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Physiological Response of Thyme Populations at Early Growth Stages to NaCl + $CaCl_2$ Induced Salinity

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ABSTRACT
Thyme is an important medicinal plant; however, in the natural environment, its growth is limited by environmental factors. So, this experiment was conducted to study the effect of salinity stress on early growth stages of seeds of three thyme populations collected from natural habitat in Iran. The experiment was conducted in factorial in the form of a completely randomized design with three replications. Treatments included: (1) thyme population in three levels including 2500, 2750 and 3050 m above the sea level in Qom province at the central part of Iran, and (2) salinity stress induced by NaCl + $CaCl_2$ in six levels including 0, 50, 100, 150, 200 and 250 mM NaCl + $CaCl_2$. Analysis of variance showed that population and salinity significantly affected plant height, root dry weight and the contents of chlorophyll, proline, soluble sugars, relative water and essential oil; their effect was not significant on shoot dry weight. The interaction of the two factors had also a significant effect on all measured traits except for the shoot dry weight and chlorophyll content. Mean comparison of the interactions indicated that plant height, root dry weight, chlorophyll content and relative water content were the highest in 2500 m × 0 mM (the lowest saline treatment); however, proline content, soluble sugars content and essential oil percentage were that highest in 3050 m × 250 mM (the highest saline treatment). Increasing the salinity from the lowest level to the highest level increased essential oil percentage from 0.14% to 0.35%.
Keywords: essential oil, proline, soluble sugars, *Thymus Kotschyanus* L.

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INTRODUCTION

In nearly all parts of the world, abiotic stresses such as temperature, drought and salinity are considered as the most limiting factors to agricultural production. Soil salinity is an important environmental stress reducing plants growth, yield and quality. Soil salinity results from two main sources: soil parent materials (primary salinity) or irrigation and leaching (secondary salinity). In saline soils, the concentration of some salts such as chlorides and sulfates of sodium is high and toxic to plants. Salinity causes nutritional problems to plants as it reduces the absorption of N, P, K and Cl etc. It also induces ion cytotoxicity and osmotic stress to plants. The result of salinity stress is metabolic imbalances and oxidative stress for plants. Sodium is an essential nutrient for some C_4 plants; however, most crops are natrophobic. Under saline condition, Na^+ and Ca^- damages plant protein structures which results in the form changes and loss of proteins function [1-4].

Different experiments have cited the effect of salinity on plants growth, yield and quality. Leilah et al. [5] tested the effect of salinity stress on canola and concluded that enhancement of salinity level significantly reduced germination speed and percentage. In another experiment on the germination of canola, Puppala et al. [6] studied the effect of five different levels of salinity from 5.4 to 26.4 ds/m and reported that increasing salinity level from 10.1 to 16.2 ds/m reduced germination percentage by about 40% compared with the control. Abbaszadeh et al. [7] studied camphor (*Comphorosma monspeliaca*) seed germination under saline condition and reported the significant effect of salinity on germination features. They reported that germination percentage reduced when the salinity increased from 0 to 500 mM.

In addition to the germination phase, other plant growth stages are also affected by salinity. Suriyan et al. [8] tested the effect of salinity on sorghum and reported the reduction of LAR, germination percentage, leaf area, dry weight and fresh weight. Afzali [9] evaluated *Melilotus officinalis* and *Trifolium fragiferum* under salinity stress and reported that the stress reduced germination rate, chlorophyll content, the number of leaves, leaf area and root and shoot length and dry weight. Dashtakian [10] also found that salinity stress resulted in the reduction of root length, shoot length, leaf area, water consumption, root weight, stem weight, leaf weight and relative growth rate of madder.

Different mechanisms enable plants to tolerate saline conditions: cellular homeostasis and osmotic balance, control and repair of damages caused by the stress, and growth regulation. [1, 4]. ABA has vital roles in all stages of plants life. It also enhances the tolerance of plants to abiotic stresses such as the salinity stress. Enhancement of ABA content in plants is one of their responses to abiotic stresses [11]. Proline concentration also increases under salinity stress. It is also a mechanism of plants tolerance to abiotic stresses [12]. Dashtakian [10] found that proline content in madder increased under salinity stress. Enhancement of soluble sugars is another plant response to abiotic stresses. It helps the balance of osmotic pressure, reduction of water loss and maintenance of turgor pressure [13]. Afzali [9] tested the effect of salinity stress on *Melilotus officinalis* and *Trifolium fragiferum* and reported that the stress increased the content of proline and soluble sugars in plants shoot. Balibrea et al. [14] also reported the enhancement of soluble sugars under saline conditions.

Plants are categorized in three levels according to their tolerance to salinity: (1) obligate halophytes grow in saline soils and their growth is limited in non-saline soils, (2) facultative halophytes are resistant to saline conditions but grow better in non-saline conditions, and (3) glycophytes only grow under non-saline conditions but some of them are somehow resistant to low salinity levels. Nearly all crop plants are glycophyte [15]. Sensitive plants such as the beans face 50% reduction of yield in 60 mM salinity; however, sugar beet tolerates the salinity level up to 260 mM [16-17]. So, the detection of salinity tolerant crop plants is important in order to cultivate the semi saline soils. Some medicinal plants have shown to be more tolerant to salinity than other crop plants.

Thyme is an important aromatic medicinal plant from the Lamiaceae family and is originated from the Mediterranean area. It affects cardiovascular and nervous systems and improves general body health. Thyme is used in medicine, cosmetic and food industries. This medicinal plant can even grow in saline habitats so it looks to have some levels of tolerance to salinity [18-19]. So, this experiment was conducted to evaluate the morpho-physiological responses of different thyme (*Thymus kotschyanus*) populations to salinity stress, to find out how resistant it is.

MATERIALS AND METHODS

This experiment was conducted to study the effect of salinity stress induced by NaCl + $CaCl_2$ on the early growth stages of the seeds of three thyme (*Thymus Kotschyanus* L.) populations collected from a natural habitat in Iran. The experiment was conducted in factorial in the form of a completely randomized design with three replications. Treatments of the experiment included:

Thyme populations: in three levels including three different altitudes from sea level (2500, 2750 and 3050 m above the sea level) in Qom province at the central part of Iran.

Salinity stress induced by NaCl + CaCl2: in six levels including 0, 50, 100, 150, 200 and 250 mM NaCl + $CaCl_2$ (50:50 ratio).

Pots and the quarts which were used to fill them were washed and held in sodium hypochlorite for 48 h in order to be sterilized. Seeds were also sterilized using 70% alcohol and were washed with distilled water. Then, they were treated with benomyl fungicide and were planted. Five pots were considered for each treatment in each replication. Pots were hold in completely controlled conditions inside a growth chamber. The growth chamber was set on 16 hours light, 8 hours darkness. Hoagland was used as the nutritional solution.

When seeds germinated and seedlings established successfully, the saline solutions were applied to the pots. After 45 days, morpho-physiological traits were evaluated. The measured traits included plant height, shoot dry weight, root dry weight, chlorophyll, proline, soluble sugars, relative water content and essential oil content.

To measure the essential oil content, 100 g of the dried shoot was poured in a clevenger balloon and essential oil was produced by hydrodistillation method in 3 hours. The essential oil was then de-hydrated by sodium sulfate and the net weight of the essential oil was measured. To measure the proline content and soluble sugars content, Irrigoyen et al. [20] method was used. First, 0.5 g of the newly produced fresh leaf was grinded in a Chinese Hawn while 20 ml of 80% acetone was being added gradually. Then, the solution was passed through a filter paper and was centrifuged in 3000 rpm for 10 minutes. Then, the upper solution was separated and its absorption rate was detected by a spectrophotometer in 663 and 647 nm. At this stage, relative water content was also measured using the following equation [21]:

$$RWC = \frac{Wf - Wd}{Wt - Wd} \times 100$$

In this equation:

Wf, plant tissue fresh weight,
Wt, swollen plant tissue weight (saturated with water),
Wd, plant tissue dry weight.

Plant chlorophyll content was also measured using the following equation:
Chlorophyll a + b (mg/l) = (7.15 × a663) – (18.71 × a647)

In this equation:

a is the absorption rate in the required wavelength.

At the end, data were analyzed using SAS and means were compared according to the Duncan's multiple range test.

RESULTS AND DISCUSSION

The effect of habitat (population)

Analysis of variance indicated that habitat significantly affected all measured traits ($P \leq 0.01$) except for the shoot dry weight which was not significantly different among the three populations (Table 1).
Mean comparison of the effect of habitat on the measured traits (Table 2) indicated that the highest plant height was achieved in 2500 m above the sea level (54.50 cm) and the lowest plant height was achieved in 3050 m above the sea level (52.11 cm). Although shoot dry weight was not significantly affected by the habitat; however, the highest shoot dry weight (200.20 g/m^2) was related to 2750 m and the lowest shoot dry weight (188.96 g/m^2) was related to 3050 m. Mean comparison showed that root dry weight was the highest (185.21 g/m^2) in 2500 m and the lowest (180.08 g/m^2) in 3050 m. Chlorophyll content was also the highest (0.983 mg/l) in 2500 m and the lowest (0.915 mg/l) in 3050 m. Results indicated that the highest proline content (0.452 mg/l) was achieved in 3050 m and the lowest proline content (0.380 mg/l) was achieved in 2500 m. The highest soluble sugars content (0.516 mg/g) was also achieved in 3050 m and the lowest soluble sugars content (0.441 mg/g) was achieved in 2500 m. Relative water content was the highest (55.86%) in 2500 m and the lowest (51.83%) in 3050 m. Finally, mean comparison showed that the highest essential oil percentage (0.27%) was related to 3050 m and the lowest essential oil percentage (0.24%) was related to 2500 m (Table 2).

Table 1. Analysis of variance of the effect of treatments on the measured traits.

SOV	df	Mean Squares							
		Plant height	Shoot dry weight	Root dry weight	Chlorophyll	Proline	Soluble sugars	Relative water content	Essential oil Percentage
Population (A)	2	**	ns	**	**	**	**	**	**
Salinity (B)	5	**	ns	**	**	**	**	**	**
A × B	10	*	ns	**	ns	*	**	**	**
Error	36	0.346	330.40	1.05	0.00021	0.00023	0.00032	0.95	0.00002
CV (%)	-	1.1	9.31	0.56	1.52	3.69	3.75	1.81	1.75

ns, non significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

Table 2. The variation of the measured traits among the populations.

Populations	Plant height (cm)	Shoot dry weight (g/m^2)	Root dry weight (g/m^2)	Chlorophyll (mg/l)	Proline (mg/l)	Soluble sugars (mg/g)	Relative water content (%)	Essential oil Percentage (%)
2500 m	54.50a	196.54a	185.21a	0.983a	0.380c	0.441c	55.86a	0.24c
2750 m	53.10b	200.20a	182.17b	0.955b	0.417b	0.481b	53.75b	0.25b
3050 m	52.11c	188.96a	180.08c	0.915c	0.452a	0.516a	51.83c	0.27a

Means in a column followed by the same letter are not significantly different at $P \leq 0.01$.

Results of this experiment showed that the measured traits were significantly different among the three populations; proving the effect of elevation, environmental and soil conditions on the collected seeds. The first habitat has the lowest elevation (2500 m) and the lowest EC; however, the third habitat (3050 m) has the highest elevation. Results showed that proline, soluble sugars and essential oil contents were the highest in the third habitat which had higher salinity level compared with the two other habitats; this may be a plant response to salinity stress [1, 4, 12].

Results of other experiments also show that the effect of environment may be noticeable on the growth, yield and physiological parameters of medicinal plants. Elevation from the sea level is an important factor of the environment which affects temperature, humidity and also salinity of soil. Krishnan et al. [22] reported that *Acalypha indica* grew better in 550 m than 350 m above the sea level. Abbaszadeh et al. [23] studied different populations of artemisia and camphor and observed significant variation in the morpho-physiological features. Abbaszadeh et al. [24] evaluated the effect of habitat on camphor seed germination and reported a significant effect on plumule length. Abbaszadeh et al. [7] collected camphor seeds from three different habitats in Iran and then tested their germination features under laboratory conditions. They reported that the habitat significantly affected germination percentage, seed vigor index, seed germination index and radicle length. Abbaszadeh et al. [25] also conducted an experiment to test the effect of habitat on early growth stages of camphor and concluded that the habitat significantly affected shoot and root growth, soluble sugars content, proline content, chlorophyll content and nutrients content including Na, K, Ca, Cl and Mg. So it is clear that the environmental conditions of the habitat affect plants growth parameters even at next generations.

The effect of salinity stress

Analysis of variance indicated the significant effect of salinity stress on all the measured traits ($P \leq 0.01$) except for the shoot dry weight which was not affected by this treatment (Table 1).

Mean comparison of the salinity stress levels (Table 3) showed that plant height was the highest (61.01 cm) in 0mM salinity level; however, it recued by 28.55% when the salinity level was increased to 250 mM. Shoot dry weight was the highest (202.7 g/m^2) in both 100 and 150 mM and it was the lowest (180.7 g/m^2) in 0 mM. The highest root dry weight (208.54 g/m^2) was achieved in 0 mM salinity level and the lowest root dry weight (162.35 g/m^2) was achieved in 250 mM. Results indicated that chlorophyll content was the highest (1.19 mg/l) in 0 mM and reduced by 39.49% when the salinity level increased to 250 mM. Mean comparison showed that the highest proline content (0.723 mg/l) was related to the highest salinity level (250 mM) and the lowest proline content (0.164 mg/l) was related to the lowest salinity level. Increasing the salinity level from 0 to 250 mM resulted in the enhancement of soluble sugars from 0.196 to 0.778 mg/g which means 296.94% enhancement. However, increasing the salinity level from 0 to 250 mM reduced relative water content from 68.9% to 41.52%. Finally, mean comparison showed that the highest essential oil percentage (0.34%) was achieved in 250 mM salinity level and the lowest essential oil percentage (0.16%) was achieved in 0 mM level which shows 112.5% enhancement of essential oil percentage as the result of salinity stress level enhancement (Table 3).

Table 3. The effect of salinity on the measured traits.

NaCl + $CaCl_2$ level	Plant height (cm)	Shoot dry weight (g/m^2)	Root dry weight (g/m^2)	Chlorophyll (mg/l)	Proline (mg/l)	Soluble sugars (mg/g)	Relative water content (%)	Essential oil Percentage (%)
0 mM	61.01a	180.7b	208.54a	1.19a	0.164f	0.196f	68.92a	0.16f
50 mM	58.00b	190.4ab	195.84b	1.08b	0.245e	0.322e	60.51b	0.21e
100 mM	55.69c	202.7a	184.38c	0.98c	0.326d	0.407d	56.06c	0.25d
150 mM	52.54d	202.7a	175.86d	0.89d	0.455c	0.550c	50.80d	0.27c
200 mM	48.57e	197.95ab	167.94e	0.81e	0.585b	0.623b	45.06e	0.30b
250 mM	43.59f	196.75ab	162.35f	0.72f	0.723a	0.778a	41.52f	0.34a

Means in a column followed by the same letter are not significantly different at $P \leq 0.01$.

Results showed that salinity stress reduced plant shoot and root growth, chlorophyll content and relative water content. In saline soils, the concentration of some salts such as chlorides and sulfates of sodium is high and toxic to plants. High concentration of salts such as NaCl and Na_2SO_4 makes the osmotic pressure of soil solution higher than the osmotic pressure of plant root cells; disturbing plants nutrients and water absorption. Salinity mainly reduces the absorption of N, P, K and Cl. When the potential of water falls below the critical level, plants face with water shortage stress which results in the reduction of germination percentage rate and seedling growth [1, 4, 26].

The effect of salinity stress on plant seed germination is reported in many experiments. Abbaszadeh et al. [7] reported the significant effect of salinity stress camphor seed germination percentage. Leilah et al. [5] tested the effect of salinity stress (50 to 200 m/cm^3 NaCl) on three canola cultivars and found the significant effect on germination speed and percentage. Tlig et al. [27] found that enhancement of salinity level negatively affected the germination percentage. Jamil et al. [28] concluded that salinity stress

reduced germination traits of sunflower including germination percentage, germination speed, radicle length, plumule length and radicle fresh weight. Khan et al. [29] also studied the effect of salinity stress on *Suaeda fruticosa* and concluded that germination percentage reduced when salinity level increased.
There are also experiments testing the effect of salinity on plants growth, yield and quality. Navarro et al. [30] conducted experiments to test the effect of salinity stress on plants endemic to Spain and reported the reduction of dry matter, leaf area, plant height, photosynthesis and intercellular space. Amirian [31] reported the reduction of whole plant growth of sorghum as the result of salinity stress. Suriyan et al. [8] observed the reduction of sorghum LAR, germination percentage, leaf area, dry weight and fresh weight as the result of salinity stress. Ryuichi et al. [32] also studied the effect of salinity stress on *Nicotiana tabacum* photosynthesis and morphology and found that the stress reduced plant stem diameter and photosynthesis.

Table 4. The effect of the interaction of population × salinity on the measured traits.

NaCl + $CaCl_2$ level	Plant height (cm)	Shoot dry weight (g/m^2)	Root dry weight (g/m^2)	Chlorophyll (mg/l)	Proline (mg/l)	Soluble sugars (mg/g)	Relative water content (%)	Essential oil Percentage (%)
2500 × 0 mM	62.57a	187.0ab	210.56a	1.23a	0.13p	0.15n	72.03a	0.14p
2500 × 50 mM	58.49cd	182.0b	200.06d	1.12a	0.22m	0.29k	62.50d	0.20m
2500 × 100 mM	56.64e	222.0a	189.30g	1.00a	0.29k	0.39i	56.86gf	0.24j
2500 × 150 mM	54.00g	196.0ab	178.43j	0.93a	0.41h	0.50g	54.60h	0.27gh
2500 × 200 mM	49.92j	177.0b	169.46m	0.84ab	0.53f	0.60e	46.40jk	0.29f
2500 × 250 mM	45.39L	212.0ab	163.43o	0.77ab	0.68c	0.71c	42.76mn	0.33c
2750 × 0 mM	61.03b	177.0b	208.50b	1.20a	0.16o	0.20m	68.53b	0.16o
2750 × 50 mM	57.93d	199.0ab	195.43e	1.10a	0.24Lm	0.31k	60.86e	0.21L
2750 × 100 mM	55.50f	199.0ab	183.03h	0.98a	0.32j	0.41hi	56.06gh	0.25i
2750 × 150 mM	52.35h	222.0a	176.26k	0.90a	0.47g	0.55f	50.16i	0.27gh
2750 × 200 mM	48.21k	210.0ab	167.70n	0.82ab	0.58e	0.61e	45.13kl	0.30e
2750 × 250 mM	43.57m	193.0ab	162.10op	0.72ab	0.72b	0.79b	41.73n	0.34b
3050 × 0 mM	59.45c	177.0b	206.56c	1.15a	0.20n	0.23L	66.20c	0.18n
3050 × 50 mM	57.59de	189.0ab	192.03f	1.04a	0.26L	0.36j	58.16f	0.22k
3050 × 100 mM	54.94gf	186.0ab	180.83i	0.95a	0.36i	0.42h	55.26gh	0.26h
3050 × 150 mM	51.27i	189.0ab	172.90L	0.86ab	0.48g	0.58e	47.63j	0.27g
3050 × 200 mM	47.58k	206.0ab	166.66n	0.79ab	0.64d	0.65d	43.66Lm	0.31d
3050 × 250 mM	41.82n	184.0b	161.53p	0.67bc	0.76a	0.83a	40.06o	0.35a

Means in a column followed by the same letter are not significantly different at P≤0.01.

Results of our experiment also showed that salinity stress increased the content of proline, soluble sugars and essential oil. These may a plant response to harsh environmental conditions, enabling plants to tolerate the condition. [1, 4, 12]. Afzali [9] reported that salinity stress increased the content of proline and soluble sugars in shoot of *Melilotus officinalis* and *Trifolium fragiferum*. Balibrea et al. [14] tested the effect of salinity stress on tomato plants and found a significant effect on the enhancement of soluble sugars. Dashtakian [10] also observed salinity stress increased proline content of madder plants.

The effect of the interaction of habitat × salinity

Analysis of variance showed that the effect of the interaction of the two factors was significant on all measured traits except for the shoot dry weight and chlorophyll content (Table 1).
Mean comparison (Table 4) indicated that plant height was the highest in 2500 m × 0 mM (62.57 cm) and the lowest in 3050 m × 250 mM (41.82 cm). Shoot dry weight was the highest in 2500 × 100 mM and 2750 × 150 mM (222.0 g/m^2) and the lowest in 2500 m × 200 mM, 2750 m × 0 mM and 3050 m × 0 mM interactions (177.0 g/m^2). The highest root dry weight (210.56 g/m^2) was related to 2500 m × 0 mM and the lowest root dry weight (161.53 g/m^2) was related to 3050 m × 250 mM. Mean comparison showed that chlorophyll content and relative water content were both the highest in 2500 m × 0 mM (1.23 mg/l and 72.03%, respectively) and the lowest in 3050 m × 250 mM (0.67 mg/l and 40.06%, respectively). However, the highest content of proline, soluble sugars and essential oil (0.76 mg/l, 0.83 mg/g and 0.35%, respectively) was achieved in 3050 m × 250 mM, and the lowest content of the measured traits was achieved in 2500 m ×0 mM (0.13 mg/l, 0.15 mg/g and 0.14%, respectively).

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