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Promotive Effect of 5-Aminolevunlinic Acid on Seed Germination of Pak Choi (*Brassica campestris* ssp. *Chinensis*) under Salt Conditions

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Abstract: Salinity is one of the most important factors affecting agricultural land in the world. Pakchoi yield declines with an increase in salinity, but the sensitivity to salts varies with salt and pakchoi seedling growth. The aim of this study was to determine the effect of 5-aminolevunlinic acid (ALA) under stress condition on germination and seedling growth, respiration and protoheme of seven pakchoi cultivars (Sa-1, Li-1, Ai-1, Qi-1, Ak-1, Ha-1, Sl-1). The experiments were undertaken with distilled water with 200mmol/l salt (NaCl alone and with five ALA) in three replicates. Five ALA concentrations ranging from 10 to 50mg/L were applied to each cultivar in experiments. Statistical analysis revealed that ALA under salt condition significantly affected germination and seedling growth. The germination response of the seven cultivars was considerable different in the germination between salt tolerant and salt sensitive cultivars. Fresh and dry weight of radicels was increased by increased ALA concentration at 30 mg/L ALA under salt condition. Cotyledons fresh and dry weight was comparatively more affected by ALA treatment than radicels and hypocotyls. These results shows that salt stress may inhibit the biosynthesis of endogenous ALA and then heme, biosynthesis ALA is necessary for seed germination and seedling growth and effect of exogenous ALA prior to germination, may be associated with the biosynthesis of heme.

Key words: Pakchoi · 5-aminolevunlinic acid · Seed germination · Salt stress

INTRODUCTION

Earth is a salty planet, with most of its water containing about 30 g of sodium chloride per liter. This salt solution has affected and continues to affect, the land on which crops are, or might be, grown. Although the amount of salt affected land (about 9003106 ha) is imprecisely known, its extent is sufficient to pose a threat to agriculture [1,2] since most plants and certainly most crop plants, will not grow in high concentrations of salt: only halophytes (by definition) grow in concentrations of sodium chloride higher than about 400 mM. Salinity affect plant growth at all stages of development however sensitivity varies from one growth stage to another [3]. Soil salinity is known to suppress the growth of most crop species [4]. Salinity also reduces imbibition of water because of lowered osmotic potentials of the medium and causes changes in metabolic activity [5].

Pakchoi (*Brassica campestris* ssp. *chinensis*) originated from China. There are a considerable number of varieties in China. Pakchoi is a glycophyte crop [6]. Transgenic techniques or traditional methods have been using to displaying some cultivars a greater degree of salt tolerance [7-10]. Low concentration (100 ppm) of ALA increased salt tolerance of young cotton seedlings [11]. ALA could significantly increase the cotton growth of plants treated with 1.5% NaCl. They compared 12 growth substances. Dry weight of control and treated with 300mg/L ALA under salt stress condition, were almost same [12]. ALA had potential to increase the crop growth in saline soils [13]. Recently, we reported that exogenous ALA promoted the chlorophyll content, antioxidative enzyme and photosynthesis rate of pakchoi seedlings under conditions of high temperature [14].

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5-aminolevulinic acid is a precursor of tetrapyrrole compounds such as chlorophyll, phycobilin, heme and vitamin B12 which are found in plants. ALA has attracted attention as a biodegradable herbicide which is not harmful to humans or animals [15].

However, very few works for the promotion by ALA in seed germination and seedling growth of pakchoi under salt stress have been reported. Wang *et al.* [6] suggested that pakchoi is rather salt tolerant and exogenous 5-aminolevulinic acid could promote seed germination of the vegetable. In this experiment, we demonstrated more evidence to show its salt tolerance and tempt to elucidate the mechanism of ALA promotion on plant salt tolerance.

MATERIALS AND METHODS

Plant Material: Seven cultivar of *Brassica campestris* ssp, *chininses* [i-e Sanchidaye (Sa-1), Lichuandasuomian (Li-1), Aijiaohuang (Ai-1), Qingyou 4 (Qi-1), Aikang 5 (Ak-1), Hanxiao (Ha-1), Shu lv (Sl-1)] were used in this study. Seeds were obtained from the Jiangpu experimental farm of Nanjing Agricultural University located at Nanjing, Jiangsu, China.

Seed Germination: For seed germination, different concentrations of ALA with NaCl (Control-Distilled water, T_1-200mmol/L NaCl, T_2- 200mmol/L NaCl+ 10mg/L ALA, T_3- 200mmol/L NaCl + 20mg/L ALA, T_4- 200mmol/L NaCl + 30mg/L ALA, T_5- 200mmol/L NaCl + 40mg/L ALA, T_6- 200mmol/L NaCl + 50mg/L ALA) were used. Two filter papers were put into Petri dishes (90mm). Ten milliliters solution was aliquoted into Petri dishes and 40 seeds were placed on the filter papers in each Petri dish. The Petri dishes were placed in a dark growth chamber at 25°C for 3 days and, then, etiolated seedlings were placed into a growth chamber with a temperature of 25°C under lighted condition with 12 h photoperiod of 60 μmol m^{-2} s^{-1} for 3 days. Each treatment consisted of 40-seeds per Petri dishes and was replicated 3 times. After 3 days of culturing, seedlings were removed from the Petri dishes and cotyledons, carefully divided into cotyledons, hypocotyls and radicels. To investigate the effects of ALA on length of radicels, fresh and dry weights of radical, hypocotyls and cotyledons under salt condition. Dry samples were obtained after drying at 65°C for 24 h.

Method of Protoheme

Extraction of Protoheme: The extraction of protoheme was carried out according to the methods of [16]. The seeds were planted into 90mm Petri dishes; in the dark chamber at 25°C for 6 days. The etiolated cotyledon (1g) was ground in a solution containing 90% acetone and 10% (v/v) of 0.1mol/L NH_3 Centrifuged the homogenate for 15 min (4000rpm), supernatant was discarded and the precipitate was washed twice with 20 ml of the 80% acetone. The final precipitate was then extracted two times with 10-15 ml acetone containing 2% HCl. The second 10-20 ml acetone-HCl extraction supernatant was added to the first and then 15-20 ml peroxide- free diethyl ether was added to the combined acetone extracts. Hundred milliliter-distilled water was added after mixing and the solution was thoroughly mixed. The protoheme was transferred to the ether phase. The ether extract was evaporated to make a final 1-2 ml in darkness at 22°C and the precipitate was transferred to a 10 ml volumetric flask, 2.5ml of pyridine and 1-2 ml of distilled water was added into the volumetric flask and then makes 10 ml of volume. For spectrophotometric analysis, $K_3Fe(CN)_6$ (5mg) was added into the reference tube sample and $Na_2S_2O_4$ (5mg) was added into the sample tube. The changes in absorbance obtained between the 557 nm peak and 541 nm trough was used to calculate the protoheme concentration, using a millimolar extinction coefficient of 20.7 [17].

Determination of Seed Respiration: For seed respiration, 20 seeds were kept in the 90mm Petri dishes. Ten milliliters salt solution (NaCl and NaCl with ALA) was aliquoted into Petri dishes and were kept in a dark chamber at 25°C for 24 h and Petri dishes were then removed from the dark chamber. Seeds were placed between two layers of wet guaze and then put into the cuvette of LI-6400 portable photosynthesis system (USA). The photosynthesis system was measured as net photosynthetic rate or respiration rate according to changes in the CO_2 concentration detected by an infrared device. The temperature of the cuvette was regulated automatically and absolute value of photosynthetic rate was used to represent seed respiration. Experiment was repeated three times.

Data Collection and Statistical Analysis: The percentage of seed germination, radicels length, fresh and dry weight of cotyledons, hypocotyls and radicels were monitored. Data from three independent experiments replicates 3 times were subjected to ANOVA (2002 by SAS Institute Inc., Cary, NC, USA 2002 Version 9.00). Means were separated by Duncan's Multiple Range Test.

RESULTS

Effect of ALA on Seed Germination and Seedling Growth under Salt Condition: Seeds were germinated in salt (50-250mmol/l NaCl) solution. Seedlings tolerated up to 200mmol/l NaCl solution. Germination percentage of seedlings in 250mmol/l NaCl solution was very low. Only two cultivars (Ai-1 and Qi-1) responded out of fifteen cultivars in highest concentration of salt solution (250mmol/l NaCl) (unpublished data). Figure 1 shows the seed germination increased with the increasing of ALA concentration. Except cultivar 'Li-1' all cultivars were affected by ALA. 'Ai-1' responded highest germination (98%) in T_6 (200mmol/L + 50mg/L ALA) (Fig. 1) and highest germination among all cultivars. 'Qi-1' and 'Ha-1' cultivars germination percentage were decreased in T_6 as compared to T_5. The data (Fig. 1) shows significant differences of seed germination among the cultivars. Cultivar 'Sa-1' responded low germination in all treatments. The germination response of the seven cultivars was considerable different between salt tolerant and salt sensitive cultivars.

The studies were carried out to observe the influence of ALA on seedling growth of germinating seeds of pakchoi cultivars. At the highest level of ALA (40-50mg/L) concentrations, radicel length did not increase as compare to low ALA (10-30 mg/L) concentration under salt stress (Table 1). Cultivars "Sl-1" had the longest radical length (5.2 cm/p) in moderate ALA concentration (200mmol + 30mg/L ALA) as compare to control (Table 1). No seedling growth was observed in cultivar 'Li-1'. The result indicated that fresh and dry weight of radicels was increased with the increasing of ALA concentration at 30 mg/L ALA under salt condition (Table 2). Radicels weight were not increased at the highest concentration of ALA (40mg/L ALA + 200mmol NaCl and 50mg/L ALA + 200mmol NaCl). Cultivars 'Ai-1', 'Ak-1', 'Ha-1' and 'Sl-1' responded higher dry radicel weight at 30 mg/L ALA + 200mmol NaCl compare to control. After 6d germination, cultivar 'Qi-1', 'Ak-1' and 'Ha-1' the fresh weight of the hypocotyls of plants treated with 200 mmol/L NaCl was higher than that of the control (Table 3). Similarly the radicels of pakchoi treated with low salinity were also better developed than control radicels (data not shown). For most cultivars the hypocotyls weight were higher at T_5 (40 mg/L ALA + 200mmol/lNaCl) as compared to T_6 (50 mg/L ALA + 200mmol/l NaCl) (Table 3).

However, NaCl concentration was 200mmol/L, the fresh and dry weight of the cotyledons of pakchoi were higher than that of the control, because the decomposition and utilization by seeds of stored nutrient may be inhibited by salt stress. Except cultivar 'Qi-1', remaining cultivars cotyledons weight were higher at T_5 (40 mg/L ALA + 200mmol/l NaCl) as compare to T_6 (50 mg/L ALA + 200mmol/l NaCl) (Table 4).

Table 1: Effect of ALA treatment on radicel length of Pakchoi. (cm/p)

Treatment	Sa-1	Li-1	Ai-1	Qi-1	Ak-1	Ha-1	Sl-1
Control	1.07a	2.30a	2.79f	3.96b	3.04a	4.96a	5.12a
T-1	0.84d	0.0b	2.85d	3.8c	2.5c	2.76d	2.9d
T-2	0.43f	0.0b	2.99c	2.77e	2.30e	2.6e	3.3c
T-3	1.01c	0.0b	4.42a	3.06d	2.40d	2.87c	4.1b
T-4	1.05b	0.0b	3.88b	3.99a	3.0b	3.2b	5.2a
T-5	0.48e	0.0b	2.81e	0.94g	0.73g	1.34g	2.2e
T-6	0.02g	0.0b	2.5g	1.70f	0.96f	1.76f	1.8f

*Means followed by the same letter in the column do not differ statistically at P= 0.05

Table 2: Effect of 5-ALA on fresh and dry weight of radicel of pakchoi. (mg/plant)

	Sa-1		Li-1		Ai-1		Qi-1		Ak-1		Ha-1		Sl-1	
Treatments	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W
Control	3.6a	0.2a	3.2a	0.04a	11e	0.8b	15.9a	0.9a	2.8g	0.3c	5.8c	0.4a	7.8c	0.7b
T-1	0.7f	0.0c	0.0b	0.0b	10.2f	0.5d	11.8e	0.6d	3.1f	0.2d	3.7f	0.2e	3.4g	0.3d
T-2	1.7d	0.1b	0.0b	0.0b	11.2d	0.7c	12.6d	0.7c	7.2c	0.4b	5.8c	0.5b	8.8c	0.7b
T-3	2.0c	0.1b	0.0b	0.0b	11.8b	0.8b	14.4c	0.8b	8.1b	0.5a	6.4b	0.5b	9.4b	0.7b
T-4	3.4b	0.2a	0.0b	0.0b	12.7a	0.9a	15.7b	0.9a	8.9a	0.5a	7.2a	0.6a	10.2a	0.8a
T-5	1.6e	0.1b	0.0b	0.0b	11.4c	0.7c	8.9f	0.4e	6.0d	0.4b	5.7d	0.5b	8.6d	0.7b
T-6	0.5g	0.0c	0.0b	0.0b	8.0g	0.3e	6.0g	0.3f	4.5e	0.3c	4.3e	0.3d	7.6f	0.5c

*Means followed by the same letter in the column do not differ statistically at P= 0.05. Fresh weight (F/W), Dry weight (D/W)

Table 3: Effect of ALA treatments on the fresh and dry weight of hypocotyls of pakchoi under salt stress. (mg/plant)

	Sa-1		Li-1		Ai-1		Qi-1		Ak-1		Ha-1		Sl-1	
	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W
Control	3.9b	0.1b	3.4a	0.05a	12.4c	0.4c	14.6g	1.0b	6.1f	0.1d	10.2f	0.3c	11.3f	0.5b
T-1	1.5g	0.0c	0.0b	0.0b	8.1g	0.4c	16.5f	0.4e	10.8e	0.4c	11.0e	0.3c	5.6g	0.2d
T-2	2.0f	0.1b	0.0b	0.0b	9.9f	0.4c	22.2e	0.6d	11.2d	0.4c	12.5c	0.4b	13.5e	0.4c
T-3	2.5e	0.1b	0.0b	0.0b	10.0e	0.4c	23.5d	0.6c	11.9c	0.5b	12.6cb	0.4b	14.2d	0.4c
T-4	3.2d	0.1b	0.0b	0.0b	10.9d	0.5b	24.3b	0.8c	12.3b	0.5b	12.7b	0.5a	14.4c	0.5b
T-5	4.3a	0.2a	0.0b	0.0b	13.2b	0.6a	25.8a	1.6a	12.9a	0.6a	12.9a	0.5a	15.9a	0.6a
T-6	3.8c	0.1b	0.0b	0.0b	14.4a	0.6a	23.8c	0.8c	11.3d	0.4c	11.3d	0.4b	14.8b	0.5b

*Means followed by the same letter in the column do not differ statistically at P= 0.05. Fresh weight (F/W), Dry weight (D/W)

Table 4: Effect of 5-ALA treatments on the fresh and dry weight of pakchoi cotyledon (mg/plant)

	Sa-1		Li-1		Ai-1		Qi-1		Ak-1		Ha-1		Sl-1	
	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W	F/W	D/W
Control	4.8a	0.4a	3.8a	0.5a	6.4g	1.1f	9.8c	1.7f	3.8g	0.5d	6.3g	0.6e	6.8g	1.0e
T-1	2.1e	0.2c	0.0b	0.0b	12.3f	1.2e	15.4bc	1.8e	10.3f	1.0c	10.3f	0.8d	15.3f	1.3d
T-2	3.2d	0.2c	0.0b	0.0b	16.6e	1.3d	19.7b	2.4d	15.0d	1.8b	13.3d	0.8d	19.1d	1.4c
T-3	3.8c	0.3b	0.0b	0.0b	17.9d	1.3d	20.6b	2.4d	16.5c	1.9b	13.9c	0.8d	20.2c	1.4c
T-4	3.9b	0.3b	0.0b	0.0b	19.9b	1.5b	21.7ba	2.5c	17.4b	2.2a	14.7b	1.0b	21.2b	1.4c
T-5	3.9b	0.3b	0.0b	0.0b	22.2a	2.3a	25.5bc	2.6b	17.8a	2.2a	15.6a	1.3a	22.1a	1.7a
T-6	1.3f	0.1d	0.0b	0.0b	18.5c	1.4c	30.4a	2.9a	13.3e	1.0c	12.9e	0.9c	18.0e	1.5b

*Means followed by the same letter in the column do not differ statistically at P= 0.05. Fresh weight (F/W), Dry weight (D/W)

Table 5: Effect of ALA on seed respiration under salt stress (imol m^{-2} s^{-1})

Treatment	Sa-1	Li-1	Ai-1	Qi-1	Ak-1	Ha-1	Sl-1
Control	1.01b	1.16a	1.14b	1.18c	1.03c	1.43a	1.30a
T1	0.50e	0.42f	0.62e	0.53g	0.64f	0.60g	0.51f
T2	0.70d	0.50e	0.87d	0.64f	0.86e	0.75f	0.64e
T3	0.90c	0.65d	0.97c	0.79e	0.97d	0.84e	0.75d
T4	0.96c	0.79c	0.99c	0.89d	1.00c	0.99d	0.90c
T5	1.00b	0.82c	1.10b	1.32b	1.10b	1.13c	1.11b
T6	1.09a	0.90b	1.23a	1.43a	1.21a	1.37b	1.28a

*Means followed by the same letter in the column do not differ statistically at P= 0.05. Fresh weight (F/W), Dry weight (D/W)

Result shows that cotyledons fresh and dry weight was comparatively more affected by ALA treatment than radicals and hypocotyls.

Effect of ALA on Heme Content of Pakchoi Cultivars under Salt Condition: Hemes are involved in respiratory and photosynthetic electron transport and occur primarily as the prosthetic group of the Cyt and the peroxidative enzymes (catalase and peroxidase). Six day old etiolated seedling were determined, Fig. 2 it can be seen that salt stress significantly depressed the heme content. In the case of cultivar 'Li-1', no germination was recorded in 6 day observation, seed germination may be sensitive to stress treatment. Cultivar 'Sa-1' heme level considerably decreased as compare to five cultivar, including Ai-1, Qi-1, Ak-1, Ha-1, Sl-1. The heme level was highest in cultivar 'Ai-1' than that of other five cultivars. In addition, cultivar 'Sl-1' the heme content was same as control in 40mmol/L ALA treatment.

Effect of ALA on Respiration of Pakchoi under Salt Condition: Respiration takes place in all viable seed. Respiration rate increases during germination. If the oxygen supply during germination is limited or reduced, germination can be severely retarded or inhibited. From Table 5, it can seen that seeds respiration of pakchoi was low in NaCl solution (T_1) and ALA increased the respiration rate. The highest respiration was observed in cultivar 'Qi-1' compared to control. There were significant ($P<0.05$) effects of ALA under salt stress.

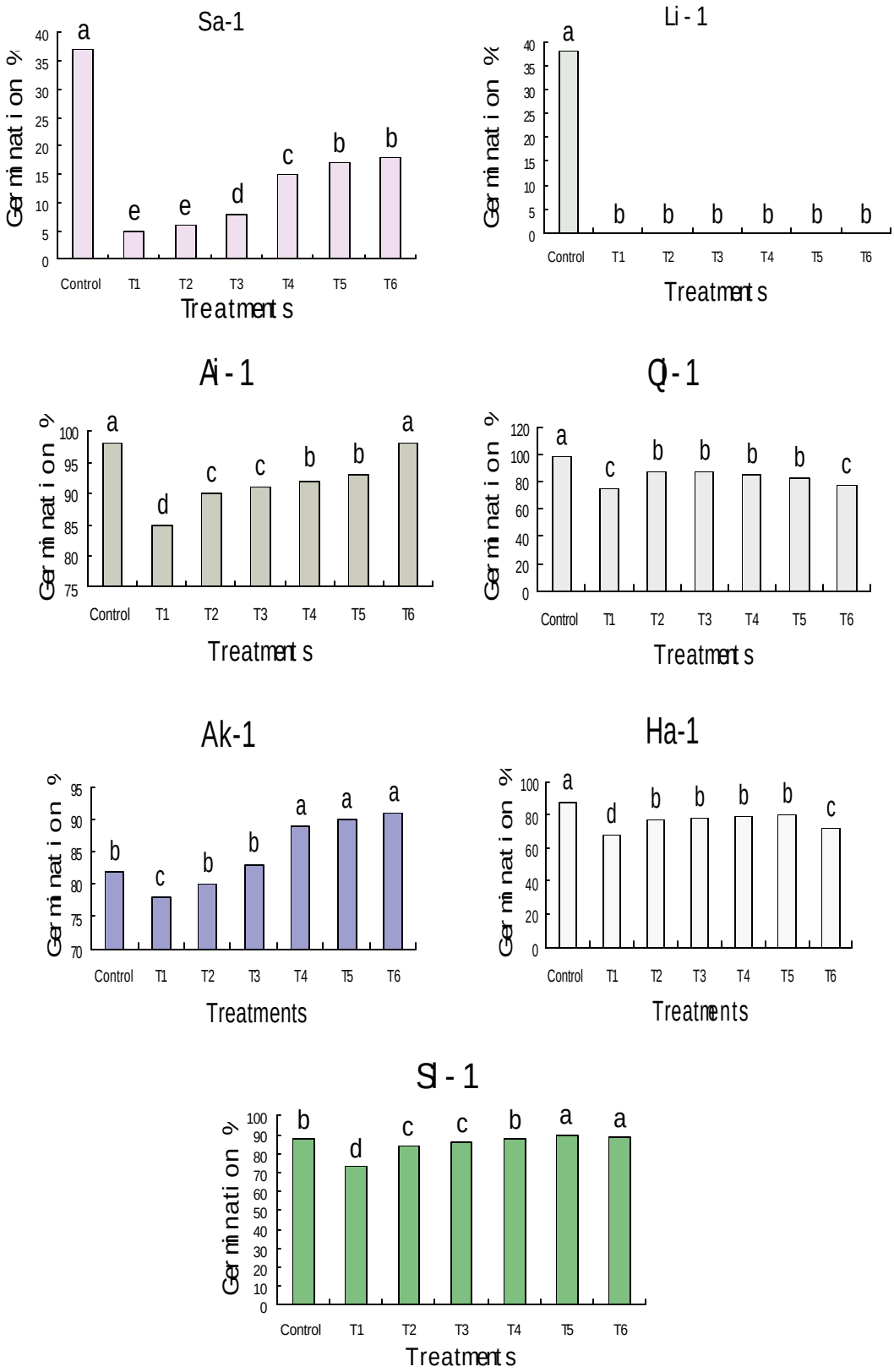

Fig. 1: Effect of 5-aminolevulinic acid (5-ALA) treatment on the seed germination of pakchoi under salt stress (C-

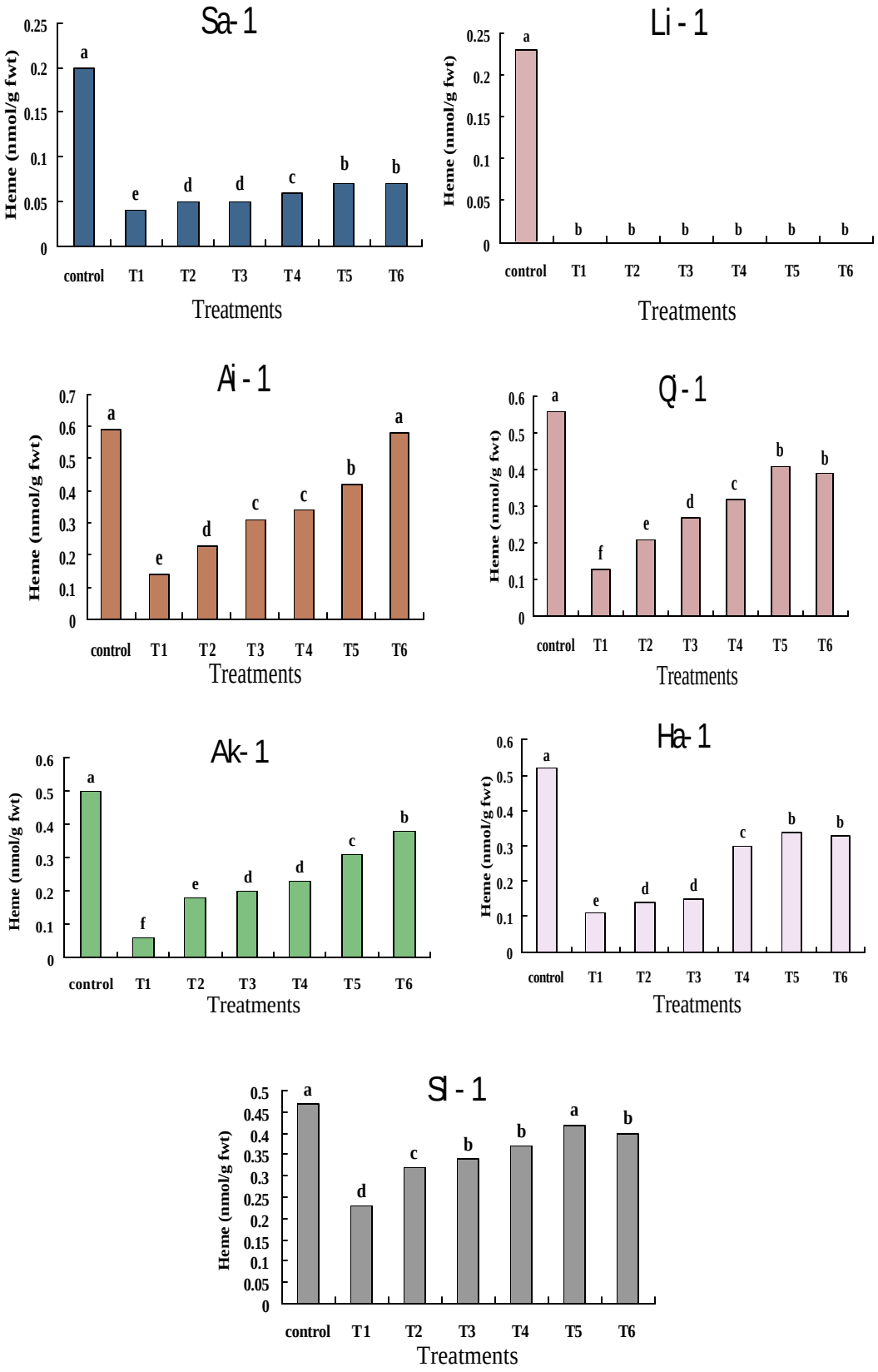

Fig. 2: Effect of 5-aminolevulinic acid (ALA) on heme content under salt stress

For example respirations of the stressed seeds were 36-62 % low as compared with control, whereas this increased to approximately 82-92% of the control followed the addition of 10-50mg/L ALA. In case of, cultivar 'Li-1' respiration rate was lowest than that of other cultivar (i-e. Sa-1, Ai-1, Qi-1, Ak-1, Ha-1, Sl-1).

DISCUSSION

Pakchoi (*Brassica campestris* ssp. *chinensis*), is one of the most important vegetable crops in China. Particularly, in South China, huge numbers of cabbages and their relatives are harvested throughout the year. Pakchoi is a salt tolerance crop [18,6]. Usually, plant salinity tolerance is evaluated based on physiological injuries or yield reduction under salinity stress. The mechanism of salinity stress injury to plants involves osmotic effects followed by specific-ion effects after prolonged exposure [19,20]. We observed that cultivar differential responded in the same salt level with different concentration of 5-aminolevulinc acid (ALA). The result indicated that ALA concentrations increase the seed germination and affect on seedling growth under salt stress (Fig. 1 and Table, 1, 2, 3, 4). ALA significantly improved the salt tolerance of cotton plants [21]. ALA promoted seed germination under salt stress [6]. We observed that different cultivars of pakchoi had ability to biosynthesis ALA under salt condition. Meanwhile, salt (T 1) decreased the levels of respiration and heme content. ALA under salt conditions was related to seed germination, implying that blockade of ALA biosynthesis by stress might be one of the causes of the inhibition of seed germination [6].

ALA is the first precursor of heme biosynthesis in all organisms [21,22]. Hemes are ubiquitous in living organisms and heme proteins are directly involved in many reactions that require oxidation-reduction, oxygenation, hydroxylation and binding of oxygen and other diatomic gases [23]. Protoheme is synthesized from the universal tetrapyrrole precursor 5-aminolevulinic acid (ALA) by seven successive enzymatic reactions with well-described intermediates [24,25]. The activities of malic and alcohol dehydrogenases and cytochrome oxidase were highly correlated with germination [26]. Throneberry and Smith [26] reported that loss of viability in corn seemed closely related to respiratory failure. The present investigation shows that salt decreases the germination and heme content in seedling (Fig. 2). So may be the respiration rate was decreased under salt stress (Table 5).

Zhang *et al.* [27] reported that at lower concentrations of ALA (0.3–3 mg/L) promoted development and growth of potato microtubers *in vitro* and enhanced protective functions against oxidative stresses, on contrary at (30 mg/L) higher concentrations was induce to oxidative damage probably through formation and accumulation of photooxidative porphyrins. In this study, we observed that in low concentrations (10-30 mg/L ALA) length, dry and fresh weight of radicel increased. In 40-50 mg/L concentration of ALA length, dry and fresh weight of radicel did not increase under salt stress. It is suggested that 30 mg/L ALA treatment was enough to increase the radicel length and weight of pak-choi. Meanwhile, hypocotyls and cotyledon weight increased with the increasing of ALA levels (Table 3, 4).

The present investigation, ALA increased the heme content in 6 day old etiolated seedling (Fig. 2). The result shows that different cultivars had increased heme content in different level of (ALA) concentrations. In case of cultivar 'Sl-1', heme content (nmol/g fresh wt) increased in T_5 more than that of T_6. Heme content significantly increased with the increase of ALA concentration. Wang *et al.* [6] compared heme content among cultivars, heme was more in salt-tolerant cultivars (Hanxiao and Shu lv) than salt sensitive cultivars (Kang 5 and Kang 6), whether under salt stress or not. In this study, we observed that ALA enhanced the heme content under salt stress.

Thus, the conclusion can be drawn that ALA had promotive effect on seed germination, seedling growth and respiration might be caused mainly by heme. And that heme is essential for the activity of cytochrome *c* in the respiration chain of the mitochondrion. By the heme biosynthesis, enhanced seed germination, aero respiration and metabolic activity.

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