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ORIGINAL PAPER

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DOES FISH FLOUR AND CALCIUM IMPROVE MENTHA DEVELOPMENT, ENZYME ACTIVITIES AND PHENOLIC COMPOUNDS UNDER HIGH SALINITY

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ABSTRACT

The use of natural and biodegradable material (such as fish flour) counteracts stress as cheaper and safer alternative for toxic chemicals (such as pesticides). The effect of calcium and fish flour (Ca and FF) (single or in combination) to improve plant tolerance against salt stress was studied. Sterilized mentha seeds were imbibed in each treatment: FF (10 g mL^{-1}), Ca (1, 3%) applied alone and in Ca+FF-combination shaking for 24 h at 150 rpm. Changes in the antioxidants (carotenoids, phenolic, and flavonoid), enzymatic system (superoxidase – SOD, guaiacol-dependent peroxidase – GPX and phenylalanine ammonia-lyase – PAL) and lipid peroxidation levels of mentha seedlings were investigated under salt stress. It was suggested that Ca and/or FF had positive regulation effects on the key enzyme activities related to phenolic compounds biosynthesis and individual phenolic contents under salt stress. Additionally, the mentha plants developed from Ca+FF-combined pre-treatments showed better response to salinity than either Ca or FF single pre-treatment. Suppression of salt injury by Ca+FF pre-treatment reduced the LPO levels, increased enzyme activities and promoted total flavonoid and phenolic contents. Ca+FF-combined pre-treatment of mentha seeds seem to be a reliable, not-expensive and easy procedure to enhance plant salt tolerance and to gain more biomass.

Key words: calcium, fish flour, mentha, phenolic contents, salinity stress

INTRODUCTION

Seed development is highly important, because almost all plant cultivation in horticulture and agriculture is based on this material. Among the seeds, mentha is the most widespread cultivated plant. However, mentha is very salt-sensitive, its growth and development are significantly affected by salt stress, which is the first reason of plant loss worldwide. It becomes an everlasting challenge to agriculture, because of the climate change and expanding area of salt-affected land (such as Turkey). For adaptation to salt stress, seeds can be primed to respond with multiple biochemical and molecular modifications. Seed pre-treatment is defined as the pre-exposure of seeds to chemical or natural agents making them more resistant to subsequent stresses. It is not only the activated series of complex metabolic reactions to increase fast and smooth emergence, but also involves rebuilding of cellular structure to provide high vigor and more yields [Bewley et al. 2013]. As a result, seed pre-treatment methods achieve low-price protection in relation to high-protection tolerance to environmental stresses.

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Our research and many studies have documented the benefits of calcium in salt tolerance [Yücel and Heybet 2016, Manaa et al. 2014]. Ca is a secondary messenger in the signal transduction between plant responses and abiotic stress. Unde salt stress, the exchange of plasma membrane-bound calcium with sodium is a principle and primary factor included in salt tolerance responses. The role of calcium in alleviating the deleterious salt stress effect *via* regulating the gene expression and carbohydrate metabolism on plant growth has been reported [Bonomelli and Ruiz 2010]. Therefore, application of $CaCl_2$ alleviates the growth inhibition of various plant species and provides recovery under salt stress [Yücel and Heybet 2016].

As another pre-treatment agents, anchovies (*Engraulis encrasicholus*) are small pelagic and the most abundant fish species in the world and also in Black Sea of Turkey. More than 60% of anchovy production is disposed as waste [Fisheries statistics 2000]. Consequently, the use of edible anchovy waste becomes highly important for economy and environmental pollution [Boscolo 2001]. The amino acids of Turkish anchovy contain in higher quantities the glutamic acid (a proline analogue), proline, aspartic acids and arginine with respect to the anchovy species of other countries [Fisheries statistics 2000]. In the plant studies, exogenous application of phenolic compounds or proline, and precursors combinations may be used to induce and improve phenolic compounds biosynthesis and related enzyme activities *via* the phenylpropanoid pathway [Shetty 2004]. Either the effects of Ca and FF or synergetic effects of them on phenolic compounds biosynthesis under salt stress have not been studied so far.

The aim of the recent study is to improve the salt tolerance of mentha seed using calcium, fish flour and their combinations as natural elicitors and to induce mentha vigor response (germination percentage, fresh weight and seedlings length) and to stimulate phenolic compounds biosynthesis (GPX and PAL enzyme activities, proline and also total flavonoids and phenolics contents).

MATERIAL AND METHODS

Mentha seeds cultivar (*Mentha piperita* L.) was provided from Ege University Research Institute. 10 g of sterilized mentha seeds were imbibed in each treatment: FF, Ca applied alone ($CaCl_2$) and in Ca+FF combination shaking for 24 h at 150 rpm. After pre-treatment process, the seeds were washed off three times with water and dried back for 48 h. Fish flour (FF) was obtained from a local anchovy fabric (Sinop Yem Fabric, 0.74 g protein g^{-1} FF). Anchovy fish flour includes 90.76% dry matter, 9.86% glutamic acid, 7.48% aspartic acid, 6.31% lysine, 5.78% leucine, 4.89% proline and 4.41% arginine. It was resized to obtain a homogeneous size particle of 0.84 mm and ground to reach the size particle of 0.5 mm. The fish flour dilutions tested were 8, 10 and 12 mL^{-1} g fish flour. 15 mentha seeds were sown in glass containers with non-cellulosic paper. The seeds and seedlings were constantly fed with Hoagland solution. Glass containers with germination seedlings were sealed to inhibit evaporation. Germinating seedlings were transferred to plastic pots of 1 L capacity and each pot with 10 seedlings was replicated three times. They were put to a plant growth cabin illuminated with 400–700 nm and 25 µmol m^{-2} s^{-1} on a 14/10 h (day/night) regime at 20°C. The salt concentration was 100 mM sodium chloride. Control group was admitted as salinity (%b0) and no exogenous treatment.

Germinated seeds were seeds that the radicle emerged through the seed coat and reached 2 mm in length. Germination percentage (GP) of all treatments was calculated using the equation: GP = n/N × 100%, where n represents the germinated seed number of g and N is the total seeds number.

For all enzyme detection, 1 g of samples with 4 mL^{-1}, 20 mM phosphate buffer (pH 7.4) was homogenized. After that, 1.5 mL^{-1} of homogenate was centrifuged (15 × g for 15 min at 4°C). Activity of phenylalanine ammonia-lyase (PAL: EC 4.3.1.24) enzyme was measured according to Hodgins method at 270 nm [1971] (E = 19.7 mM^{-1} cm^{-1}). Assay concentrations contained distilled water, 150 mM Trizma base buffer pH 8.5, 3 mM phenylalanine and sample extract (enzyme). The guaiacol peroxidase (GPX: EC 1.11.1.7) activity assay was determined by the method of Nakano and Asada [1981]. Total reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 18 mM guaiacol, 10 mM hydrogen peroxide (H_2O_2) and sam-

ple extract (enzyme). Activity was determined in bsorbance at 470 nm because of guaiacol oxidation (E = 26.6 mM^{-1} cm^{-1}) [Nakano and Asada 1981].

Total phenolic content of mentha seedlings was determined according to McCue and coworkers method [2000] with Folin-Ciocalteu phenol reagent. The absorbance of mixtures was recorded in a spectrophotometer at 725 nm. Standard calibration curve of gallic acid (0–100 mg mL^{-1}) was drawn and used as a phenolic standard. Flavonoid content of mentha seedlings was measured using aluminum chloride colorimetric method according to Du and coworkers' method [2009]. The concentration of total flavonoid was measured from a standard calibration curve of rutin in the concentration from 0–160 µg mL^{-1} at 510 nm. The amount of lipid peroxidation was calculated by spectrophotometric method based on thiobarbituric acid (TBA) reactivity (E = 153 mM^{-1} cm^{-1}) [Buege and Aust 1978].

Chlorophylls and carotenoids concentrations (µg cm^{-2} FW) were calculated by Lichtenthaler and Wellburn method [Lichtenthaler and Wellburn 1983]. The amount of proline of mentha seedlings was measured based on reaction of ninhydrin with proline at 520 nm [Bates and Waldren 1973]. The total protein concentration of mentha seedlings was measured according to Bradford method [Bradford 1976].

Mentha seedlings of HPLC analysis of individual phenolics were measured according to Lee and Scagel [2009]. Analyses were achieved using Agilent 1100 HPLC system with UV detector (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples were separated on C18 Kinetex column (100 × 2.1 mm) with 2.6 µm particle size. The mobile phase was composed of eluent A: 0.1% trifluoroacetic acid (TFA) in water and eluent B: acetonitirile. The elution gradient was launched using 90% solvent A: 10% solvent B and after 5 minutes 20% solvent A: 80% solvent B at constant flow rate of 0.4 mL^{-1} min. The absorbance was scanned at 280 nm by the UV detector. Each identified compound amount was calculated by comparing integrated peak areas to calibration curves prepared with their analytical standard (obtained from Sigma), and then expressed as µg phenolic per g dry weight.

Tukey test was performed for statistical significance analysis. The data were expressed as the mean ±SD of 3 independent experiments and 3–4 replicates of biochemical tests. Also Pearson correlation comparison was made for each substrate and/or enzyme.

RESULTS

Mentha seeds germinated with different pre-treatment, calcium (Ca: 1, 3%), fish flour (FF: 8, 10, 12 g mL^{-1}) and Ca+FF-combination under non-saline and salt stress conditions (100 mM NaCl) were analyzed and compared with one another and a control during mentha growth on the 1st, 6th and 11th days. As part of this study, fresh weight and the percentage of seed germination, lengths of shoot – root were investigated (Tabs. 1 and 2). FF and Ca improved all the investigated parameters under high salinity. Salt treatment decreased the fresh weights from 120 ±3 mg to 70 ±6 mg on the 11th day ($p < 0.01$). However, the fresh weight levels were able to be restored with Ca-alone, FF-alone and Ca+FF--combined pre-treatment under non-saline and salt stress conditions. Fresh weights of mentha seeds pre-treatment with 1% Ca + g 10 mL^{-1} FF resulted in a 1.5-fold increase under salt stress compared to non-saline control on the 11th day.

Salt stress decreased the seed germination rate from 100 to 70% on the 11th day. The rates, however, recovered in all pre-treatment groups under salt stress ($p < 0.05$). The maximum increases of germination rate were obtained the FF-alone and 1% Ca + g 10 mL^{-1} FF-combined pre-treatment mentha seeds under salt stress on the 11th day. Germination percentage of mentha seeds pre-treatment with Ca+FF did not change significantly under non-saline conditions ($p > 0.05$).

Shoot lengths of all treatment groups increased with time under non-saline and salt stress conditions (Tab. 2). The shoot lengths of control group under salt stress dropped from 8.1 ±2.0 to 3.7 ±0.6 cm on the 11th day. However, the levels increased with Ca-alone, FF-alone and Ca+FF-combined pre-treatment under salinity and non-saline conditions when compared with salt treatment alone group. However, the increases were not dependent on Ca concentration. The maximum shoot length of the 1% Ca + g 10 mL^{-1} FF-combined pre-treatment group was determined as 7.9 ±0.1 cm under salt stress.

Table 1. Growth parameters: fresh weight and % germination of mentha for different treatments; control, FF (g 10 mL^{-1}) and 1, 3% Ca + FF under non-saline and FF (g 10 mL^{-1}), 1, 3% Ca + FF (g 8 mL^{-1}, g 10 mL^{-1}, g 12 mL^{-1}) under salt stress 100 mM NaCl

Growth parameters	Salinity %	Treatments (% + g 10 mL^{-1})	Plant growth (in days)		
			1	6	11
Fresh weight (mg)	0	control	40 ±2	120 ±2	120 ±3
		FF	50 ±2	100 ±2$^{\delta}$	180 ±8$^{\varepsilon}$
		1% Ca^{2+}	40 ±2	90 ±2$^{\delta}$	160 ±5$^{\varepsilon}$
		3% Ca^{2+}	40 ±2	90 ±2$^{\delta}$	160 ±5$^{\varepsilon}$
		1% Ca^{2+} + g 10 mL^{-1} FF	50 ±2	90 ±2$^{\delta}$	170 ±9$^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	50 ±2	90 ±2$^{\delta}$	160 ±5$^{\varepsilon}$
	100	control	40 ±2	60 ±10	70 ±6
		FF	50 ±2	130 ±08$^{\varepsilon}$	170 ±5$^{\varepsilon}$
		1% Ca^{2}	40 ±1	90 ±11$^{\delta}$	130 ±4$^{\varepsilon}$
		3% Ca^{2+}	40 ±2	90 ±11$^{\delta}$	130 ±4$^{\varepsilon}$
		1% Ca^{2+} + g 8 mL^{-1} FF	50 ±2	110 ±09$^{\varepsilon}$	130 ±4$^{\varepsilon}$
		1% Ca^{2+} + g 10 mL^{-1} FF	50 ±2	90 ±11$^{\delta}$	180 ±11$^{\varepsilon}$
		1% Ca^{2+} + g 12 mL^{-1} FF	50 ±2	100 ±08$^{\varepsilon}$	160 ±12$^{\varepsilon}$
		3% Ca^{2+} + g 8 mL^{-1} FF	40 ±2	100 ±09$^{\varepsilon}$	130 ±6$^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	40 ±2	110 ±11$^{\varepsilon}$	150 ±5$^{\varepsilon}$
		3% Ca^{2+} + g 12 mL^{-1} FF	50±2	90 ±08$^{\varepsilon}$	140 ±4$^{\varepsilon}$
% Germination	0	control	0.2	87	100
		FF	–	100$^{\delta}$	100$^{\delta}$
		1% Ca^{2+}	–	80	82
		3% Ca^{2+}	–	83	84
		1% Ca^{2+} + g 10 mL^{-1} FF	0.1	79	79
		3% Ca^{2+} + g 10 mL^{-1} FF	–	80	80
	100	control	0.2	70	70
		FF	–	93$^{\delta}$	100$^{\varepsilon}$
		1% Ca^{2+}	–	90	90
		3% Ca^{2+}	–	91	94
		1% Ca^{2+} + g 8 mL^{-1} FF	–	87$^{\delta}$	87$^{\delta}$
		1% Ca^{2+} + g 10 mL^{-1} FF	–	100$^{\varepsilon}$	100$^{\varepsilon}$
		1% Ca^{2+} + g 12 mL^{-1} FF	–	93$^{\delta}$	93$^{\delta}$
		3% Ca^{2+} + g 8 mL^{-1} FF	0.2	80$^{\delta}$	87$^{\delta}$
		3% Ca^{2+} + g 10 mL^{-1} FF	0.1	93$^{\delta}$	93$^{\delta}$
		3% Ca^{2+} + g 12 mL^{-1} FF	–	80$^{\delta}$	87$^{\delta}$

Data are 'mean ±SD' and are averages of 10 seedlings
$^{\delta}p < 0.05$ (probably significant)
$^{\varepsilon}p < 0.01$ (definitely significant)

Table 2. Growth parameters: shoot and root length of mentha for different treatments; control, FF (g 10 mL^{-1}) and 1, 3% Ca + FF under non-saline and FF (g 10 mL^{-1}), 1, 3% Ca + FF (g 8 mL^{-1}, g 10 mL^{-1}, g 12 mL^{-1}) under salt stress 100 mM NaCl

Growth parameters	Salinity %	Treatments (mM + g 10 mL^{-1})	Plant growth (in days)		
			1	6	11
Root length (cm)	0	control	0.2	5.7 ±1.1	6.0 ±2.0
		FF	–	6.8 ±0.7	7.7 ±0.5
		1% Ca^{2+}	–	4.7 ±0.1 $^{\delta}$	5.0 ±0.2 $^{\delta}$
		3% Ca^{2+}	–	4.6 ±0.1 $^{\delta}$	5.0 ±0.2 $^{\delta}$
		1% Ca^{2+} + g 10 mL^{-1} FF	0.1	4.7 ±0.1 $^{\delta}$	5.2 ±0.2 $^{\delta}$
		3% Ca^{2+} + g 10 mL^{-1} FF	–	4.5 ±0.2 $^{\delta}$	4.7 ±0.1$^{\delta}$
	100	control	0.2	1.2 ±0.3	1.4 ±0.6
		FF	–	4.6 ±0.2$^{\varepsilon}$	4.9 ±0.5$^{\varepsilon}$
		1% Ca^{2+}		4.2 ±0.2$^{\varepsilon}$	4.6 ±0.2$^{\varepsilon}$
		3% Ca^{2+}		4.1 ±0.2$^{\varepsilon}$	4.6 ±0.2$^{\varepsilon}$
		1% Ca^{2+} + g 8 mL^{-1} FF	–	4.9 ±0.1$^{\varepsilon}$	6.2 ±0.4$^{\varepsilon}$
		1% Ca^{2+} + g 10 mL^{-1} FF	–	4.8 ±0.2$^{\varepsilon}$	5.7 ±0.1$^{\varepsilon}$
		1% Ca^{2+} + g 12 mL^{-1} FF	–	2.7 ±0.2$^{\varepsilon}$	5.0 ±1.2$^{\varepsilon}$
		3% Ca^{2+} + g 8 mL^{-1} FF	0.2	4.4 ±0.3$^{\varepsilon}$	4.8 ±0.6$^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	0.1	5.4 ±0.2$^{\varepsilon}$	6.0 ±0.5$^{\varepsilon}$
		3% Ca^{2+} + g 12 mL^{-1} FF	–	2.5 ±0.1$^{\delta}$	4.1 ±0.4 $^{\delta}$
Shoot length (cm)	0	control	0.2	5.2 ±1.1	8.1 ±2.0
		FF	–	3.7 ±0.7 $^{\varepsilon}$	8.5 ±0.5
		1% Ca^{2+}		3.2 ±0.7 $^{\varepsilon}$	5.9 ±0.7 $^{\varepsilon}$
		3% Ca^{2+}		3.4 ±0.7 $^{\varepsilon}$	5.5 ±0.7 $^{\varepsilon}$
		1% Ca^{2+} + g 10 mL^{-1} FF	0.1	3.3 ±0.1 $^{\varepsilon}$	6.4 ±0.2 $^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	–	2.8 ±0.2 $^{\varepsilon}$	6.0 ±0.1$^{\varepsilon}$
	100	control	0.2	3.2 ±0.3	3.7 ±0.6
		FF	–	4.6 ±0.2 $^{\varepsilon}$	7.4 ±0.5 $^{\varepsilon}$
		1% Ca^{2+}		3.0 ±0.2	6.1 ±0.4 $^{\varepsilon}$
		3% Ca^{2+}		3.1 ±0.2	6.1 ±0.4 $^{\varepsilon}$
		1% Ca^{2+} + g 8 mL^{-1} FF	–	2.1 ±0.1 $^{\delta}$	6.5 ±0.4 $^{\varepsilon}$
		1% Ca^{2+} + g 10 mL^{-1} FF	–	2.9 ±0.2	7.9 ±0.1 $^{\varepsilon}$
		1% Ca^{2+} + g 12 mL^{-1} FF	–	2.3 ±0.2 $^{\delta}$	6.9 ±1.2 $^{\varepsilon}$
		3% Ca^{2+} + g 8 mL^{-1} FF	0.2	2.1 ±0.3 $^{\delta}$	6.6 ±0.6 $^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	0.1	3.3 ±0.2	7.4 ±0.5 $^{\varepsilon}$
		3% Ca^{2+} + g 12 mL^{-1} FF	–	1.7 ±0.1 $^{\varepsilon}$	6.7 ±0.4 $^{\varepsilon}$

Data are 'mean ±SD' and are averages of 10 seedlings
$^{\delta}p < 0.05$ (probably significant)
$^{\varepsilon}p < 0.01$ (definitely significant)

Table 3. Total phenolic and flavonoid contents of mentha for different treatments; control, FF (g 10 mL^{-1}) and 1, 3% Ca + FF under non-saline and FF (g 10 mL^{-1}), 1, 3% Ca + FF (g 8 mL^{-1}, g 10 mL^{-1}, g 12 mL^{-1}) under salt stress 100 mM NaCl

	Salinity %	Treatments (mM + g 10 mL^{-1})	Mentha organs		
			shoot	root	seed
Total soluble phenolic content (mg g^{-1} FW)	0	control	14.6 ±1.4	7.8 ±0.8	8.9 ± 0.2
		FF	18.8 ± 2.0$^{\varepsilon}$	11.9 ±0.6$^{\varepsilon}$	13.4 ±0.5$^{\varepsilon}$
		1% Ca^{2+}	15.6 ±1.4	8.8 ±0.8	9.1 ± 0.2
		3% Ca^{2+}	15.9 ±1.4	8.5 ±0.8	9.0 ± 0.2
		1% Ca^{2+} + g 10 mL^{-1} FF	22.7 ±0.7$^{\varepsilon}$	14.4 ±1.1$^{\varepsilon}$	15.2 ±0.2$^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	25.7 ±0.7$^{\varepsilon}$	16.6 ±0.9$^{\varepsilon}$	16.3 ±0.1$^{\varepsilon}$
	100	control	22.1 ±3.2$^{\varepsilon}$	20.1 ±1.8	14.1 ±0.6
		FF	23.8 ±2.4	21.5 ±1.2	16.8 ±0.5$^{\varepsilon}$
		1% Ca^{2+}	22.2 ±3.2	20.4 ±1.8	14.8 ±0.5
		3% Ca^{2+}	22.0 ±2.2	20.4 ±1.5	14.2 ±0.5
		1% Ca^{2+} + g 8 mL^{-1} FF	24.0 ±2.2$^{\varepsilon}$	22.4 ±0.9$^{\delta}$	14.9 ±0.4
		1% Ca^{2+} + g 10 mL^{-1} FF	27.3 ±1.6$^{\varepsilon}$	22.6 ±1.5$^{\delta}$	16.6 ±0.7$^{\varepsilon}$
		1% Ca^{2+} + g 12 mL^{-1} FF	27.6 ±2.3$^{\varepsilon}$	29.9 ±0.7$^{\delta}$	21.0 ±1.2$^{\varepsilon}$
		3% Ca^{2+} + g 8 mL^{-1} FF	25.1 ±2.7$^{\varepsilon}$	23.0 ±1.2$^{\varepsilon}$	10.4 ±0.4$^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	24.4 ±1.8$^{\varepsilon}$	25.0 ±2.1$^{\varepsilon}$	14.3 ±0.1
		3% Ca^{2+} + g 12 mL^{-1} FF	23.5 ±2.2$^{\varepsilon}$	23.6 ±2.1$^{\varepsilon}$	11.8 ±1.2$^{\delta}$
Total flavonoid content (µg g^{-1} FW)	0	control	176 ±12	150 ±03	543 ±25
		FF	214 ±11$^{\varepsilon}$	187 ±11$^{\varepsilon}$	586 ±25$^{\delta}$
		1% Ca^{2+}	186 ±12	155 ±03	546 ±25
		3% Ca^{2+}	189 ±12	152 ±03	547 ±25
		1% Ca^{2+} + g 10 mL^{-1} FF	225 ±13$^{\varepsilon}$	204 ±13$^{\delta}$	676 ±32$^{\varepsilon}$
		3% Ca^{2+} + g 10 mL^{-1} FF	224 ±15$^{\varepsilon}$	198 ±11$^{\varepsilon}$	589 ±21$^{\varepsilon}$
	100	control	200 ±13	170 ±13	600 ±6
		FF	214 ±11	177 ±13	634 ±5$^{\delta}$
		1% Ca^{2+}	205 ±13	172 ±13	605 ±6
		3% Ca^{2+}	202 ±13	171 ±13	604 ±6
		1% Ca^{2+} + g 8 mL^{-1} FF	238 ±13$^{\delta}$	193 ±11$^{\delta}$	624 ±4$^{\varepsilon}$
		1% Ca^{2+} + g 10 mL^{-1} FF	258 ±10$^{\varepsilon}$	226 ±13$^{\varepsilon}$	662 ±1$^{\varepsilon}$
		1% Ca^{2+} + g 12 mL^{-1} FF	310 ±13$^{\varepsilon}$	279 ±12$^{\varepsilon}$	695 ±12$^{\varepsilon}$
		3% Ca^{2+} + g 8 mL^{-1} FF	252 ±11$^{\varepsilon}$	214 ±11	604 ±4
		3% Ca^{2+} + g 10 mL^{-1} FF	257 ±11$^{\varepsilon}$	224 ±13$^{\varepsilon}$	623 ±1$^{\delta}$
		3% Ca^{2+} + g 12 mL^{-1} FF	270 ±13$^{\varepsilon}$	229 ±11$^{\varepsilon}$	650 ±12$^{\varepsilon}$

Data are 'mean ±SD' and are averages of 10 seedlings
$^{\delta}p < 0.05$ (probably significant)
$^{\varepsilon}p < 0.01$ (definitely significant)

The maximum root length in 1% Ca + g 10 mL^{-1} FF-combined pre-treatment was determined as 6.2 ±0.4 cm under salt stress. The root length decreased under salt stresses during the treatment period and reached their minimum (by a 4.3-fold decrease) on the 11th day. In our results, the shoot – root length, fresh weight and germination percentage were determined in salt stressed seedlings and this plant showed a greater biomass indicating greater tolerance than saline-control. FF and Ca improved the total amount of phenolic compounds and flavonoids under high salinity. Total amount of phenolic compounds increased with salt stress, while the levels varied similarly for both Ca concentrations under non-saline and salt stress conditions (Tab. 3). The effect of the pre-treatment on the increase of the total phenolic contents of shoots under non-saline and salt stress was, in order of strength, Ca < FF < Ca+FF-combined.

The highest phenolic contents for shoot, root and seed were obtained from the 3% Ca + g 10 mL^{-1} FF-combined pre-treatment group, which showed a 1.8, 2.1 and 1.8-fold increase when compared to that of the control under non-saline conditions. Under salt stress, the maximum phenolic contents for shoot, root and seed were found to have increased by 1.9, 2.9 and 1.9-fold, in mentha shoots grown from seeds primed with 1% Ca + g 10 mL^{-1} FF, as compared to the same parameters in the non-saline control. Salt stress increased total flavonoid content of mentha seedlings. The contents of the mentha seedling organs increased significantly in the FF and Ca+FF treatment group ($p < 0.01$). However, the beneficial effects of FF pre-treatment on the total flavonoid contents of all mentha organs grown from the Ca+FF pre-treatment group were induced by increasing FF concentrations under salt stress (data not shown). However, the contents did not increase significantly by increasing the Ca concentration ($p > 0.05$). The maximum flavonoid levels in mentha that germinated from the seeds primed with a 1% Ca + g 10 mL^{-1} FF-combined pre-treatment resulted in 1.5, 1.5 and 1.2-fold increases under salt stress, as compared to non-saline control, for shoot, root and seed, respectively.

Table 4. Parameters: chlorophyll, carotenoid and LPO content, PAL and GPX activities; control, FF (g 12 mL^{-1}), 1% Ca, 1% Ca + FF (g 12 mL^{-1}), and 3% Ca + FF (g 12 mL^{-1}) under non-saline and salt stress 100 mM NaCl on the 11th day

Parameter	No salinity control	Salinity stress, 100 mM NaCl				
		non-treatment	1% Ca	combined pretreatment Ca + FF (g 12 mL^{-1})		
				FF (g 12 mL^{-1})	1% Ca +FF	3% Ca +FF
Chlorophylls (µg cm^{-2})	148 ±10	68 ±10$^{\varepsilon}$	70 ±10$^{\varepsilon}$	320 ±11$^{\varepsilon}$	340 ±21$^{\varepsilon}$	330 ±12$^{\varepsilon}$
Carotenoids (µg cm^{-2})	5.5 ±0.3	3.5±0.3$^{\delta}$	4.5 ±0.3$^{\delta}$	7.7 ±0.3$^{\varepsilon}$	9.1 ±0.2$^{\varepsilon}$	8.5 ±0.2$^{\varepsilon}$
PAL (U mg^{-1} protein)	32 ±1	98 ±3$^{\varepsilon}$	105 ±1	148 ±18$^{\varepsilon}$	152 ±12$^{\varepsilon}$	145 ±14$^{\varepsilon}$
GPX (U mg^{-1} protein)	0.5 ±0.1	2.5 ±0.3$^{\varepsilon}$	3.5 ±0.3$^{\varepsilon}$	4.4 ±0.3$^{\varepsilon}$	5.9 ±0.2$^{\varepsilon}$	4.9 ±0.4$^{\varepsilon}$
LPO (nmol MDA g^{-1})	3.5 ±0.5	6.5 ±0.6$^{\varepsilon}$	6.0 ±0.6$^{\varepsilon}$	5.4 ±1.0$^{\delta}$	4.4 ±1$^{\delta}$	5.1 ±1.0$^{\varepsilon}$
Proline (µmol g^{-1} FW)	3.4 ±0.1	6.9 ±0.1$^{\varepsilon}$	6.8 ±0.1$^{\varepsilon}$	6.8 ±0.1$^{\varepsilon}$	5.9 ±0.1$^{\varepsilon}$	4.5 ±0.1$^{\delta}$

Data are 'mean ±SD' and are averages of 10 seedlings
$^{\delta}p < 0.05$ (probably significant)
$^{\varepsilon}p < 0.01$ (definitely significant)

Table 5. 1% Ca +FF (g 12 mL^{-1}), pretreatment and salt stress on the content of individual phenolic compounds in the shoot of mentha

Phenolic compounds (µg g^{-1})	No salinity, 0 mM NaCl		Salinity stress, 100 mM NaCl	
	control	Ca + FF	control	Ca + FF
Scopelletin	0.79 ±0.02	4.04 ±0.15$^{\varepsilon}$	0.99 ±0.04	4.60 ±0.11$^{\varepsilon}$
Rutin	2.99 ±0.05$^{\varepsilon}$	19.38 ±0.22$^{\delta}$	9.48 ±0.18	24.00 ±0.15$^{\varepsilon}$
Syringic acid	4.19 ±0.06$^{\delta}$	12.30 ±0.22$^{\varepsilon}$	5.99 ±0.05	15.30 ±0.01$^{\varepsilon}$
Gallic acid	1.28 ±0.01$^{\delta}$	2.78 ±0.03$^{\delta}$	1.88 ±0.01	4.81 ±0.01$^{\varepsilon}$
Salicylic acid	1.30 ±0.03	1.20 ±0.03$^{\varepsilon}$	0.49 ±0.02	1.29 ±0.03

Data are 'mean ±SD' and are averages of 10 seedlings
$^{\delta}p < 0.05$ (probably significant)
$^{\varepsilon}p < 0.01$ (definitely significant)

Table 4 shows the chlorophyll-carotenoid, proline contents, PAL, GPX enzyme activities and the LPO levels of mentha shoots primed with Ca+FF under control and salt stress on the 11th day. Chlorophyll content decreased significantly under salt stress, whereas with Ca, FF and Ca+FF-combined pre-treatment, the chlorophyll contents were enriched. Salt stress resulted in a decrease ($p < 0.05$) in β-carotene level, while the Ca, FF and Ca+FF-combined pre-treatment induced an additional increase ($p < 0.05$) in β-carotene contents under salt stress. Ca+FF-combined pre-treatment increased significantly ($p < 0.01$) PAL activities of mentha under salt stress compared to non-saline control. The 1% Ca+FF pre-treatment appeared to be the best effective treatment in alleviating the deleterious effects of salinity on PAL activities. GPX activity increased significantly under salt stress. FF and 1% Ca+FF pre-treatment resulted in approximately 8.8 and 11.8-fold increases, respectively, in peroxidase activity under salt stress. Salt stress increased the proline content from 3.4 to 6.9 µmol mg^{-1} FW on the 11th day. However, these rates were recovered by all pre-treatment groups under salt stress ($p \leq 0.05$). Application of Ca at 1% + FF and 3% + FF resulted in a 1.7 and 1.4-fold increase in proline content under salt stress when compared to non-saline. Salt stress generated roughly 2-fold increase in LPO levels. The LPO levels, however, did recover with Ca+FF > FF > Ca pre-treatment.

Rutin, scopoletin, syringic, gallic, and salicylic acids were identified in the mentha samples and quantified using HPLC (Tab. 5). Ca+FF-combined pre-treatment resulted in a significant enhancement of the endogenous individual phenolic compound levels in the shoot under salt stress ($p < 0.01$). Furthermore, salt stress and Ca+FF-combined pre-treatment induced additional increases ($p < 0.01$).

DISCUSSION

FF and Ca improved all the investigated parameters under high salinity. Fresh weights and germination percentages of mentha seedlings increased gradually from day 1 to 11 in Ca-alone, FF-alone and Ca+FF-combined pre-treatment under non-saline and salt stress conditions. With this study, we have sought to provide insight into how seed pretreatment can protect plants against salt stress. Today, salinity stands as one of the most serious environmental stresses and is globally widespread due to human activities, particularly in both semi-arid and arid regions [Mackova et al. 2013]. Mentha growth and productivity is very deeply affected by salinity. Mentha, the most important herbal plant, is very susceptible to salinity stress [Schachtmann and Munns 1992]. Moreover, it has been well established that during the germination process and early seedling development, mentha is more sensitive to salt stress than during later developmental stages. On account of this reason, improving tolerance to salt stress during seed development is of great significance for crop production. The pre-treatment of seeds before stress exposure can

induce a tolerance mechanism to subsequent stresses [Wang et al. 2014].

The effect of the pre-treatment on increasing the growth of the mentha seedling under salt stress was, in order of strength, Ca < FF < Ca+FF. The sum up, these findings reveal that an additive, or even synergistic manner between calcium and fish flour exists in the modulation of seed germination under conditions of salt stress. It may thus be suggested that Ca and FF act as important molecules inducing ameliorative effects on the growth potential of salt stressed mentha plant.

At present, the potential contribution of calcium in seed defenses against various biotic and especially abiotic stresses has also been attracting an increasing attention [Wahid et al. 2007, Yücel and Heybet 2016]. Much evidence shows that exogenous calcium pre-treatment can increase a plant's tolerance mechanisms to adverse environments by regulating different pathways [Antosiewicz 2004, Arfaoui 2016, Wahid et al. 2007, Yücel and Heybet 2016]. Yet, under environmental stresses to the various regulatory mechanisms of Ca may be detrimentally different. For example, the membrane-protecting mechanism of calcium is most prominent under stress conditions. In addition, $CaCl_2$ pre-treatment has been shown to increase the accumulation of amino acids in rice roots under abiotic stress [Aurisano et al. 1995]. Increased accumulation of several proteins in pea seeds imbibed with $CaCl_2$ may occur in the Ca-regulated transcription process during seed germination [Wang et al. 2014]. The protective and growth promoting effects of calcium foster healthy and non-stressed seeds under salt stress, which result in an increase in germination percentage and better seedling growth. In our study, Ca primed mentha plants resulted as 2-fold of fresh weight and 58% increase of germination rate under salt stress. As of recently, much effort has been focused on the potential use of natural, cheap pre-treatment substances for the purpose of improving the plant development and for alleviating the harmful effects of stresses on plant growth. Related to these efforts, the results from this study demonstrated that salt exposed plants pre-treated with both calcium and fish flour exhibited increased plant tolerance and growth. In addition to the efficacy of combined treatment, we found that fish flour alone was also effective in counteracting the deleterious effect of salt stress on seed development process.

Salt stress also reduced carotenoids and chlorophyll levels; nevertheless, this reduction was partially overcome in mentha seedlings germinated from the seeds primed with FF and Ca+FF-combined pre-treatments. The reduction of chlorophylls by salt stress might result as a consequence not only of photo-oxidative destruction of existing pigments, but also of inhibition of biosynthesis. Nemat Alla et al. [2008], in their study, indicated that the induced photo destruction of chlorophylls in maize seedlings was a result of the inhibition of carotenoids. Carotenoids protect chlorophylls and chloroplasts from photo-oxidation.

PAL is an enzyme at the entry-point of the phenylpropanoid pathway, and the activation of PAL under stress circumstances has been recognized as being a part of the plant defense mechanisms; GPX plays an important role in secondary metabolism and in addition to polymerizing phenolic monomers together, it plays a role in the lignification process [Randhir et al. 2004]. In our study, combined calcium and fish flour at suitable concentrations not only stimulated phenolic production by PAL activity, but also facilitated structural development, as indicated by the peroxidase activity. In general, increased GPX activity induced lignin biosynthesis and then subsequent lignification process of the cell wall, the results of which could be related to increased plant growth.

Additionally, Ca+FF-combined pre-treatment applications on mentha caused a significant enhancement in the contents of individual phenolic compounds, including an eight-fold increase of rutin, a six-fold increase of scopoletin, and an approximately four-fold increase of phenolic acids in the shoot of Ca+FF-primed mentha under salt stress. In agreement with our findings, rutin, scopoletin, syringic, gallic and salicylic acids have all been determined in other study [Saleh and Madany 2015] to be the major phenolic compounds in mentha. Proline, as a typical stress response in plants, is one of the abundant compounds especially against salt stress. The proline plays a major role in osmoregulation under abiotic stress and acts as a detoxification of reactive oxygen species, contribution to cellular osmotic regulation, and equalization of proteins and enzymes [Verbrug-

gen and Hemas 2008]. In our study, the low proline content of salt stressed plants exposed to the combined Ca+FF pre-treatment suggests that these plants experienced lower degree of stress intensity.

In our study, it was found that Ca+FF-combined pre-treatment caused an increase ($p < 0.05$) in all detected free phenolic compounds, the findings of which could suggest that Ca+FF-combined pre-treatment has a direct or indirect roles for the regulation of the shikimic acid biosynthetic pathway.

CONCLUSIONS

From our results, high phenolic content related to high PAL activity leads to better seed vigor such as fresh weight, germination percentage, seedling length and also greater lignification related to high peroxidase activity was achieved from the Ca+FF combined pre-treatment group. Also, Ca+FF-combined pre-treatment resulted in an increase ($p < 0.05$) in all the detected individual phenolic compounds, chlorophyll-carotenoid and proline content when compared to saline-control. The results suggest that the detrimental effects of salinity might be reduced by calcium and fish flour treatment.

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