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ORIGINAL ARTICLE

Priming with 5-SSA, Glutamine and Thyme oil Improves the Emergence and Early Seedling Growth in Pea (*Pisum sativum* L.)

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ABSTRACT
Salinity is one of the major abiotic stresses, and high concentrations of salts in irrigation water is a common environmental problem affecting plant growth, decrease carotenoid and induce reduction in chlorophyll and photosynthetic activity and yield. In this study, the effect of 0.5, 1.5 and 2.5 mM 5-SSA, 2 and 4 mM glutamine, 100 and 150 ppm thyme oil priming on Pea (Pisum sativum L.) subjected to 100 mM of NaCl at germination and seedling growth stages was analyzed. The seeds were sterilized by using 30% hypochlorite for 5 minutes and then washed 3 times with distilled water. For priming, seeds were soaked in aerated solutions of 5-sulfosalicylic acid, glutamine and essential oils of Thyme for 24 h. Results showed that saline stress significantly reduced dry weight and water content of root and shoot. Photosynthetic rate, relative water content, stomatal conductance and chlorophyll contents reduced by the NaCl while MDA conent and free proline content intensified. 5-SSA, glutamine and thyme oil priming alleviated salt-induced oxidative stress by reducing malondialdehyde (MDA) content and increasing SOD activity. 5-SSA treated plants had greater shoot and root dry weights compared to untreated plants when exposed to salt stress. Result showed 2.5mM 5-SSA application significantly increased photosynthetic rates, stomatal conductance, relative water content, total chlorophyll (a+b) content and leaf area in salt stressed plants. It was concluded that 5-SSA, glutamine and thyme oil treatment could alleviate the adverse effects of salinity on pea (Pisum sativum L.).
Keywords: priming, Pea, salt stress, germination, seedling growth
***Abbreviations**: 5-SSA, 5-sulfosalicylic acid; Pn, photosynthesis rate; SOD, superoxide dismutase; MDA, malondialdehyde; NRA, nitrate reductase activity; NiRA, nitrite reductase activity; GLU, glutamine*

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INTRODUCTION

Salinity is one of the major abiotic stresses, and high concentrations of salts in irrigation water is a common environmental problem affecting plant growth, decrease carotenoid and induce reduction in chlorophyll and photosynthetic activity. Salinity can affect plant physiological processes resulting in reduced growth and yield [1]. In addition, salinity stress can trigger a secondary oxidative stress that leads to an increase of reactive oxygen species (ROS). One effect of ROS accumulation in plant cells under stress is lipid peroxidation via oxidation of unsaturated fatty acids leading to membrane damage and electrolyte leakage [2]. MDA, a decomposition product of polyunsaturated fatty acids, have been utilized as a biomarker for lipid peroxidation. MDA content can serve as an indicator of the rate of oxidative processes in plant cells under stress [3]. Rapid accumulation of free proline is also a typical response to salt stress and when plants are exposed to drought or a high salt content in the soil, many plants accumulate high amounts of proline, in some cases several times the sum of all the other amino acids [4]. The role of proline in protection of cell membranes against salt injury has been discussed [5]. Also, Poor germination and seedling establishment are the results of salinity. Azooz [6] reported during the course of salinity stress, active solute accumulation of osmotic solutes such as soluble carbohydrates, proteins and free amino acids is claimed to be an effective stress tolerance mechanism. Enhancing salt tolerance in plants has major implications in agriculture. Salicylic acid is a plant phenol, and today it is in use as internal regulator hormone, because its role in the defensive mechanism against biotic and a biotic stresses has been confirmed. Sedghi et al., [7] and Farooq et al., [8] reported seed priming with salicylic acid increased germination under low temperature condition and improved chilling tolerance faster, synchronous emergence of maize by activation of antioxidants, maintenance of tissue water contents and

reduced membrane permeability. Gautam and Singh, [9] reported seed priming with ascorbic acid and salicylic acid induced salinity tolerance. Glutamine is important as a constituent of proteins and as a central metabolite for amino acid transamination via α-ketoglutarate and glutamate. Glutamine plays an important role in the nitrogen and carbonskeleton exchange among different tissues, where this amino acid fulfils many different physiological functions. When glucose levels are low and energy demands are high, cells can metabolize amino acids for energy. Also, glutamine is one of the most readily available amino acids for use as an energy source and it is a major source of energy for many rapidly dividing cell types. Essential oils are complex components with different kinds of chemical substances including: hydrocarbons, alcohols, cetons, and aldehydes [10]. Thymus essential oil is known to contain more than 40% of phenolic compositions (thymol and carvacrol), that have strong antiseptics effect. In addition to thymole, caffeic acid and thanin existing in essential oil can effectively prevent growth of bacteria, fungus and viruses. The objective of this study was to the physiological responses (photosynthesis, transpiration and membrane functioning) associated with enhanced tolerance resulting from the application of 5-sulfosalicylic acid, glutamine and essential oils of Thyme to plants grown under saline condition.

MATERIALS AND METHODS

Plant material and treatments

The investigation was conducted in season 2011-2012. Seed of Pea (*Pisum sativum* L.) were obtained from Ilam, Iran (Ilam: Elevation 1339 m, Latitude East 33.638, Longitude North 46.431). The seeds were sterilized by using 30% hypochlorite for 5 minutes and then washed 3 times with distilled water. For priming, seeds were soaked in aerated solutions of 5-sulfosalicylic acid (0.5, 1.5 and 2.5 mM), glutamine (2 and 4 mM) and essential oils of Thyme (100 and 150 ppm) for 24 h. After soaking period the seeds were air dried. Primed seeds were sown in plastic pots filled with soil composed of clay and sand (6 seeds in each pot), The plastic pots were divided into two sub-groups; the pots of the first subgroup were irrigated with normal water only to serve as control, while the pots of second sub-group were irrigated with 100 mM NaCl. Relative humidity was maintained at 65%. The plants were harvested at seedling stage and mature plant stage (30 days after sowing) to analyze various morpho-physiological biochemical. They were rinsed with deionized water and blotted on paper towels before being weighed (fresh weight) [6].

Measurement of plant growth parameters

Dry weight (DW) and Na^+/K^+ ratio of root and shoot (g $plant^{-1}$), percentage water content (WC%), leaf area (cm^2), chlorophyll a+b and percentage relative water content (RWC%), MDA content and SOD activity were measured. Total chlorophyll (a+b) content was measured by chlorophyll meter (SPAD-502, Minolta Co. Japan) which is presented by SPAD value. Average of 3 measurements from different leaves was considered. Oxidative damage to lipids was measured based on the method of Heath and Packer [11]. The activity of superoxide dismutase was measured based on the method of Beauchamp and Fridovich [12]. Proline was quantified by using ninhydrin reagent and measured according to Bates et al., [13]. Leaf area was measured by digital planimeter. To determine dry weight; the freshly harvested roots and shoots were dried in an aerated oven at 80°C until constant weight. The samples were ground into fine powder and stored in sealed glasses at room temperature for the chemical analysis. The RWC of leaves was calculated by the following equation of schonfeld et al., [14]. Na^+ and K^+ were determined by the flame photometric method [15]. The germination percentage was calculated as under;

Germination % = Number of seed germinated / Total number of seed sown × 100

Nitrate reductase activity (NRA) of pea was calculated by the protocol of Sym [16]. Nitrite reductase activity (NiRA) of pea was calculated by the protocol of Ramarao et al., [17]. Photosynthesis rate (Pn) and stomatal conductance (gs) were measured from the second fully expanded young leaves. Plots were arranged in a completely randomized design with 4 replications. Analysis of variance was performed on the data collected using the general linear model (GLM) procedure of the SPSS software (Version 16, IBM Inc.). The mean separation was conducted by Duncan analysis in the same software ($p= 0.05$).

RESULTS AND DISCUSSION

The results revealed that 5-SSA, glutamine and Thyme oil significantly affected the germination and early seedling growth in *Pisum sativum* (Table 1-3) ($p\leq0.05$). NaCl caused a considerably greater decrease in germination (53%) as compared with control (85%) treatment (Table 1). Under normal conditions, maximum germination (98.8%) ($p\leq0.05$)was achieved in seeds primed with 2.5mM 5-SSA (Table 1). However, lowest germination was achieved in seeds primed with 100 ppm Thyme treatment (78%) (Table 1). The results revealed that under saline conditions, maximum germination was obtained in seeds primed with 2.5 mM 5-SSA +4 mM GLU +150 ppm Thyme (86.4%) and lowest germination was achieved

in seeds primed with 0.5 mM 5-SSA treatment(57%)(Table 1) (p≤0.05). However, maximum reduction in percentage was recorded in seeds primed with 0.5 mM 5-SSA under saline conditions as compared to remaining treatments including control (Table 1).

Table 1. Effect of NaCl, 5-SSA, GLU, Thyme and their combination on germination (%), Photosynthesis rate, Stomatal conductance, nNRA and NiRA on Pisum sativum after 30 days of sowing

TREATMENTSMEANCOMPARISON(NUMBERS REPRESENT THE MEAN)					
Treatment	Germination %	Photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	Stomatal conductance (mmol m^{-2} s^{-1})	NRA (NO2 g^{-1} F.wt. h^{-1}.)	NiRA (μmol NO2 g^{-1} F.wt. h^{-1})
Control	85	45.14	55.45	5.8	3.11
5-SSA (0.5 mM)	90	56.8	59.68	5.87	3.15
5-SSA (1.5 mM)	91	65.69	72.36	6.12	3.21
5-SSA (2.5 mM)	98.8	76.45	87.36	8.36	5
Glu (2mM)	90	59.68	61.25	6	3.47
Glu (4mM)	92	63.5	68.78	6.74	3.96
Thyme (100 ppm)	78	55.63	63.54	5.7	3
Thyme (150 ppm)	90	59.78	67.11	6.3	3.7
NaCl (50mM)	53	39.96	50.45	3.11	2.31
5-SSA (0.5 mM) +NaCl (50mM)	57	40.11	51.25	4.56	2.6
5-SSA (1.5 mM) +NaCl (50mM)	73.5	43.6	54.8	5.86	3.14
5-SSA (2.5 mM)+NaCl (50mM)	85.3	66.8	70.12	7.51	4.15
GLU (2 mM) +NaCl (50mM)	69.8	48.9	53.14	3.89	3
GLU (4 mM) +NaCl (50mM)	76.3	52.36	60.7	5.45	3.47
Thyme (100 ppm)+NaCl (50mM)	50.9	38.9	55.68	3.49	2.9
Thyme (150 ppm)+NaCl (50mM)	84.8	55.87	59.78	5.14	3.59
5-SSA (2.5 mM)+GLU (4 mM) + Thyme (150 ppm)+NaCl (50mM)	86.4	70.45	82.4	6.98	4.36
F -TESTPROBABILITIES(FACTOREFFECTS)					
5-SSA	0.001	0.000	0.002	0.000	0.000
Glu	ns	0.03	0.05	ns	ns
Thyme	0.02	0.05	0.05	ns	ns

ns=non-significant (α≥0.01). Means in each column followed by similar letters are not significantly different (Duncan's test, α<0.05).

NaCl treatments (100 mM) caused a significant reduction photosynthesis rate and stomatal conductance but 1.5 and 2.5 mM 5-SSA treatments showing the maximum photosynthesis rate (40.11 and 43.6), stomatal conductance (51.25 and 54.8) as compared to control (Table 1). 5-SSA, glutamine, Thyme and their combination proline content as compared to control under salt stress. Under normal conditions, maximum photosynthesis rate and stomatal conductance (76.45 and 87.36) was achieved in seeds primed with 2.5mM 5-SSA (Table 1). The activities of nitrate reductase and nitrite reductase (Table 1) were decreased while superoxide dismutase (SOD) (Table 3) were significantly increased under NaCl stress. Under saline conditions, the plants grown from seeds germinated with 2.5 mM 5-SSA and 2.5 mM 5-SSA +4 mM GLU +150 ppm Thyme showed the highest enzymatic activities than control and other treatment . Shoot and root dry weights were reduced under NaCl stress, the main effects of salinity on shoot and root dry weights were statistically significant as compared with control (Table 2). The results revealed that 2.5 mM 5-SSA application under NaCl stress and normal significantly enhanced roots dry weights and shoot (Table 1-2). Under normal conditions, water content roots and shoot were significantly increased in seedlings raised from seeds primed with 2.5 mM 5-SSA+4 mM glutamine+150 ppm Thyme (Table 2). Under saline conditions, lowest water content roots and shoot was achieved in seeds primed with 0. 5 mM 5-SSA (Table 2). Contents of MDA and K^+/Na^+ ratio reveal that, salinity increased contents of MDA and decreased K^+/Na^+ ratio in root and shoot (Table 2-3). K^+/Na^+ ratio was significantly increased in seedlings raised from seeds primed with 2.5 mM 5-SSA+4 mM glutamine+150 ppm Thyme (Table 2 and 3). salinity stress significantly reduced total chlorophyll (*chl* a+b) contents, leaf area and relative water content (Table 3). Seed priming with 2.5 mM 5-SSA+4 mM glutamine+ 150ppm Thyme and their combination significantly improved total chlorophyll (*chl* a+b) contents, leaf area and relative water content, especially in plants subjected to salt stress (p≤0.05).The present study revealed that 5-SSA, glutamine and Thyme priming can be employed to improve early emergence and seedling growth in *Pisum sativum*. Salinity stress results in a clear stunting of plant growth, which results in a considerable

decrease in the dry weights of leaves, stems and roots [18]. Zribi *et* al. [19] reported immediate response of salt stress is reduction in the rate of leaf surface expansion leading to cessation of expansion as salt concentrations increases. Increased tolerance to salinity stress in crop plants is necessary in order to increase productivity with limited water supplies and high salinity [6]. Our results showed under saline condition, 5-SSA, glutamine, Thyme or their combination significant increased in leaf area of pea plants.

Table 2. Effect of NaCl,5-SSA, GLU, Thyme and their combination on Dry weight(g plant-1), water content(%), Stomatal conductance (mmol m-2 s-1), K+/Na+ (mg g-1 DW) of root and shoot on Pisum sativum after 30 days of sowing

TREATMENTSMEANCOMPARISON(NUMBERSREPRESENTTHEMEAN)					
Treatment	ROOT		SHOOT		
	Dry weight (g plant^{-1})	K+/Na+ (mg g^{-1} DW)	Dry weight (g plant^{-1})	water content(%)	K+/Na+ (mg g^{-1} DW)
Control	0.052	2.36	0.22	80.2	9.33
5-SSA (0.5 mM)	0.062	2.6	0.31	83.45	10
5-SSA (1.5 mM)	0.067	2.98	0.4	86.87	10.36
5-SSA (2.5 mM)	0.12	4.89	0.45	96.36	13.69
Glu (2mM)	0.068	2.6	0.23	90.12	10.26
Glu (4mM)	0.07	3.12	0.28	93.54	10.9
Thyme (100 ppm)	0.6	2	0.22	83.69	9.35
Thyme (150 ppm)	0.067	2.08	0.25	87.14	10
NaCl (50mM)	0.043	1.68	0.17	65.3	6.36
5-SSA (0.5 mM) +NaCl (50mM)	0.045	2	0.19	67.36	6.89
5-SSA (1.5 mM) +NaCl (50mM)	0.049	2.06	0.21	69	6.9
5-SSA (2.5 mM)+NaCl (50mM)	0.086	3.86	0.32	86.36	8.96
GLU (2 mM) +NaCl (50mM)	0.054	2.24	0.18	80.12	6.59
GLU (4 mM) +NaCl (50mM)	0.06	2.56	0.2	82.36	6.8
Thyme (100 ppm)+NaCl (50mM)	0.046	1.9	0.17	66.36	6.4
Thyme (150 ppm)+NaCl (50mM)	0.053	2.05	0.2	68	6.59
5-SSA (2.5 mM)+GLU (4 mM) +Thyme (150 ppm)NaCl (50mM)	0.09	3.7	0.3	87.69	7.12
F -TESTPROBABILITIES(FACTOREFFECTS)					
5-SSA	0.000	0.002	0.000	0.001	0.000
Glu	ns	0.02	0.000	0.02	0.05
Thyme	ns	ns	ns	0.01	ns

ns=non-significant (α≥0.01). Means in each column followed by similar letters are not significantly different (Duncan's test, α<0.05).

Al-Hakimi and Hamada [20] reported improved germination rate and percentage by ascorbate and sodium salicylic acid treatments in wheat (*Triticum aestivum* L.). It may be that NaCl reduced the rate of germination due to the reduced water potential and the resulting slower rate of imbibition. SA significantly stimulated the activities of enzymes involved in germination such as transkelolase, enolase, malate dehydrogenase, phosphoglycerate kinase, glyceraldehyde 3 phosphate, dehydrogenase, fructose 1,6-diphosphatase, and pyruvate decarboxylase [21]. In this study, we found that the presence of NaCl reduced the germination rate compared to the control as a consequence of salt osmotic effects, which reduced water availability. Salt stress also affects starch mobilization by reducing amylase activity [22] and lipid storage breakdown through a reduction in the activity of glyoxysomal cycle enzymes [23].

The results related to germination percentage can be related to earlier findings in which El-Tayeb [24] found an improvement in seeds pretreated with SA solution than those of un-treated seeds. Improved seedling fresh and dry weights might be due to increased cell division within the apical meristem of seedling shoots and roots, which caused an increase in plant growth [25]. Sakhabutdinova et al., [26] reported that salicylic acid treatments maintain the IAA and cytokinin levels in the plant tissues, which enhanced the cell division. Singh and Ushu [27] also found that SA application increased the dry weight of wheat seedlings under water stress. The decrease in total chlorophyll content observed in peas is related to the ROS accumulation, which is known to promote the degradation of these pigments as well as reduce their biosynthesis [28]. Ashraf and Bhatti, [29] reported decreased in chlorophyll content under salinity stress is a commonly reported phenomenon in various studies, because of its adverse effects on membrane stability. Our results showed under saline condition, 5-SSA, glutamine, Thyme or their combination significant increased in chlorophyll and SOD activity of pea plants. Similarly, SA might be involved in mobilization of internal tissue NO^{3-} and chlorophyll biosynthesis to increase the functional state of the photosynthetic machinery in plants [30], or it may induce accumulation of α-amino levulinic acid (α-ALA) in cotyledons. Kazemi et al., [31-32-33-34] that treatment with salicylic acid, essential oils

and glutamine significantly extends the vase life with reduced the MDA, proline content and increasing chlorophyll content and superoxide dismutase activity. 5-SSA, glutamine and Thyme combination had a pronounced ameliorative as well as, growth promoting effect under both saline and non-saline conditions. The ameliorative effect of 5-SSA, glutamine and Thyme might be linked to the observable increase in WC, RWC and photosynthetic pigments as well as, leaf area. Increases in chlorophyll content, indicates that the SA plays a regulatory role during the biosynthesis of active photosynthetic pigments. Although the direct effect of SA on chlorophyll biosynthesis in plants is not clearly understood, α- ALA mediated enhancement in chlorophyll biosynthesis by benzyladenine (synthetic SA) [48]. Salinity increased the membrane permeability of sensitive rice varieties. Ashraf and Bhatti, [29] reported decreased in chlorophyll content under salinity stress, because of its adverse effects on membrane stability. Under salinity stress, leaf pigments, studied in nine genotypes of rice, reduced in general [35]. Our results are in agreement with those of Rajasekaran et al., [36] and Shakirova et al., [37] , who showed a promotion in seed germination and reduction membrane permeability with SA application. kazemi et al. [31-32-33-34] reported that treatment with salicylic acid, glutamine and essential oils significantly reduced the membrane permeability. Similar results were obtained by Lutts et al., [38] who reported that salinity increased the membrane permeability of sensitive rice varieties. NaCl severely reduces K^+ uptake and translocation from roots to shoots (Table 2). Consequently, selectivity in favour to potassium decrease with salinity (Table 2 and 3).

Table 3. Effect of NaCl,5-SSA, GLU, Thyme and their combination on Dry weight(g plant-1), water content(%), Stomatal conductance (mmol m-2 s-1), K+/Na+ (mg g-1 DW) of leaves on *Pisum sativum* after 30 days of sowing

TREATMENTSMEANCOMPARISON(NUMBERSREPRESENTTHEMEAN)

treatment	SOD (U g^{-1}Protein)	MDA (µmol/mg protein)	leaf area(cm2)	Proline (µmol.g^{-1} FW)	Chl a+b (SPAD reading)
Control	61.56	87	21.21	0.41	0.87
5-SSA (0.5 mM)	70.15	61.12	22	0.46	1
5-SSA (1.5 mM)	78.69	55.36	23.1	0.48	1.11
5-SSA (2.5 mM)	110.36	30.12	25.12	0.8	2.64
Glu (2mM)	65.89	60.36	21	0.53	1
Glu (4mM)	83.25	52.36	23.3	0.55	1.3
Thyme (100 ppm)	73.8	59.69	20	0.56	0.87
Thyme (150 ppm)	80.14	50.12	22	0.61	0.97
NaCl (50mM)	52.17	135.45	17.36	0.63	0.43
5-SSA (0.5 mM) +NaCl (50mM)	55.4	121.15b	17.6	0.65	0.49
5-SSA (1.5 mM) +NaCl (50mM)	58.96	111.5c	17.9	0.68	0.58
5-SSA (2.5 mM)+NaCl (50mM)	90.36	66.3b	20.11	0.92	1.01
GLU (2 mM) +NaCl (50mM)	58.69	130.56	18	0.63	0.68
GLU (4 mM) +NaCl (50mM)	68.12	120.3	19.3	0.68	0.91
Thyme (100 ppm)+NaCl (50mM)	56.98	127.36	17.84	0.6	0.55
Thyme (150 ppm)+NaCl (50mM)	60.12	120	18.36	0.62	0.7
5-SSA (2.5 mM)+GLU (4 mM) +Thyme (150 ppm)+NaCl (50mM)	87.69	67.32	21	1	1.84
F -TESTPROBABILITIES(FACTOREFFECTS)					
5-SSA	0.001	0.003	0.000	0.000	0.000
Glu	ns	0.05	0.01	ns	0.03
Thyme	0.002	0.04	ns	0.001	0.03

ns=non-significant (α≥0.01). Means in each column followed by similar letters are not significantly different (Duncan's test, α<0.05).

Whereas 5-SSA addition ameliorates this parameter. 5-SSA treatments reduced Na^+, while increased K^+ and K^+/Na^+ ratio in pea plants. This indicates that seed priming with SA, GLU and Thyme oil induced a reduction of Na^+ absorption and toxicity. This could explain the mitigation effect of SA, GLU and Thyme oil on growth of seedling. Further, the antagonistic relation between Na^+ and K^+ as a result of SA, GLU and Thyme oil treatments indicates that, SA, GLU and Thyme oil could play a role in modifying K^+/Na^+ selectivity under salt stress, which is reflected in lowering membrane damage and higher water content in pea especially under salinity stress. Dry weights of roots and shoots decreased progressively due to salinity as compared to control (Table 2). Results indicated that concentrations of SA at 2.5 mM might

induce NR synthesis by mobilization of intracellular NO^{3-}, and provide protection to NR degradation in absence of NO^{3-} [39]. Fariduddin et al. [40] reported increased NR activity due reduced concentrations of SA while higher concentrations were observed to be inhibitory to NR activity in *Brassica juncea Czern* and Coss cv. Varuna. Our results showed NaCl induced oxidative stress in pea seedlings, which resulted in an increas in the H_2O_2 concentration. This increase was particularly marked in the photosynthetic organs, which are a major source of ROS in plants [41] and are sites known for being very sensitive to NaCl stress. At these sites, salt-induced ROS accumulation induces plastid structural alterations, which cause damage to photosynthetic pigments, and in particular chlorophyll [42]. ROS cause chlorophyll degradation and membrane lipid peroxidation. So, malondialdehyde (MDA) accumulation as product of lipid peroxidation and chlorophyll retention are two oxidative stress indicators that are tested tools for determining salt tolerance in plants [43]. To scavenge ROS, plants posse's specific mechanisms, which include activation of antioxidant enzymes and non enzymatic antioxidants such as, carotenoids and ascorbic acid [2]. Azevedo et al., [44]; Athar et al., [45] reported that a correlation between the antioxidant enzyme activities and salinity tolerance was demonstrated by comparison of tolerant cultivar with sensitive cultivar in several plant cultivars. The protective function of SA includes the regulation of ROS and antioxidant enzymes [46-47]. In Conclusion, data obtained from the present study suggest that exogenous application of 5-SSA, glutamine, Thyme or their combination can ameliorate the deleterious effects of salt stress by increasing chlorophyll contents, water content, decreasing MDA content, thus inducing salt tolerance in pea plants. So we can suggest that 5-SSA, glutamine, Thyme or their combination could to improve plant growth and under salt stress.

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