Salt stress mitigation by seed priming with olive waste of *Phaseolus vulgaris* L.

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Abstract: Bean seeds were presoaked in 0.75% (W/V) aqueous olive waste extract (OWE) then were irrigated with distilled water up to 21days, then were irrigated with 50 mM NaCl and 100 mM NaCl to investigate the potential effects of this extract. OWE alleviated the adverse effects of NaCl stress to varying degrees. The concentrations of free proline (Pro), reduced glutathione (GSH), ascorbic acid (AsA), hydrogen peroxide and lipid peroxidation [as malondialdehyde (MDA)], were increased. The activity of the antioxidant enzymes [catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and ascorbate peroxidase (APX)] were enhanced while glutathione peroxidase (GPX) and soluble protein were decreased compared to their corresponding controls. Obtained results suggest that, the potential of OWE can be applied to alleviate the harmful effects of NaCl stress and offers an opportunity to increase the resistance of common bean plants to grow under saline conditions.
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1. Introduction

High salinity, drought, cold and heat are the chief environmental factors affecting adversely up to 70% on the existence, biomass production and yield of vital food crops therefore, threatening the global food security (Ahmad *et al*., 2012). Shabala *et al*. (2015) reported that salinity stress is one of the most serious abiotic stress factor restraining global crop productivity and quality. Parida and Das (2005) ascribed the adverse effect of salinity due to osmotic stress, ionic imbalance, ion toxicity and nutrient deficiency that were produced due to salinity. Consequently, secondary stresses such as oxidative damage frequently occur that generates reactive oxygen species (free radicals) which involve in stimulating membrane lipid peroxidation as well as membrane leakage (Gunes *et al*., 2007), finally scratch chloroplast and mitochondria by disrupting their cellular structures (Mittler, 2002). All these factors cause hostile effects on plant growth and development at physiological and biochemical levels (Munns and James, 2003) and at the molecular level (Tester and Devenport, 2003).

Several strategies were conducted for solving the salinity issue like seed or seedling priming (Azooz, 2009) and/or the application of stress metabolites that could be recognized and integrated by plants as components of a stress-induced adaptation response (Ashraf and Foolad, 2007). Application of different wastes on *Phaseolus vulgaris* grown under saline condition was investigated in several studies, e.g. Rady *et al*. (2013) studied the effect of the leaf extract of *Moringa oleifera*, while Semida and Rady (2014) studied the effect of the aqueous extract of maize grain. Application of olive wastes on many plants were studied as well as their effects were discussed. Such effects may be attributed to their satisfactory C/N ratio (Alburquerque *et al*., 2007); high content of polyphenols, polyalcohols, and volatile fatty acids (Linares *et al*., 2003); source of numerous antioxidants (Bouaziz *et al*., 2005) and a lot of phenolic compounds (Owen *et al*., 2003). These phenolics and antioxidants may shield the organism against oxidative damage caused by ROS (Aruoma, 2003). Diacono and Montemurro (2015) reported that fertilizers made from byproducts of the olive oil industry and poultry manure enhance the content of soluble and exchangeable K^+ (therefore restraining the entry of Na^+) as well as cation exchange capacity (CEC).

Bayuelo-Jiménez *et al*. (2002) reported that, along with the Nile valley (including Egypt and Sudan), common bean (*Phaseolus vulgaris)* is a principal vegetable crop, but unfortunately about 20–30% of its production areas were injured by soil salinity. Common bean categorized as extremely salt sensitive crop that agonizes yield reduction at salinity levels less than $2dSm^{-1}$ (Maas and Hoffman, 1977).

In this investigation authors studied if priming of *Phaseolus vulgaris* L. seeds by OWE can enhance its tolerance against salinity stress.

2. Materials and methods

Materials

Seeds:

The seeds of common bean (*Phaseolus vulgaris* L., cv. Nebraska) were provided from Sids station of the Agricultural Research Center (ARC), Beni-Suef,

Egypt. All seeds were surface-sterilized with 0.5% NaOCl solution for 1 min.

Soil:

Experiment was performed in an open field condition (in squares 3m X 3m) in September, 2016 up to January, 2017, when temperature range was (25^0C±2) at Alfashn fields, Alfashn Center, Beni-Suef governorate, Egypt. The soil texture was a clay loam type with organic carbon 0.91%, total N 0.12%, C/N ratio 8.3, total P 0.072, and CaCO3 3.4%.

Determination of salinity dose:

In a preliminary experiment, seeds were germinated in serial NaCl concentrations and after 7 days germination percentage was calculated. Calculation showed that 50 mM and 100 mM can be used as modrate and severe stress, respectively.

Determination of OWE dose:

The selection of the OWE which will be applied was investigated in a pot experiment. After 14 days and according to morphological growth criteria (seedling length, fresh weight and dry weight) the selected dose was (0.75%) after 8-hrs soaking which gave the optimum growth

The experiment design:

It was a random multifactorial design experiment. According to the preliminary experiments, the seeds were divided into two sets; one set was soaked for 8 hr. in distilled water (group A) while the second set was soaked for 8hr. in 0.75% OWE (group B). Seeds of each set were allowed to air dry overnight prior sowing into the field. All seeds were cultivated and irrigated with distilled water up to 21 days when the first true leaves were established. After then, seeds of each group were divided into three sub-groups (1, 2 and 3). First sub-group of each was irrigated with distilled water; the second was irrigated with 50 mM NaCl and the third was irrigated with 100 mM NaCl. Samples were harvested at (zero, one, three and five weeks) after application of salt stress, to follow up different plant stages; seedling, vegetative, flowering and fruiting stage, respectively. All samples were subjected for the measurements of the different biochemical parameters. Each sample had five plants as replicates.

Methods

1. Estimation of Proline, GSH, AsA, MDA and H_2O_2

Proline, GSH, AsA, MDA and H_2O_2 were estimated in the fresh leaves according to methods of Bates *et al*. (1973), Beutler *et al*. (1963), Jagota and Dani (1982), Elavarthi and Martin (2010) and Jessup *et al*. (1994) respectively.

2. Determination of protein and enzymatic antioxidant activities:

The crud protein extract was prepared according to procedure of Semida and Rady (2014). Total soluble proteins were estimated according to method of Bradford (1976).

Determination of enzymatic antioxidant activities:

The specific activities of the antioxidant enzymes CAT, POX, SOD, APX and GPX were determined in the crude extract at 25 ºC following the methods of Gong *et al.* (2001), Nakano and Asada (1981), Marklund and Marklund (1974) and Matkovics *et al*. (1998) respectivly.

Statistical Analyses: Data were analyzed by using SPSS V20. Both of one-way and 2-wayes analyses of variance (ANOVA) were applied. Data presented in the study were means of five replicates (n=5) ± SD.

3. Results and discussion

1. Proline:

The obtained data (table 1 and fig.1a**)** showed that proline increased significantly under salinity and this was agreed with the results obtained by Kibria *et al*. (2017). Ashraf and Foolad (2007) showed that plants, under salinity stress, respond with a several tolerance mechanisms, mainly accumulation of compatible solutes and proline is the most mutual one. Also, proline plays several roles as it, under stress conditions, acts as an osmoprotectant (Hartzendorf and Rolletschek, 2001); membrane stabilizer (Bandurska, 2001) and as a scavenger of ROS (Matysik *et al.*, 2002). Therefore proline accumulation in stressed plants is associated with enhanced salt tolerance (Sripinyowanich *et al*., 2013) and prompt tolerance to oxidative stress by modifying the activities of antioxidant enzymes (Saeedipour, 2013). In the present experiment OWE enhanced proline production which eased salt stress. Such enhancement may be explained due to proline may act as protecting for enzymes (Khedr *et al.,* 2003), reducing lipid peroxidation (Okuma *et al.,* 2004), enhanced CAT, POX and SOD and APX activities (Hoque *et al.,* 2007), increasing phenolic contents (Posmyk and Janas, 2007) and decreasing the photo-damages of thylakoid membranes by scavenging the superoxide radicals (Banu *et al.,* 2009).

2. Ascorbic acid:

The obtained data (table 1 and fig.1c**)** showed a significant increase in ascorbic acid content which agreed with data obtained by Yan *et al.* (2017). Also accumulation of ascorbic acid under salinity stress was reported by other authors like Mohamed *et al.* (2010) and Davey *et al.* (2000) who ascribed that, the accumulation of ascorbic acid is correlated with the activities occurred in its producing enzymes as they were highly enhanced under stressful environment. Several studies have revealed that ascorbic acid plays an important role in improving plant tolerance against abiotic stress (Ahmad and Umar, 2011). Ascorbic

acid, like total phenols, is an vital antioxidant, crucial for plant defense against oxidative stress i.e. it reacts not only with H_2O_2 but also with O^{2-}, OH and lipid hydroperoxides (Ahmad *et al*., 2011). Pastori *et al*. (2003) reported that besides the antioxidant capacity of ascorbic acid, it can act as a signaling molecule involved in the regulation of complex processes such as the senescence of plants and their reaction to O_3, photo-oxidative situations or pathogen invasion.

3. Glutathione:

In the present work and as shown in (table 1 and fig.1b**)** glutathione accumulated under salinity levels and this accumulation agreed with the investigations of by Rady *et al*. (2013). The accumulation of GSH under salt stress was observed, also, by Mullineaux and Rausch (2005) who ascribed this accumulation to the role of GSH in the defensive mechanism against oxidative damage. On the other hand, Ahmad *et al*. (2011) attributed the accumulation of GSH to its powerful antioxidant property which protect the plant cell from the oxidative stress generated due to production of ROS. It was also reported that under stress conditions GSH involved in the ascorbate/glutathione cycle and in the regulation of protein thiol-disulphide redox status of plants (Yousuf *et al*., 2012). Also Noctor *et al*. (2002) reported that glutathione is a substrate of glutathione peroxidase (GPX) and glutathione-*S*-transferases (GST), which are also intricate in the elimination of ROS. It was reported that GSH increase under salinity is essential for signal transduction and protection against reactive oxygen species by various control mechanisms including direct initiation of GSH biosynthetic enzymes encoding genes (Srivalli and Khanna-Chopra, 2008). Therefore, GSH acts as a redox sensor of environmental signs, and the accumulation of GSH aids plants to tolerate oxidative stress. Hussain *et al*. (2008) followed the enzymatic mechanisms linked in GSH production in wild type and salt-tolerant *Brassica napus* plants. They pointed to integration of sulfur and the biosynthesis of cysteine and GSH to alleviate salt-induced oxidative stress.

4. Protein:

The obtained results (table 1 and fig.1d) showed a decline in protein content due to salinity which agreed with the results of experiments were performed on *Oryza sativa* by Saeidi-Sar *et al*. (2013). Similar results were found by Mittal *et al*. (2012)*, in Beta vulgaris* and Rahdari *et al*. (2012) *in Portulaca oleraceae*. Mittler (2002) reported that under salinity stress, the damage caused by ROS is due to enzyme inhibition, oxidation and peroxidation of proteins and lipids respectively causing finally cell death. According to Ahmad *et al*. (2011), oxidative damage of proteins may be caused due to damage occurred in site specific amino acid alterations, disintegration of the peptide chain, accumulation of cross linked reaction products and increased liability to proteolysis. Also Zhu *et al*. (2004) reported that ROS accumulation upsets typical metabolism via oxidative damage to photosynthetic pigments, proteins, nucleic acids and lipids. It was established that the process of protein formation involves great amounts of K^+, as it is essential for the binding of tRNA to ribosome and possibly other facets of ribosome purpose (Tester and Davenport, 2003). Therefore interruption of protein production by raised concentrations of Na^+ that compete with K^+, acts to be an chief cause of injury by NaCl.

5. Lipid peroxidation:

In the present experiment, the level of lipid peroxidation which measured as MDA showed a marked increase in response to salinity (table 1 and fig.1e). This result was approved by many authors in several plants such as, Hasanuzzaman *et al*. 2011(a) in *Brassica napus* and Hasanuzzaman *et al*. 2011(b) in *Triticum aestivum.* They ascribed the increased level of lipid peroxidation due to oxidative stress caused by the highly accumulated ROS under salinity. This finding was approved by the work of Ahmad *et al*. (2010) who linked between the injury related with salt stress increases the lipid peroxidation, electrolyte leakage and hydrogen peroxide with larger level in salt sensitive genotypes or species.

6. Hydrogen peroxide:

In the present experiment and as illustrated in (table 1 and fig.1f) salinity caused a significant increase in H_2O_2 content in all plants and this result agreed with those results observed by Talukdar (2013). For several years, it was believed that hydrogen peroxide is ROS and undesirable aerobic respiration byproduct, However recent studies report that H_2O_2 has a vital role in redox signaling in normal processes regulation, together with oxidative stress therefore it has been recognized as an essential evil for cell signaling (Rhee, 2006). Hernandez *et al*. (2010), also studied hydrogen peroxide role as a signaling molecule in transduction of stress signals to the modification of expression profiles of objective genes. Cheeseman (2007) recognized the link between H_2O_2 and signaling networks of several stress responses. Petrov and Van Breusegem (2012) concluded that hydrogen peroxide acts a regulating agent for various vital metabolic processes. But, Anjum *et al*. (2015) showed that the accumulation of H_2O_2 being harmful to cell structures; therefore, steady-state level of cellular H_2O_2 is necessary to be firmly controlled.

7. Antioxidant enzymes

7.1. Catalase:

In the present investigation, the activity of CAT increased under salinity (table 1 and fig.2a) and this agreed with data obtained by Semida and Rady (2014).

This induction of CAT activity may be attributed to the principal function of CAT as catalysing the breakdown of hydrogen peroxide into H_2O and O_2 (Willekens *et al.*, 1997). As mentioned before, salinity causes over production of ROS, and the plant, to overcome this stress, it generates a powerful antioxidant system containing antioxidant enzymes such as superoxide dismutase, catalase and peroxidase. Kolbert *et al*. (2012) reported that under stress, plants exploit most of their capitals, for the progress in defense mechanisms rather than growth and development. Some studies have shown that the antioxidants activity increment of SOD, CAT, APX and GR is preventing the damage caused by salinity (Plaut *et al*., 2013). It was found that, under salinity stress, catalase turnover rate is very high as it can catalyze the breakdown of millions of hydrogen peroxide molecules every second (Deisseroth and Dounce, 1970).

7.2. Peroxidase:

The results obtained in this work (table 1 and fig.2b) showed that salinity enhanced the activity of peroxidase and this finding confirmed by the results of Yusuf *et al.* (2012). According to Fagerstedt *et al.* (2010), POXs are a group of isoenzymes able to scavenge hydrogen peroxide mainly in the apoplastic space. Conversely, Kawano (2003) reported that generation of POXs can yield reactive oxygen species which have been revealed to do an efficient extracellular signal transduction role for stomatal closer and cell elongation. The occurred activity of POXs is concomitant with the increment occurred in H_2O_2 and this finding can be attributed to the role of H_2O_2 in signaling or scavenging of ROS (Jithesh *et al*., 2006).

7.3. Superoxide dismutase:

Viewing the data of our investigation (table 1 and fig.2c), it was cleared that the activity of SOD increased under salinity and this increment is congruent with results of Yan *et al*. (2011). SODs dismutate $O^{2\cdot-}$ into H_2O_2, so SODs have been considered to act as the first line of defense against oxidative stress in plants (Alscher *et al.*, 2002). Maksimovic *et al.* (2013), reported that glycophytes under salinity stress showed a high activity of SOD, but on the other side, halophytes have an extraordinary aptitude for using superoxide dismutase to protect themselves under stress conditions (Ozgur *et al.*, 2013).

7.4. Ascorbate peroxidase:

The activity of APX (as shown in the table 1 and fig.2d) was increased under salinity and this increment agreed with the data of Yan *et al*. (2011). Jithesh *et al*. (2006) reported that the ability of both salt sensitive and salt tolerant plants to tolerate to salinity stress increased as APX expression/activity increased. APXs necessary for the plant defense system as they are similar to CATs which scavenge hydrogen peroxide. Shigeoka *et al.* (2002), discussed that the APX isozymes are found in stroma of chloroplasts, mitochondria, cytosol, and the membrane of peroxisomes and chloroplasts, act to consume ascorbate as a reductant.

7.5. Glutathione peroxidase:

It was stated that plant glutathione peroxidases, accomplish several roles including regulation of cell cycle (Kadota *et al*., 2005), hydrogen peroxide detoxification, signal transduction and redox sensing (Delaunay *et al*., 2002). In this experiment the activity of GPX decreased under salinity (table 1 and fig.2e) and this was agreed with results of Srinieng *et al*. (2015) who ascribed the reduction in GPX activity under salinity might be due to hydrogen peroxide consumption due to the increasing of the activity of CAT. Apel and Hirt (2004) reported that APX, GPX and CAT are the key of enzymes responsible for scavenging the accumulated hydrogen peroxide. The fact that GPX is one of the enzymes non-heme containing peroxidase families was approved by Bela *et al*. (2015). Due to GPXs broad spectrum of substrate specificity and higher affinity towards H_2O_2; GPxs they have been subjected to studies concerned with the oxidative stresses. Brigelius-Flohe and Flohe (2003) reported that, GPXs mostly protect the plant from the oxidative damage resulting from the accumulated ROS. According to Jung *et al*. (2002) plant GPXs have been reported to be involved in various biotic and abiotic stress adaptation pathways. GPXs in the process of H_2O_2 scavenging might use three different reductants; GSH, NADPH and Thioredoxin (TRX).

Finally, the enhancing role of OWE has been approved by many authors like Tejada and Gonzalez (2003) and El-Darier *et al*. (2015) who reported that olive waste increased leaf soluble carbohydrates, pigments (chl a, chl b and carotenoids) and leaf minerals concentration (N, K, Fe, Mn and Zn). This enhancing role may be attributed to the composition of this waste. Analysis of olive waste showed that, it consists of high contents of polyphenols and polyalcohols (Linares *et al*., 2003), antioxidants and flavonoids (Bouaziz *et al*., 2005) and a lot of phenolic compounds (Owen *et al*., 2003). Both of flavonoids and phenolic compounds play an important role in enhancement of antioxidant system by scavenge free radicals and other active species by their hydrogen atom and/or electron donation, therefore they shield the organism against oxidative damage (Waśkiewicz *et al.,* 2013). As salinity caused a disturbance in the balance of minerals ratio, while OWE rebalanced this disrurbance, so the enhancing effect of OWE may be referred to the return of metabolic roles of these

rebalanced minerals especially for anabolic activities (as protein biosynthesis), for different enzymatic activities (as antioxidant enzymes) and their imprtant roles in the different activities of the cell (Diacono and Montemurro, 2015).

Table 1: Two way ANOVA analysis showed the effects of OWE, salinity and OWE*salinity interaction on proline, GSH (glutathione), AsA (ascorbic acid), total soluble protein, MDA (malondialdehyde), H_2O_2(hydrogen peroxide), CAT (catalase), POX (peroxidase), SOD (superoxide dismutase), APX (ascorbate peroxidase) and GPX (glutathione peroxidase), at zero, one, three and five weeks sampling times. The data shown in the table including df and F-value superscripted with P-value designated as *** ($p \leq 0.001$), ** ($p \leq 0.01$) and * ($p \leq 0.05$).

Treatment	Time (weeks)	Df	Proline	GSH	AsA	Protein	MDA	H2O2	CAT	POX	SOD	APX	GPX
OWE	0	1	524.91^{***}	2669.1^{***}	169.1^{***}	6363.59^{***}	272.88^{***}	34.51^{***}	177.17^{***}	465.7^{***}	646.48^{***}	648.5^{***}	388.35^{***}
	1	1	193.4^{***}	593.2^{***}	41.43^{***}	2091.98^{***}	189.63^{***}	242.15^{***}	178.46^{***}	1.21^{NS}	345.05^{***}	29.54^{***}	797.91^{***}
	3	1	953.78^{***}	47.16^{***}	32.1^{***}	715.23^{***}	65.87^{***}	58.79^{***}	1246.27^{***}	44.39^{***}	48.93^{***}	1563.23^{***}	48.25^{***}
	5	1	1130.21^{***}	622.55^{***}	742.13^{***}	5824.39^{***}	85.1^{***}	0.85^{NS}	144.61^{***}	21.29^{***}	543.42^{***}	127.77^{***}	335.18^{***}
Salinity	0												
	1	2	189.4^{***}	148.87^{***}	85.17^{***}	469.4^{***}	729.69^{***}	351.52^{***}	136.55^{***}	0.69^{NS}	735.29^{***}	114.39^{***}	214.75^{***}
	3	2	494.26^{***}	179.54^{***}	2.82^{NS}	665.52^{***}	374.95^{***}	220.14^{***}	277.26^{***}	444.05^{***}	405.23^{***}	1538.25^{***}	158.16^{***}
	5	2	1177.22^{***}	497.24^{***}	245.6^{***}	1109.98^{***}	370.25^{***}	154.92^{***}	90.54^{***}	161.95^{***}	1243.58^{***}	453.21^{***}	342.46^{***}
OWE *Salinity	0												
	1	2	1.55^{NS}	5.86^{**}	1.64^{NS}	22.95^{***}	12.57^{***}	6.69^{**}	50.36^{***}	2.28^{NS}	21.65^{***}	636.85^{***}	26^{***}
	3	2	36.75^{***}	0.09^{NS}	7.46^{**}	14.54^{***}	19.76^{***}	0.28^{NS}	109.34^{***}	7.97^{**}	7.048^{**}	818.66^{***}	1.36^{NS}
	5	2	39.92^{***}	1.27^{NS}	42.03^{***}	40^{***}	3.5^{*}	3^{NS}	54.15^{***}	10.46^{***}	138.01^{***}	2.03^{NS}	19.27^{***}

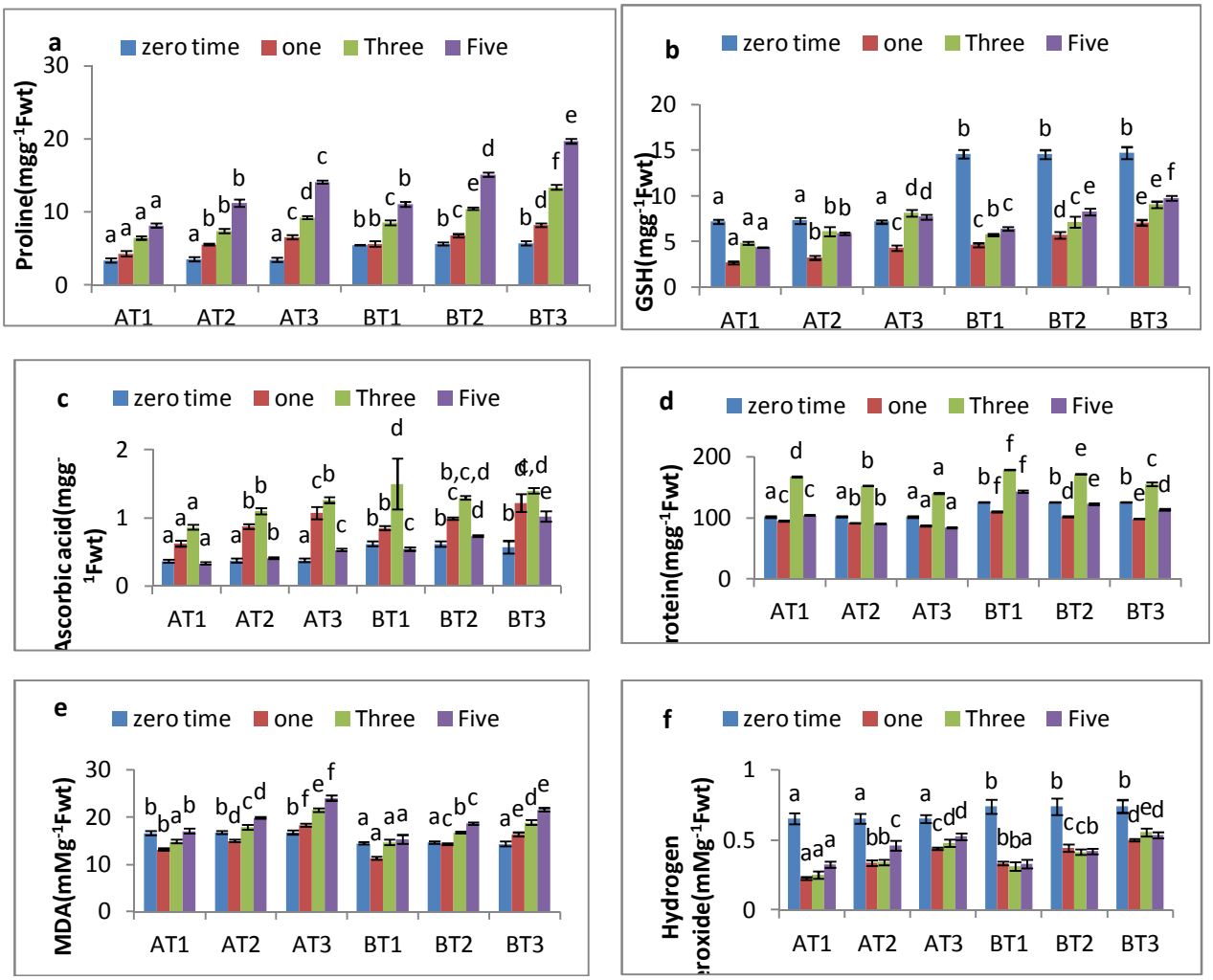

Fig.1. Effect of different treatments (AT1: Seeds were soaked in dist.water and irrigated with tap water; AT2: Seeds were soaked in dist.water and irrigated with 50mMNaCl; AT3: Seeds were soaked in dist.water and irrigated with 100mMNaCl; BT1: Seeds were soaked in 0.75% OWE and irrigated with tap water; BT2: Seeds were soaked in 0.75% OWE and irrigated with 50mMNaCl and BT3: Seeds were soaked in 0.75% OWE and irrigated with 100mMNaCl) on proline ($mgg^{-1}Fwt$), ascorbic acid ($mgg^{-1}Fwt$), Reduced glutathione ($mgg^{-1}Fwt$), Protein ($mgg^{-1}Fwt$), MDA ($mMg^{-1}Fwt$) and Hydrogen peroxide ($mMg^{-1}Fwt$). The same litters mean non-significant while the different litters mean significant at $p \leq 0.05$.

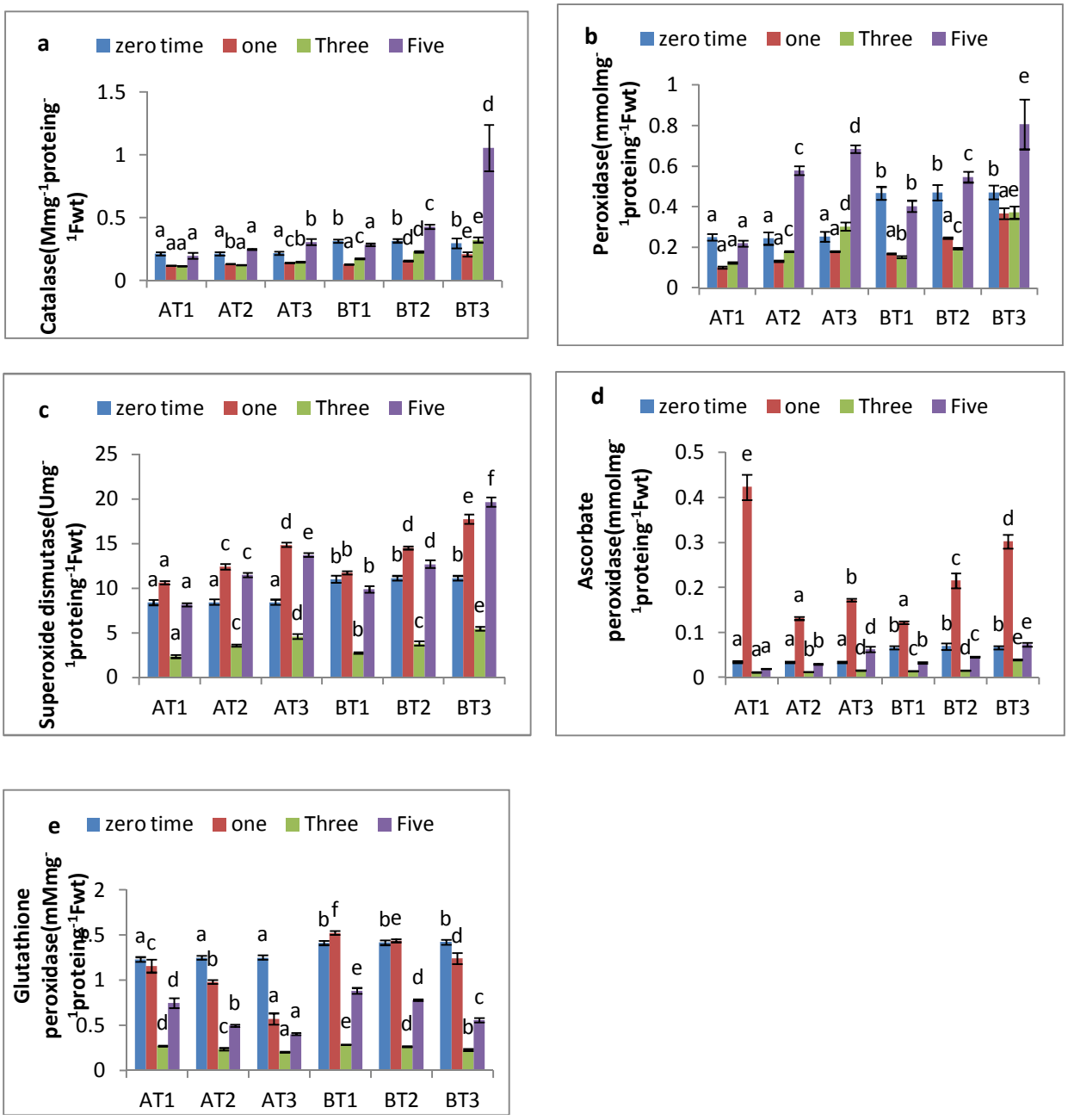

Fig.2. Effect of different treatments (AT1: Seeds were soaked in dist.water and irrigated with tap water; AT2: Seeds were soaked in dist.water and irrigated with 50mMNaCl; AT3: Seeds were soaked in dist.water and irrigated with 100mMNaCl; BT1: Seeds were soaked in 0.75% OWE and irrigated with tap water; BT2: Seeds were soaked in 0.75% OWE and irrigated with 50mMNaCl and BT3: Seeds were soaked in 0.75% OWE and irrigated with 100mMNaCl) on CAT ($mMmg^{-1}proteing^{-1}Fw$), POX ($mMmg^{-1}proteing^{-1}Fw$), SOD ($Umg^{-1}proteing^{-1}Fw$), APX ($mmolmg^{-1}$ $proteing^{-1}$ Fw) and GPX ($mMmg^{-1}proteing^{-1}Fw$). The same litters mean non-significant while the different mean significant at $p \leq 0.05$.

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