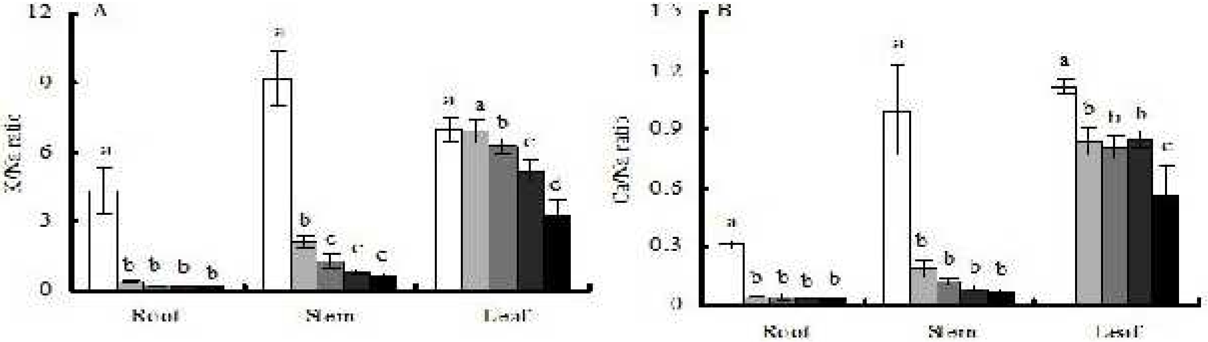


Fig.2. Effects of seawater stress on K^+/Na^+ (A) and Ca^{2+}/Na^+ (B) ratios in roots, stems and leaves of Basilplants

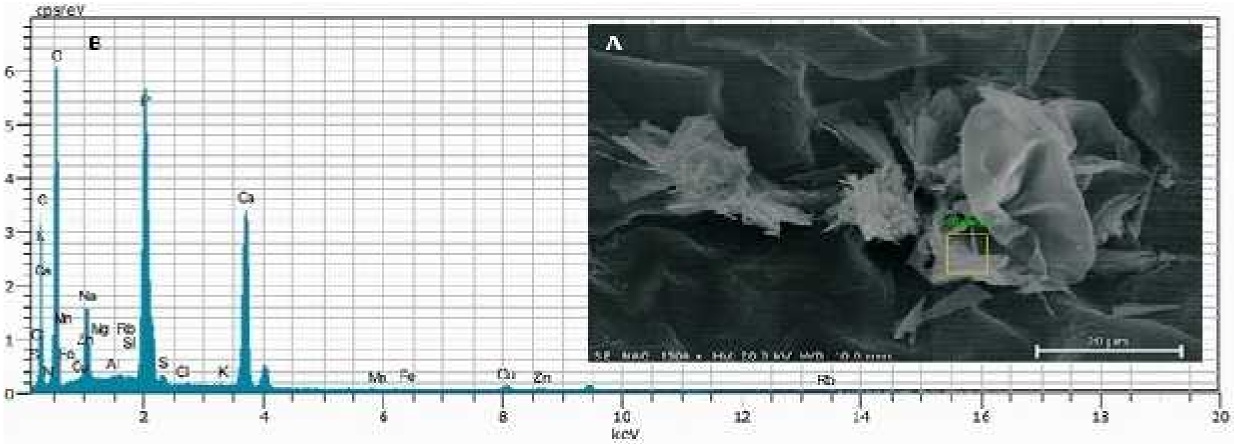


Fig.3. Capitate gland with secreted materials on adaxial leaf side of Basil plants treated by 40% seawater (A, magnification, 1500×,bar, 20µm) and the EDS spectrum (B) was taken from the area indicated by the square

Table 2.Relative percentage elemental composition of gland secretions on both sides of Basil leaf surface under seawater treatments

Elements	Seawater concentration (%)				
	Control	5	10	20	40
C	18.2±8.7 ab	21.3 ±9.8ab	24.8±9.4 a	15.0± 7.0 b	20.9±7.2ab
N	2.0 ± 0.8 ab	2.6 ± 2.0ab	1.5 ±0.6 b	1.8 ±0.5ab	3.9 ±3.3 a
O	55.1± 3.0ab	53.9±1.3ab	52.6±4.5 b	56.2 ±5.0 a	52.2 ±2.1 b
Na	4.9 ±2.1c	5.3 ±1.7 c	6.6 ±3.3 bc	8.8 ±3.7 ab	9.5 ±2.0 a
P	10.1±2.1 a	10.6±2.6 a	9.4 ±2.8 ab	10.7±2.8 a	6.9±3.3 b
Ca	10.6 ±4.3 a	8.5 ± 5.2ab	4.4 ± 4.1 b	5.4 ±4.9 b	4.9 ±3.4 b

DISCUSSION

Responses of plant growth to seawater stress: Salinity depressed the plant's capability of utilizing water and resulted reduction in plant growth rate, as well as changes in many metabolic processes (Munns, 2002). Basil has been identified as a salt tolerant glycophyte in previous study (Attia *et al.*, 2009). In present investigation, Basil shoot weight decreased significantly under seawater stress, whereas the root, the first injury site of salt stress, was not affected until the highest salinity (40% seawater) (Table 1). These results indicated that shoot growth was more inhibited by seawater than that of root. Similar responses were also found in tomato (Maggio *et al.*, 2007), cowpea (Murillo-Amador *et al.*, 2006) and prosopis (Meloni *et al.*, 2004). Thus, the increased root/shoot ratio in salt-stressed plants appeared to be an acclimation to salinity, resulting in a more efficient water and nutrient absorption under saline conditions (Maggio *et al.*, 2007).

Responses of ion distribution and balance to seawater stress: A major consequence of salt stress is salinity-induced nutritional disorders. To alleviate the ionic toxicities induced by Na$^+$, plants tend to restrict root Na$^+$ absorption and transportation, thus retain low level of Na$^+$in the leaves (Imada *et al*., 2009). Results in this study showed remarkable increase in Na$^+$ and Cl$^-$ in salt stressed plants with increasing salinity level (Fig. 1 A, B). However, it was found that Basil exhibited a mechanism for salt tolerance based on partitioning of most of the Na$^+$ (57-65% of the total) in the roots, thereby maintaining low level of Na$^+$ in the leaves (8-11% of the total) (Fig. 1A). As compared with Na$^+$, Cl$^-$ uptake and transport appeared to be less controlled in some plant species such as cucumber (Colla*et al*., 2012), *Bruguieraparviflora* (Parida *et al*.,2004) and sugar beet (Ghoulam *et al*., 2002). Similar phenomenon was also found in the current study where Cl$^-$ concentration in the shoot especially in the leaves was higher than that of Na$^+$ (Fig. 1 A, B). The incapability of Basil to restrict Cl$^-$ shoot uptake might be linked to the mechanisms related with NaCl toxicity. Excessive salt (e.g. Na$^+$ and Cl$^-$) uptake competes with the uptake of other nutrient ions and produce extreme ratios of Na$^+$/Ca^{2+}, Na$^+$/K$^+$, Cl$^-$/NO$_3^-$ (Grattan and Grieve, 1999).In common with previous studies concerning other plant species (Hakim *et al*., 2014; Colla *et al*., 2012; Ghoulam *et al*., 2002), tissue concentrations of K$^+$ and Ca^{2+} were generally reduced in salt-stressed plant. Present study showed that seawater stress caused significant decrease in K$^+$ and Ca^{2+} in roots or stems (Fig.1 C, D). Interestingly, the foliar concentration of two cations remained stable or increased under salinity (Fig.1 C, D). A similar response in foliar K$^+$ or Ca^{2+} was also found in *Salvadorapersica* (Ramoliya*et al*., 2004), *Cassia montana*(Patel and Pandey, 2007) and strawberry (Korona) (Keutgen and Pawelzik, 2009). In contrast with Na$^+$, most of the K$^+$ (51-58% of the total) and Ca^{2+} (56-62% of the total) were distributed in the leaves (Fig. 1 C, D) under elevated seawater level, suggesting a more efficient K$^+$, Ca^{2+} uptake in Basil compared to other plants. According to Ramoliya *et al.* (2004), the accumulation of K$^+$in the shoot and decrease of K$^+$ in the root in salt-stressed plant, may attribute to: (i) transfer of K$^+$ from roots to leaves, (ii) exchange of K$^+$ with Na$^+$ in root tissues, or (iii) Na$^+$ interferes uptake of K$^+$. Moreover, the predominance of Ca^{2+} in leaves under salt conditionsmight be due to its preferential absorption andtranslocation *via* xylem and it being immobile in phloem is trappedin the leaves (Bhivare and Nimbalkar, 1984). It has been well established that K$^+$ and Ca^{2+} play important role in many key physiological metabolisms in plant cells (Grattan and Grieve, 1999). The maintenance or accumulation of two cations in salt-affected Basil leaves may represent plant adaptation to salinity.

Not only K$^+$ and Ca^{2+} content, but also a suitable K$^+$/Na$^+$ or Ca^{2+}/Na$^+$ ratio is necessary for salt tolerance in many plants (Parida and Das, 2005; Hakim *et al*., 2014). In current study, we have observed that though the leaves of salt stressed plants contained greater quantities of K$^+$ and Ca^{2+}, it failed to overcome salinity detriment due to the disturbed mineral homeostasis as observed from the decrement in K$^+$/Na$^+$ (Fig.2 A) and Ca^{2+}/Na$^+$ ratio (Fig.2 B). This may be a major cause of salinity toxicity occurred in Basil plant. However, Basil was characterized by a higher K$^+$/Na$^+$ and Ca^{2+}/Na$^+$ ratio in leaves in comparison with those in roots and stems (Fig. 2). Particularly, the foliar K^+/Na^+ ratio in salt-stressed plants ranged from 3.3 to 6.9 (Fig. 2 A), which was far greater than 1, a minimum value suggested for optimal efficiency of protein synthesis and normal growth of glycophyte plants under saline conditions (Imada*et al*., 2009). The higher Ca^{2+}/Na^+ ratio in the leaves than in roots and stems (Fig. 2 B) may contribute to maintenance of high foliar K^+/Na^+ ratio. This is because Ca^{2+}plays an important role in the selective transport and exclusion of Na^+ by plant cellmembranes (Imada*et al*., 2009).

Composition of Basil gland secretions and the identification of its salt secretion: It has been reported that glandular trichomes play additional or alternative roles in the detoxification of toxic chemicals (e.g. heavy metals and salts) and in response to various other stress conditions (McCaskill and Croteau, 1999; Choi *et al*., 2004). As noted above, the flowering glycophyte plants generally secrete various organic compounds through glandular trichome, while some halophytes mainly secrete inorganic ions through their salt glands or salt bladders (Khan *et al*., 2000; Marcum *et al*., 1998; Ding *et al*., 2010). Being an aromatic glycophyte, Basil secretes lipophilic and polysaccharidic substances composed by C, H and O elements (Klimánková *et al*., 2008).In present study, it was found that Basil gland secretions were predominately C and O, with a certain amount of P, N, Na and Ca (Fig.3), which may be organic substances mixed a variety of ions. As might be expected, salt ions especially Na^+ and Ca^{2+} were identified in the gland secretions (Fig.3). Similarly, other plant species generally assumed to be glycophytes such as *Hermanniaicana* Cav. (Appidi *et al*., 2008), *Solanumpseudocapsicum* (Aliero *et al*., 2006) and tobacco (Choi *et al*., 2004) were found to secrete substances containing inorganic ions. Calcium is generally found in plant tissues in the formation of insoluble calcium oxalate (Webb *et al*., 1995). Some early studies have indicated the presence of calcium in certain plant trichomes, such as in the organic acid-secreting trichomes of chickpea, in trichomes of *Centaureascabiosa* and *Leontodonhispidus* (De Sikva *et al*., 1996). According to Choi *et al*. (2004), the detection of Ca^{2+} in Basil gland secretions indicated that Ca^{2+} played an important function in salt detoxification of basil plant. The present study showed that Na^+ percentage in the secretions increased while Ca^{2+} percentage decreased with increasing seawater levels (Table 2).The results agree with data of chemical analysis on the Na^+ content in Basil leaves showed in Fig.1 A. However, the change of Ca^{2+} content differed from the data of chemical analysis on leaves showed in Fig.1 C, which indicated that Basil Ca^{2+} secretion may be inhibited with increasing salt levels. The detection of mineral elements especially Na^+ and Ca^{2+}in the secretions supports our hypothesis that Basil possesses the salt secretion capabilities. To our knowledge, this is the first time to report the salt secretion in Basil. The role of gland salt secretion, probably contributes to the maintenance of lower Na^+ level and the regulation of salt balance in Basilleaves.

In conclusion, results obtained in present study revealed that salt tolerance of Basilis associated with low accumulation of Na^+ but high uptake of K^+ and Ca^{2+}, and maintenance of high K^+/Na^+ and Ca^{2+}/Na^+ ratios in the leaves. Moreover, our investigations disclosed that secretions from Basil leaf glands contained various inorganic ions and organic substances. The Na^+ and Ca^{2+} were presented in a certain amount in secreted materials, which supported our original hypothesis that Basil gland has the capability of salt secretion. The results indicated that salt secretion might be involved as an efficient additional mechanism of salt tolerance in Basil. However, further investigations are required to reveal the mechanisms of gland Na^+ and Ca^{2+} secretions as well as the impacts of salt secretion on essential oil production in this species.

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