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Salicylic Acid and Fish Flour Pre-treatments Affect Wheat Phenolic and Flavonoid Compounds, Lipid Peroxidation Levels under Salt Stress

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The potential effect of combined salicylic acid and fish flour to improve plant tolerance to salt stress was investigated. This pre-treatment improved the growth of wheat seedlings under salinity when compared to control (untreated wheat seedlings). Moreover, combined pre-treatment improved significantly phenylalanine ammonia lyase (PAL) and peroxidase (POD) enzyme activities, also phenolic-flavonoid content in the shoots of salt stressed seedlings. One of the most important consequences of increase in salt stress is the oxidative tissue damage. In our study, salt stress increased lipid peroxidation levels (LPO) and also the loss of chlorophylls levels during stress might also be related to photo-oxidation resulting from oxidative stress. Whereas phenylalanine ammonia-lyase (PAL) activities of wheat shoots increased by a 2.1-fold under salt stress, the activities of shoots grown from seeds primed with salicylic acid and fish flour (SA+FF) increased by a 4-fold for 0.05 mM SA+FF, 4.8-fold for 0.1 mM SA+FF and 3.7-fold for 2.5 mM SA+FF combined pre-treatment under salt stress. Also, the combined salicylic acid+fish flour primed seedlings showed higher content of the scopoletin, and salicylic, syringic, vanilic and gallic acids under both salt and non-salinity stress conditions.

Keywords: phenolic contents, fish flour, salicylic acid, salt stress, wheat

Introduction

Among abiotic stresses, salt stress is a major environmental factor that inhibits crop productivity. Nowadays, salt stress affects deeply large areas of Turkey soils especially in semi-arid and irrigated areas of Mediterranean region with respect to the climate change. Adaptation of crops to saline conditions is very important. Seed priming is one of the useful approaches could adapt crops to salt stress. Seed priming enhances seed vigour and then multiple biochemicals and molecular mechanisms must be activated synergistically (Iyengar and Reddy 1996).

Wheat is an important and the most economic food crop world over. However, wheat is not rich in secondary compound and it is also salt-sensitive its growth and grain yield are significantly affected by salt stress. According to traditional agriculture, seed vigour methods include germination percentage and root/shoot length (Ventura et al. 2012).

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More recently, there is also strong evidence to support a link between certain biochemical characteristics and seed vigour. These biochemical parameters include the biosynthesis of secondary metabolites, such as phenolics. In the evaluation of seed priming, there is a positive correlation between phenolic synthesis and seed vigour response (Randhir and Shetty 2003).

Salicylic acid (SA) is admitted one of the key plant hormone involved in the activation of many plant defence responses. Application of SA could significantly induced resistance against a variety of abiotic and biotic stresses (Shah and Klessig 1999). However, abiotic tolerance induced by SA is less understood than biotic tolerance. SA priming have been used in seeds against a range of stress conditions such as bean priming with SA against salt stress (Azooz 2009), tomato priming with SA and Ca against salt stress (Manaa et al. 2014) and soybean priming with SA and Ca against Al stress (Lan et al. 2016). Like our study, they found that SA and Ca co-ordinately regulate root elongation and increased stress tolerance.

Anchovy *(Engraulis encrasicholus)* is the most abundant fish species in Black Sea of Turkey (Anonymus 2000). The fish waste during processing, including the head and other residues, represents over 60% of its production. Therefore, using edible fish waste becomes highly important for economy and environmental pollution (Boscolo 2001). Mechanically separated fish waste may be used to produce fish flour through drying. The amino acids of Turkish anchovy (72–74% protein) found in higher quantity were glutamic acid (a proline precursor), proline, aspartic acids and arginine with respect to the species of other countries (Dincer et al. 2010). Fish flour contains sufficient concentrations of glutamic acid and proline and in the plant cells; both amino acids may be involved in the regulation of phenolic synthesis via the phenylpropanoid pathway (Shetty 1997). Both salicylic acid (SA), a likely signal in resistance responses of plants to stresses (Waseem et al. 2006) and fish flour (FF) a likely rich sources for phenylpropanoid pathway product, might be perform defence-related functions. However, whether to regulate phenylpropanoid pathway synthesis by FF has been no data obscure. There are only a few studies about fish protein hydrolysates, which is different fisheries industry product (Horii et al. 2007). Exogenous plant hormone as salicylic acid (SA) and fish flour (FF) applied alone and also combined effects of SA + FF on plant growth and development have received little attention under salt stress.

The objectives of this study were to determine exogenous elicitor may result in an increase in both biochemical (individual phenolic compounds, PAL and POD enzyme activities) and traditional agronomic indicators (germination percentage, shoot and root length) of seed vigour.

Materials and Methods

Plant material and seed treatment

Ten g of wheat cultivar (*Triticum durum* Desf. cv. Yelken) seeds were soaked in each treatment (fish flour (FF), salicylic acid (SA) and SA + FF combined pre-treatment's)

with shaking at 150 rpm overnight. Fish flour (FF) was purchased from a local sardine fabric (protein content 0.74 protein g/FF g). 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 sieved to obtain a homogeneous size particle of 0.84 mm and ground to reach particle size of 0.5 mm. To examine the effects of salinity on germination percentages replicates of 15 seeds were sown on non-cellulosic paper in 500 mL plastic containers. The seeds were constantly based-watered. Containers were sealed to prevent evaporation and maintained at 20 °C in a growth cabinet illuminated (25 µmol m^{-2} s^{-1}, 400–700 nm) on a 14-h day/10-h night regime. The salinity concentration is 100 mM sodium chloride. Seeds were considered to be germinated when the radicle emerged through the seed coat and reached more than 2 mm in length. The number of germinated seeds for each cultivar and treatment was recorded every day. Germination percentages (GP), the weight of whole seedlings and the root–shoot length were measured.

Enzyme determinations

For enzyme determination: one gram of germinating seeds and seedlings were ground and homogenized in 4 ml, 20 mM phosphate buffer (pH 7.4). The homogenate was filtered and then centrifuged at 15,000 × *g* for 15 min. The enzymatic assay of phenylalanine ammonia-lyase (EC 4.3.1.5) activity was measured Hodgins method (Hodgins 1971). Assay concentrations contained 150 mM Tris-base pH 8.5, 3 mM L-phenylalanine and enzyme. The guaiacol-dependent peroxides (EC 1.11.1.7) activity assay was composed of 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2, and enzyme. Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation (E = 26.6 mM^{-1} cm^{-1}) (Nakano and Asada 1981).

Analytical methods

Total phenolic content in wheat samples were taken on first, third, fifth day of germination and was determined according to McCue (McCue et al. 2000) based Folin-Ciocalteu reagent. The flavonoid content in the crude extract was determined Du and co-workers method with some modifications (Du et al. 2009). Lipid peroxidation levels (LPO) was estimated based on thiobarbituric acid (TBA) reactivity (Buege and Aust 1978). Concentrations of chlorophyll (a + b) and carotenoids were measured as described by Lichtenthaler and Wellburn after extraction with 80% acetone (Lichtenthaler and Wellburn 1983). The protein content was determined by Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976).

Quantitative analysis of individual phenolic compounds by HPLC

The phenolic acids and rutin standard solution were prepared at 1 mg/mL in methanol. They were diluted into seven concentrations (5.0, 2.5, 1.0, 0.5, 0.25, 0.125 and 0.0625 µg/mL) for a calibration curve. Each extract was dissolved in methanol (1 mg/mL).

All solutions were filtered through a 0.2 mm membrane filter. Samples were extracted as described in Lee and Scagel (2009) with some modifications. A 0.5 g dried wheat shoot tissues was ground in a mortar with acidified methanol (0.1% formic acid, v/v) and were waited in a boiling water bath for 5 min, then immediately kept in ice bath for 10 min. All the mixtures filtered with Whatman paper and the pellet was re-extracted. Analyses were carried out using Agilent 1100 HPLC system with a UV detector (Agilent Technologies, CA, USA). Samples were separated on C18 Kinetex column (100 × 2.1 mm) with 2.6 µm particle size. The mobile phase consisted of eluent A: 0.1% TFA in water and eluent B: acetonitirile. The absorbance of the eluent was scanned at 280 nm by the UV.

Results

Growth parameters

The priming treatments with fish flour (FF) in the order of fresh weight of the wheat seedlings were 10 mL > 12 mL > 13 mL ≥ 15 mL/g FF ($p < 0.01$, data not shown). Seed pre-hydration with fish flour of 10 mL/g showed that the average plant height and weight resulted in 10-fold increase (10 cm) and 2.6-fold increase (189 mg) compared to control, respectively (data not shown). Because of these results, 10 mL/g FF concentration was used for further study. The seeds of wheat germinated in different pre-treatment's, control, salicylic acid (SA; 0.05, 0.1, 2.5, 5.0 mM), and SA + FF combined pre-treatment under both non-saline and salt stress (100 mM NaCl) were analyzed during plant growth on the 1st, 6th and 11th days. Percentage of seed germination, shoot-root lengths and also fresh weight were studied (data not shown). Germination percentages, FW and shoot-root lengths of wheat seedlings increased gradually from day 1 to 11 in SA-alone and SA + FF-combined pre-treatment under both non-saline and salinity conditions. Salt stress decreased the all the growth parameters. However, the rates were recovered by SA-alone and SA + FF-combined group under salt stress ($p < 0.01$ and $p < 0.05$, data not shown). The maximum growth parameters was found in wheat plants that germinated from the seeds primed with 0.1 mM SA + FF under salt stress ($p < 0.01$).

The total phenolic and flavonoid content

The total phenolic contents of the wheat seedlings organs (root, shoot and seed) increased in all treatment group, and salt-stressed group when compared to control, whereas the increases were not dependent on SA concentration (Table 1). At the same time, the exogenous stimulators were ordered with respect to the rates of increase in the phenolic contents of wheat shoots as SA < SA + FF under both non-saline and salt conditions. The highest total phenolic content was found in wheat plants that germinated from the seeds primed with 0.1 mM SA + FF under salt stress ($p < 0.01$). The amounts of maximum content under salt stress were determined as 31.1 ± 2.5 mg/g for shoot, 29.0 ± 2.1 mg/g for root and 29.0 ± 2.9 mg/g for seed, respectively.

Table 1. Total soluble phenolic content of wheat for different treatments; control, 0.05, 0.1, 2.5, 5.0 mM SA and 0.05 mM SA+g/10 mL FF; 0.1 mM SA+g/10 mL FF, 2.5 mM SA+g/10 mL FF, 5.0 mM SA+g/10 mL FF under non-saline and 100 mM NaCl

	Salinity %	Treatments (mM +g/10 mL)	Wheat organs		
			Shoot	Root	Seed
Total soluble phenolic content (µg/g FW)		Control	13.2±2.1	6.3±0.5	7.8±0.5
		0.05 SA	15.4±1.8$^{\delta}$	7.7±0.6$^{\delta}$	10.6±0.9$^{\varepsilon}$
		0.1 SA	20.4±2.3$^{\varepsilon}$	13.9±1.1$^{\varepsilon}$	10.9±0.2$^{\varepsilon}$
		2.5 SA	15.6±1.5$^{\delta}$	12.7±1.3$^{\varepsilon}$	8.0±1.1$^{\delta}$
		5.0 SA	14.9±0.5	10.6±2.1$^{\varepsilon}$	9.6±1.2$^{\delta}$
	100	Control	15.7±2.1$^{\delta}$	8.0±0.8$^{\delta}$	7.8±0.6
		0.05 SA	16.8±1.8	20.2±2.1$^{\varepsilon}$	10.3±0.5$^{\varepsilon}$
		0.1 SA	22.2±2.7$^{\varepsilon}$	14.6±2.4$^{\varepsilon}$	9.9±0.4$^{\varepsilon}$
		2.5 SA	21.9±2.5$^{\varepsilon}$	13.9±1.8$^{\varepsilon}$	9.8±0.9$^{\varepsilon}$
		5.0 SA	17.8±1.9$^{\delta}$	13.9±1.9$^{\delta}$	9.0±1.2$^{\varepsilon}$
	0	0.05 SA+FF	17.9±1.6$^{\varepsilon}$	8.5±0.8$^{\delta}$	8.8±0.6$^{\delta}$
		0.1 SA+FF	25.3±2.8$^{\varepsilon}$	13.8±1.3$^{\varepsilon}$	8.2±0.5$^{\delta}$
		2.5 SA+FF	26.7±2.1$^{\varepsilon}$	13.4±1.1$^{\varepsilon}$	9.5±0.4$^{\delta}$
		5.0 SA+FF	27.1±1.5$^{\varepsilon}$	12.3±0.7$^{\delta}$	8.5±0.9$^{\delta}$
	100	0.05 SA+FF	20.1±1.8$^{\varepsilon}$	23.2±1.5$^{\varepsilon}$	23.2±0.9$^{\varepsilon}$
		0.1 SA+FF	31.1±2.5$^{\varepsilon}$	29.0±2.1$^{\varepsilon}$	29.0±2.9$^{\varepsilon}$
		2.5 SA+FF	26.7±2.4$^{\varepsilon}$	23.9±2.1$^{\varepsilon}$	23.9±0.1$^{\varepsilon}$
		5.0 SA+FF	27.9±1.5$^{\varepsilon}$	19.0±2.0$^{\varepsilon}$	19.0±1.1$^{\varepsilon}$
Total flavonoid content (µg/g FW)		Control	20.7±1.7	9.3±0.5	17.3±0.9
		0.05 SA	24.0±1.5$^{\delta}$	25.3±1.2$^{\varepsilon}$	45.3±2.5$^{\varepsilon}$
		0.1 SA	26.7±1.5$^{\varepsilon}$	18.0±1.3$^{\varepsilon}$	32.6±1.2$^{\varepsilon}$
		2.5 SA	30.7±0.9$^{\varepsilon}$	19.3±1.4$^{\varepsilon}$	48.7±2.1$^{\varepsilon}$
		5.0 SA	24.6±0.6$^{\delta}$	19.1±1.9$^{\varepsilon}$	34.7±1.2$^{\varepsilon}$
	100	Control	32.0±1.1$^{\varepsilon}$	16.0±0.5$^{\varepsilon}$	20.1±1.6$^{\delta}$
		0.05 SA	28.0±1.2$^{\delta}$	24.0±1.3$^{\varepsilon}$	21.3±1.5$^{\delta}$
		0.1 SA	27.1±1.3$^{\delta}$	15.3±0.3$^{\varepsilon}$	42.0±1.4$^{\varepsilon}$
		2.5 SA	46.0±2.1$^{\varepsilon}$	18.6±0.9$^{\varepsilon}$	57.3±3.1$^{\varepsilon}$
		5.0 SA	29.8±1.7$^{\delta}$	19.1±1.1$^{\varepsilon}$	55.3±1.2$^{\varepsilon}$
	0	0.05 SA+FF	20.7±0.9	25.3±1.7$^{\varepsilon}$	26.7±0.6$^{\varepsilon}$
		0.1 SA+FF	21.0±0.8	24.0±1.5$^{\varepsilon}$	32.7±0.5$^{\varepsilon}$
		2.5 SA+FF	24.7±1.3$^{\delta}$	26.0±2.1$^{\varepsilon}$	82.0±0.4$^{\varepsilon}$
		5.0 SA+FF	24.7±1.2$^{\delta}$	28.0±2.5$^{\varepsilon}$	79.0±3.1$^{\varepsilon}$
	100	0.05 SA+FF	38.0±2.1$^{\varepsilon}$	23.1±1.0$^{\varepsilon}$	42.0±2.5$^{\varepsilon}$
		0.1 SA+FF	38.0±2.2$^{\varepsilon}$	25.3±1.1$^{\varepsilon}$	61.3±3.0$^{\varepsilon}$
		2.5 SA+FF	44.0±2.5$^{\varepsilon}$	26.0±1.2$^{\varepsilon}$	68.0±1.0$^{\varepsilon}$
		5.0 SA+FF	36.7±1.8$^{\varepsilon}$	29.3±1.4$^{\varepsilon}$	70.0±0.1$^{\varepsilon}$

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

As it can be seen in Table 1, the total flavonoid contents of the wheat organs increased in all treatment group (SA and SA + FF), and salt-stressed group when compared to control. However, the beneficial effects of SA pre-treatment on the total flavonoid contents of root and seed was induced with increasing SA concentrations ($p < 0.05$ and $p < 0.01$). The levels of wheat shoot that germinated from the seeds primed with 2.5 mM SA and 2.5 mM SA + FF resulted in 2.2- and 2.1-fold increases under salt stress as compared to non-saline control, respectively ($p < 0.01$). The flavonoid content of wheat seed that germinated with 5.0 mM SA + FF were obtained in a 4.0-fold increase (max. value) under salt stress ($p < 0.01$).

Chlorophyll-carotenoids contents, LPO levels and enzyme activities

Table 2 shows chlorophyll-carotenoid contents, PAL and Gua-dep POD enzyme activities and also LPO levels of wheat seedlings primed with SA + FF under control and salt stress on the 11th day. Chl pigment content decreased significantly under salt stress ($p < 0.01$), whereas the contents alleviated by SA + FF-combined pre-treatment with decreasing SA concentration. Salinity stress resulted in massive increase ($p < 0.05$) in the non-photosynthetic pigment content (β-carotene), whereas combined-SA + FF pre-treatment induced additional increase in β-carotene. Salt stress and SA + FF-combined pre-treatment markedly increased ($p < 0.05$) PAL activities of wheat seedlings grown under salt stress and 0.1 mM SA + FF-combined treatment appeared to be the most effective treatment in counteracting the negative effects of salt stress on PAL activities. Gua-dep POD activity of shoots grown from seeds primed SA + FF treatment showed a positive correlation with ($r = 0.670$, $p < 0.01$) decreasing SA concentrations and reached their maximum (by a 6.8-fold increase for 0.05 mM SA + FF treatment group) under salt stress on the 11th day (Table 2). Salt stress and the SA + FF-combined pre-treatment of wheat shoots were ordered with respect to the rates of increase in the LPO levels as 0.1 mM SA + FF < 0.05 mM SA + FF < salt stress < 2.5 mM SA + FF.

Table 2. Parameters: Chlorophyll, Carotenoid and LPO content, PAL and Gua-dep POD activities; control, SA (0.05; 0.1; 2.5 mM) + FF (10 mL/g) combined treatment under non-saline and salt stress on the 11th day

Parameter	No salinity	100 mM NaCl, Salinity stress			
	Control		SA mM + FF (10 mL/g) combined treatment		
			0.05 mM	0.1 mM	2.5 mM
Chlorophylls (μg · cm^{-2})	168 ± 10	95$^{\varepsilon}$	320 ± 11$^{\varepsilon}$	291 ± 21$^{\varepsilon}$	193 ± 12$^{\delta}$
Carotenoids (μg · cm^{-2})	2.5 ± 0.3	3.5$^{\delta}$	6.7 ± 0.3$^{\varepsilon}$	6.1 ± 0.2$^{\varepsilon}$	5.5 ± 0.2$^{\varepsilon}$
PAL (IU · mg^{-1} protein)	32 ± 1	98$^{\varepsilon}$	128 ± 18$^{\varepsilon}$	152 ± 12$^{\varepsilon}$	118 ± 14$^{\varepsilon}$
Gua-dep POD (IU · mg^{-1} protein)	0.5 ± 0.1	2.5$^{\varepsilon}$	3.4 ± 0.3$^{\varepsilon}$	2.9 ± 0.2$^{\varepsilon}$	2.5 ± 0.4$^{\varepsilon}$
LPO (nmol MDA · g^{-1})	5 ± 1	7.5$^{\varepsilon}$	6.4 ± 1.0$^{\delta}$	6 ± 1$^{\delta}$	8.1 ± 1.0$^{\varepsilon}$

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

Table 3. SA+FF pre-treatment and salt stress on the content of individual phenolic compounds in the shoot of wheat

Phenolic compunds	No salinity, 0 mM NaCl		Salinity stress, 100 mM NaCl	
	–SA+FF+SA+FF		–SA+FF+SA+FF	
Scopoletin	1.19±0.04	2.84±0.15$^{\delta}$	0.69±0.02$^{\varepsilon}$	3.60±0.11$^{\varepsilon}$
Caffeic acid	8.48±0.18	15.38±0.22$^{\varepsilon}$	4.99±0.05$^{\varepsilon}$	19.00±0.15$^{\varepsilon}$
Vanillic acid	4.99±0.05	9.30±0.22$^{\varepsilon}$	3.19±0.06$^{\delta}$	11.30±0.01$^{\varepsilon}$
Gallic acid	1.38±0.01	2.28±0.03$^{\delta}$	0.98±0.01$^{\delta}$	3.31±0.01$^{\varepsilon}$
Salicylic acid	0.49±0.02	1.12±0.03$^{\delta}$	1.30±0.03$^{\varepsilon}$	1.32±0.03$^{\varepsilon}$

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

Scopoletin and phenolic acids such as caffeic, vanillic, gallic, salicylic acids were identified in the wheat samples and quantified using HPLC (Table 3). SA + FF-combined pre-treatment cause a significant increase in the level of endogenous individual phenolic compound levels in the shoot of wheat seedlings under salt stress ($p < 0.01$). On the other hand, salt stress and SA + FF-combined pre-treatment induced additional increases ($p < 0.01$) (by the 3-fold increases of scopoletin, approximately 2-fold increases of caffeic, vanillic, gallic and salicylic acids) in all detected free phenolic compounds. There was not a significant difference in salicylic acid content between SA + FF-combined pre-treatment groups under salt stress. This result indicates that the soaking seeds with SA + FF did not affect plant SA content negatively or positively.

Discussion

Salt stress is very detrimentally effective on world land; 34 million hectares of these lands are salt affected and 1.5 million hectares of them out of production each year. Thus, it is accepted that half of the cultivable lands will be lost by the mid-21st century. For enhancing crop tolerance capacity salt-stress is a strategic goal for governments all over the world. It is well established that salt stress has negative correlation with seed germination and growth parameters. Seed priming is a widely accepted the practice to enhance seed vigour and plant growth against stress conditions. Upon imbibitions, the embryonic cells switch from quietness to a highly activate metabolic state (Bradford 1986). The positive effects of priming against salt stress have been reported in many crops, such as maize (Tabatabaei 2014), pea (Naz et al. 2014) and soybean (Miladinov et al. 2015).

The present study clearly indicated that salt stress (100 mM NaCl for 11 days) severely inhibited the growth of wheat, as shown by significantly decreased FW, shoot-root lengths and GP. Thus, pre-treatment of seeds improved salt tolerance. In this study, only the best elicitor concentrations of FF determined during wheat germination (10 mL/g fish flour). The maximum recovery were determined the effect of addition of FF to SA treatment as a potential seed vigour enhancer. Combined effects of the varying salicylic acid

levels and fish flour (SA + FF), the most effective levels for promoting growth parameters were 0.1 mM SA + FF under salt stress. Effects of SA and FF were additive for growth parameters and also total phenolic and flavonoid content especially under salt stress. The phenolic content generally increased salt stress when compared to non-saline conditions especially for SA + FF-combined pre-treatment group, indicating that salt and SA + FF-combined pre-treatment together had also synergistic effect.

Also, total soluble phenolic compounds show the same effect and the most effective levels for promoting phenolic levels were 0.1 mM SA + FF under salt stress, whereas total flavonoid content increased generally with increasing SA levels under salt stress. The total phenolic content of wheat organs that germinated from the seeds primed with 0.1 mM SA + FF increased almost two-, five- and fourfold for shoot, root and seed, respectively. This treatment had a better effective response than SA-alone especially under salt stress. Shetty and Randhir had been investigating the effect of fish protein hydrolysates (FPH, Iceland) on seedling vigour (Shetty and Randhir 2005). They found that FPH treatments the most stimulated soybean seedling vigour via increasing the phenolic content (48%). When compared to these studies, our common by-product of fisheries industry, fish flour was more effective than FPH on seed vigour. FPH is known to be rich in amino acids common to proline metabolism. But FF is known to be rich in amino acids, and also other primary metabolism product. Our results show that a potential stimulation of pentose-phosphate pathway activity by fish product as a source of sugar-phosphate precursors may needs amino acids and also other primary metabolism product via synergistic effect to support phenolic synthesis. Therefore, we hypothesize that FF and also SA as a seed pre-treatment may be the best appropriate for food crops that are naturally rich in phenolic compounds. Plant phenolics could be utilized in agriculture as eco-friendly alternatives for weeds and pest management (Saleh and Madany 2015). From our results, maximum PAL activities and maximum phenolic content were observed in shoots grown from seeds primed 0.1 mM SA + FF combined pre-treatment. It is seen that this concentration stimulated as well as synchronize PAL activities and phenolic content. Salt stress could result in β-carotenoid oxidative destruction and also lipid peroxidation accumulation in wheat seedlings, which is toxic to the cell. Our results show that salicylic acid and fish flour pre-treatment can protect cell membranes structure against the toxic and destructive effects of radicals during salt stress. But 2.5 mM SA level might not be effective protection for membranes under salt stress. Accumulation of phenolic compounds can inhibit lipid peroxidation, where they were proven to trap the lipid alkoxyl radical depending on their structure (Milic et al. 1998). In addition, phenolics such as flavonoids have the tendency to bind with the polar head groups of phospholipids, thus accumulated at the membrane surfaces, helping in maintaining membrane integrity (Verstraeten et al. 2003). They limit the diffusion of free radicals to the hydrophobic region of the bilayer and protect membrane structure (Arora et al. 2000). The salt injury incidences in terms of GP, FW, shoot length decreased and LPO levels increased sharply on the 11th day when compared to control. The 0.1 mM SA + FF combined pre-treatment decreased salt stress injury incidence. Because transient increases PAL activities and phenolic content might prevent the protection of wheat against oxidative injury. These results seen that the pre-

vention of damage to membrane can be achieved by co-operative effects of PAL, POD and phenolic content. In the coming years, wheat crops could be engineered for the salicylic gene and fish flour could be used as priming agent, thereby enhancing phenolic contents, and producing crops that overcome easily salt stress.

Additionally, as the plant growth regulators, SA + FF-combined pre-treatment applications on wheat resulted in a significant increases in the contents of individual phenolic compounds. Thus, exogenous growth regulators may be metabolized as a result of phenolic acids and scopoletin. This statement may be supported by the threefold increases of scopoletin, approximately twofold increases of phenolic acids in the shoot of SA + FF-primed wheat under non-salinity conditions. Scopoletin and caffeic and vanillic acids has been determined as the major phenolic compounds in wheat by the other researches (Saleh and Madany 2015).

In our study, all the contents of phenolic acids decreased under salt stress, except for salicylic acid. The reason for this increase in salicylic acid, salicylic acid plays the important defensive role via modulating the plant metabolism and growth to stresses such as salinity. Thereby, the significant accumulation of salicylic acid in the shoot of salt-stressed wheat, SA + FF combined pre-treatment seedlings can contribute to the growth ameliorative effect of SA and FF. Application of 100 mM NaCl increased the level of ferulic, cholorogenic, salicylic acids, decreased syringic, vanillic, gallic and *trans*-cinnamic acids in previous studies (Saleh and Madany 2015). In our study, it is determined that SA + FF-combined pre-treatment resulted an increase ($p < 0.05$) in all the detected free phenolic compounds and this result could bring out a direct or indirect role of SA + FF-combined pre-treatment in regulation of shikimic acid biosynthetic pathway.

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