Effects of penconazole on hormonal crosstalk and fatty acids from salt-stressed safflower

Fatemeh Shaki, Hasan Ebrahimzadeh Maboud, and Vahid Niknam

Department of Plant Biology, School of Biology, College of Science, University of Tehran, Tehran, Iran

Abstract

Salinity of soil is a major abiotic stress limiting the crop production and growth of safflower. To mitigate stress, the effects of penconazole (PEN) on the growth of safflower were studied to understand the underlying mechanisms of salt tolerance. PEN, a triazole derivative, which has both fungicidal and plant growth regulator properties, protects plants from several types of abiotic stresses. The purpose of this work was to assess the effect of sodium chloride (0, 100, and 200 mM) and PEN (15 mg/L) on some biochemical responses of safflower. Results revealed that salicylic acid (SA) content increased under salinity while indol-3-acetic acid (IAA) and gibberellic acid (GA) contents decreased. Further, in terms of fatty acids, palmitic and oleic acids contents decreased while stearic, linoleic, and linolenic acids contents increased under salinity. Exogenous PEN had a positive effect on SA and GA contents as well as palmitic and stearic acids content, but it decreased IAA, linoleic acid, and linolenic acid contents in safflower plants. Safflower is a viable alternative for use in rotations where saline irrigation water limits productivity of non-tolerant crops. Our data provided new insights into mechanisms that help regulate salinity resistance in safflower. PEN may be considered as foliar application to ameliorate salinity effects due to its low price and availability.

Keywords: safflower; salinity; penconazole; fatty acids; phytohormones

Shaki, F., H. Ebrahimzadeh Maboud and V. Niknam. 2020. 'Effects of penconazole on hormonal crosstalk and fatty acids from salt-stressed safflower'. *Iranian Journal of Plant Physiology* ,10 (3), 3213-3221.

Introduction

Global climate change is one of the major challenges of our time and the socio-economic consequences are alarming. One consequence of climate change is a shift in rainfall distribution with less rainfall in some regions, which in turn may increase the salinity of soils. As a result, plant nutrient availability reduces, and therefore, growth, development, and productivity decreases (Wang et al., 2015; Azimian and Roshandel, 2016; Ali and Yun, 2017; Samadi et al., 2019; Shaki et al., 2019).

Further, salinity is a problem in safflower (*Carthamus tinctorius* L.) plants in many areas around the world (Gengmao et al., 2015). Safflower is an oilseed crop grown for its flowers and seeds, which have numerous biological properties (Peiretti, 2009; Peiretti et al., 2017; Chavoushi et al., 2019). Safflower is also known as a salt-tolerant plant even though its growth and yield decreases as the salinity level increases (Bassil and Kaffka, 2002; Gengmao et al., 2015).

Plant responses to stresses are coordinated by arrays of growth and

*Corresponding author
E-mail address: fatemeshaki373@ut.ac.ir,
Received: July, 2019
Accepted: March, 2020

developmental programs, which involves a variety of mechanisms that allow them to adapt to adverse conditions (Du et al., 2013). To mitigate environmental stresses, plants induce production of some key hormones such as salicylic acid (SA), as well as desaturation of fatty acids in cytosol. Phytohormones play central roles in adaptation to changing environments by mediating nutrient allocation, growth, and development. It has been reported that plant hormones are able to reduce symptoms of environmental stresses in plants (Hayat et al., 2010). The ability of exogenous SA to enhance antioxidant protection, to increase accumulation of osmolytes, and to maintain optimum Na^+/K^+ ratio under saline conditions has been suggested as potential mechanisms of salt tolerance in plants (Hayat et al., 2010; Shaki et al., 2017). SA regulates various aspects of plant responses to stress through extensive signaling cross-talk with other hormones such as gibberellic acid (GA) and indol-3-acetic acid (IAA). Further, hormonal cross-talk results in synergetic or antagonistic interactions that play crucial roles in plant response to stress (Jayakannan et al., 2013).

The ability to respond to environmental stimuli is among the most fundamental processes that enable plants to survive. Various agents such as penconazole (PEN) have been proposed as signal transducers in plant responses to environmental stresses. PEN, considered a triazolic fungicide, causes several responses in plants (Merati et al., 2014). This compound could cause induction of root growth, reduction in reactive oxygen species damage, and increase in antioxidant potential under stress conditions (Hassanpour et al., 2012; Merati et al., 2014). These qualities make that an ideal chemical to increase plant resistance to stress conditions such as salinity.

Our previous work revealed that PEN had a positive effect on safflower plant growth in saline conditions, and could minimize the negative effects of salt stress by increasing some antioxidant compounds, as well as the regulation of compatible solutes observed in such conditions, and could be used for amelioration of stress in safflower plants (Shaki et al., 2018). To date, there is a little information available about the effect of PEN on phytohormones and fatty acids profile in plants and particularly, there is no information on safflower. Thus, the working hypothesis for this study was that the beneficial effects of PEN during salt stress may be related to the existence of a crosstalk between phytohormones in stress conditions, as well as desaturation of fatty acids in the membrane. Therefore, the purpose of this work was to assess the effect of PEN on some key biochemical parameters in safflower. Revealing the mechanisms underlying salt tolerance of safflower which is mediated by PEN, might provide a basis to improve safflower growth in saline areas around the world.

Materials and Methods

Plant cultivation and chemical treatments

Seeds from safflower plants were sown in Tref peat in a greenhouse with 15 h light/9 h dark photoperiod, 27±2 ℃ temperature, and 70% relative air humidity. Seedlings were thinned to five per free-draining plastic pot (15 cm in diameter, 15 cm deep) filled with perlite, 4 weeks after sowing. Based on the studied PEN concentrations (0, 5, 10, 15, and 20 mg L^{-1}), 15 mg L^{-1} of PEN was selected as the optimum level for further work. Each pot was considered as one replicate and there were 3 replicates of each treatment (0, 100, and 200 mM NaCl) with or without PEN (15 mg L^{-1}). The pots were irrigated every other day with 100 ml half-strength Hoagland solution (pH 6.8-7) (Hoagland and Arnon, 1950). There were six groups of pots and each group started to receive Hoagland solution containing different concentrations of NaCl (0, 100 and 200 mM).

The PEN was dissolved in distilled water and 3 to 4 mL of the solution was sprayed (once a week, for 3 weeks) uniformly at the vegetative stage of the plants. The final harvest was performed 21 days after the start of treatments, and fresh leaves were stored at -70 ℃ until performing analysis.

Salicylic acid (SA) quantification by HPLC

Quantification of salicylic acid (SA) was performed according to the method of Wen et al. (2005). Leaf tissues (1g) were extracted with methanol/water/trifluoroacetic acid (TFA)

(50:50:0.1) mixed solvent, and the volume of the turbid fluid was adjusted to 10 ml. Chromatographic separations were performed on an Agilent 1200 series high-performance liquid chromatography, including a quaternary pump and a degasser equipped with a G1321A fluorescence detector and a G1315D diode array detector. Separation process was carried out on a C18 column (250 9 4.6 mm, with 5.0 m particle size) from Waters Company (Massachusetts, USA). The injection volume was 10 µl and samples were detected at 305 nm.

Determination of indol-3-acetic acid (IAA)

The method for determination of IAA production was described by Malik and Singh (1980). Fresh leaf tissue (0.1 g) was extracted in 3 ml ethanol 96%. The IAA concentration was determined using UV–Vis spectrophotometer (UV-160, Shimadzu, and Tokyo, Japan) at 535 nm.

Determination of gibberellic acid (GA)

Determination of GA was based on the method described by Berrios et al. (2004). Fresh leaf tissue (0.1 g) was extracted in 3 ml ethanol 96%. The absorbance of the solution was measured by spectrophotometer at 254 nm. The concentration of GA in the sample was determined using linear regression equation of standard graph.

Extraction and analysis of fatty acids

Leaf samples (1 g) were extracted with chloroform: methanol (2:1 v/v) following the modified procedure of Bligh and Dyer (1959). For gas chromatography analysis, a Shamadzu GC-17A gas chromatograph (Shimadzu Italia, Milan, Italy) equipped with a flame ionization detector (FID) and an electronic pressure control (EPC) injector was used. The injection volume was 1 µL. Identification of FAMEs was made by comparing their retention times with those of reference standards (Steinheim, Germany).

To evaluate the efficiency of the desaturation pathway during salt treatment, the desaturation ratios from oleic to linoleic (ODR: oleic desaturation ratio), linoleic to linolenic acid (LDR: linoleic desaturation ratio), and oleic acid/linoleic acid ratio (OLR) were calculated as follows:

ODR = [(% $C_{18:2}$ + % $C_{18:3}$) / (% $C_{18:1}$ + % $C_{18:2}$ + % $C_{18:3}$)] × 100

LDR = [(% $C_{18:3}$) / (% $C_{18:2}$ + % $C_{18:3}$)] × 100

OLR = % $C_{18:1}$ / % $C_{18:2}$

The magnitude of desaturation ratios represents the amount of substrate which is desaturated from C18:1 to C18:2 and C18:3, thus providing a proportional measure of the desaturating enzymes' activities during salt treatment (Mondal et al., 2010).

Statistical Analysis

This experiment was performed in a completely randomized design (CRD) and each data point was the mean of three replicates (n=3) in each group. Statistical calculations were performed with SPSS (version 18). Tests for significant differences were conducted using analysis of variance (ANOVA) with Duncan's multiple range tests at the 0.05 level of confidence. The principal component analysis (PCA) was laid out using the XLSTAT (version 2018.7).

Results

HPLC analysis in safflower showed that SA content in leaves of salt-stressed plants (both 100 and 200 mM NaCl) was significantly higher in comparison with controls (Fig. I). Following PEN treatment of plants, their SA content was significantly induced, in salt-stressed plants.

In general, NaCl treatment resulted in a remarkable increase of linoleic and linolenic acids as the critical unsaturated fatty acids in salt-

treated safflower plants, which reflects the higher efficiency of the desaturation system from oleic to linoleic acid. There was also a reduction in the OLR, showing a reduction in oleic acid and an increase in linoleic acid in salt-treated plants.

The correlation matrix indicated correlation coefficients between sets of variables measured in this study (Table 1). Further, principal component analysis (PCA) showed that principal component 1 (F1) described 57.54% of total variation and principal component 2 (F2) described 22.77% (Fig. IV) with a cumulative percentage of 80.31%.

Discussion

In this work, some biochemical parameters were investigated to better understand the impacts of exogenous PEN application on safflower plants in saline conditions. The present study, for the first time, reports the positive effects of PEN on salt tolerance of safflower, in terms of hormonal crosstalk and unsaturation of fatty acids.

There are some reports on the role of phytohormones in stress conditions; however, much more information is needed on the changes in the metabolism of these components in plants. In our experiment, the SA content increased, while the IAA and GA contents decreased under salt stress. Our results are in agreement with other findings in various plant species under stress conditions (Rubin et al., 2002). Further, enhancement of growth and productivity of salt-treated plants under exogenous GA application has been previously observed by other researchers (Prakash and Prathapasenan, 1990; Amzallag et al., 1992; Aharoni et al., 1977; Hamayun et al., 2010). It is also reported that SA may have a role in some of the physiological processes associated with GA since exogenous SA was able to improve seed germination in *Arabidopsis* plants under saline conditions (Alonso-Ramírez et al., 2009).

Exogenous application of PEN prevented, to some extent, the negative effects of stress and allowed increased SA and GA contents, as well as IAA content reduction in safflower. Our data support the idea that enhanced levels of SA following exogenous application of PEN can have

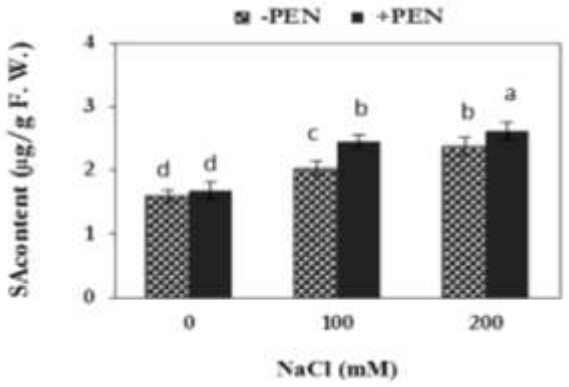

Fig. I. Effects of salinity and penconazole (PEN) on salicylic acid (SA) content in safflower 21 days after the start of treatments; the groups are -PEN (plants with no PEN treatment) and +PEN (plants sprayed with 15 mg/L PEN once a week). Columns indicate mean ± SE.

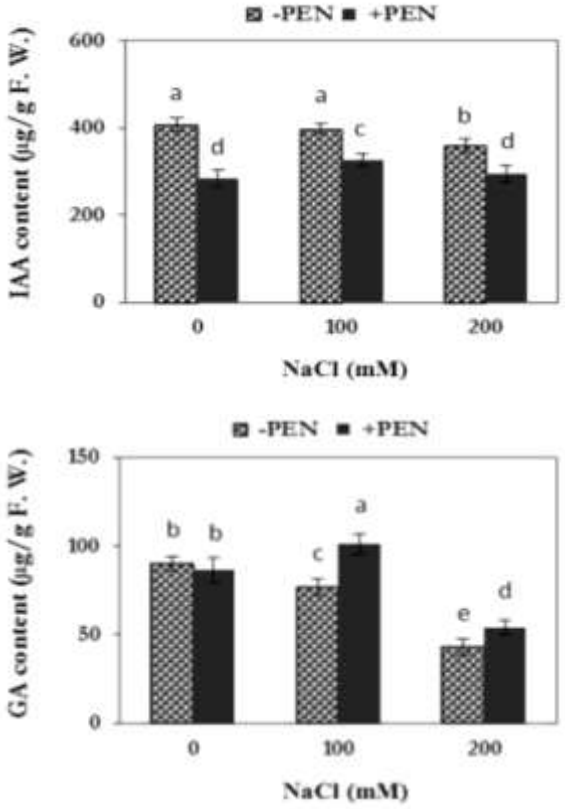

Fig. II. Effects of salinity and penconazole (PEN) on content of indol-3-acetic acid (IAA) and gibberellic acid (GA) in safflower 21 days after the start of treatments; the groups are -PEN (plants with no PEN treatment) and +PEN (plants sprayed with 15 mg/L PEN once a week). Columns indicate mean ± SE.

an important role in GA biosynthesis and action, and that some of the physiological effects of this hormone may be mediated by GA. It can also be hypothesized that the lower IAA content in PEN-

Table 1
Correlation matrix (Pearson) analysis; results obtained from the biochemical data of safflower subjected to salinity and penconazole (PEN)

Variables	SA	IAA	GA	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	ODR	LDR	OLR
SA	1										
IAA	-0.346	1									
GA	-0.486	0.083	1								
Palmitic acid	-0.580	-0.408	0.550	1							
Stearic acid	0.751	0.139	-0.564	-0.734	1						
Oleic acid	-0.638	0.387	0.923	0.513	-0.534	1					
Linoleic acid	0.225	0.625	-0.395	-0.912	0.431	-0.296	1				
Linolenic acid	0.197	0.330	-0.804	-0.699	0.304	-0.660	0.764	1			
ODR	0.423	0.128	-0.899	-0.773	0.499	-0.841	0.723	0.947	1		
LDR	-0.071	-0.405	-0.611	0.303	-0.165	-0.535	-0.331	0.353	0.340	1	
OLR	-0.529	-0.258	0.752	0.940	-0.636	0.722	-0.872	-0.863	-0.938	0.000	1

treated plants could be the result of triazole influence on the endogenous cytokinin content in cytosol. Although the change in cytokinin content was not obtained in this work but, it can be suggested that the reduction in IAA content could interfere with increased cytokinin content under PEN treatment in treated plants (Hassanpour et al., 2013). In summary, our results show the existence of a crosstalk between these hormones in stress conditions, showing another junction in the complex mechanism of hormone interactions.

Salinity also modified fatty acids composition in safflower, which is considered to be very important in stress tolerance (Azachi et al., 2002). In our experiment, increased content of linoleic and linolenic acids were observed under salinity. Further, a redirection of the lipidic metabolism towards synthesis of unsaturated fatty acids was observed. Taken together, such composition could be related to the importance of maintaining a high degree of unsaturation to control membrane properties to cope with saline conditions. It is well established that fatty acids are important in salt resistance of plant species, and therefore, the extent of unsaturation of fatty acids is thought to maintain the membrane fluidity crucial for proper functions of membrane in plants (Azachi et al., 2002). Supporting our results, Gigon et al. (2004) reported increased unsaturation index in *Arabidopsis* plants under drought stress. Similarly, increased level of unsaturation was observed in *Brassica oleracea* under salinity (López-Pérez et al., 2009). They reported that drought stress caused an increase in linolenic acid content, which is resulted from the activation of desaturase enzymes activities.

Salt-induced harmful effects on fatty acids composition in safflower plants were reduced by PEN. Exogenous PEN caused a marked change in key saturated and unsaturated fatty acids of safflower (Fig. III). It can be assumed that PEN, in some extent, modulates stress impacts on fatty acids profile of plants. Similarly, the role of triazols in amelioration of stress effects on fatty acids of oilseed rape (*Brassica napus* L.) was investigated by Leul and Zhou (1999), which can support our results.

Principal component analysis in this study helped to identify the important parameters responsible for plant mechanisms changes in

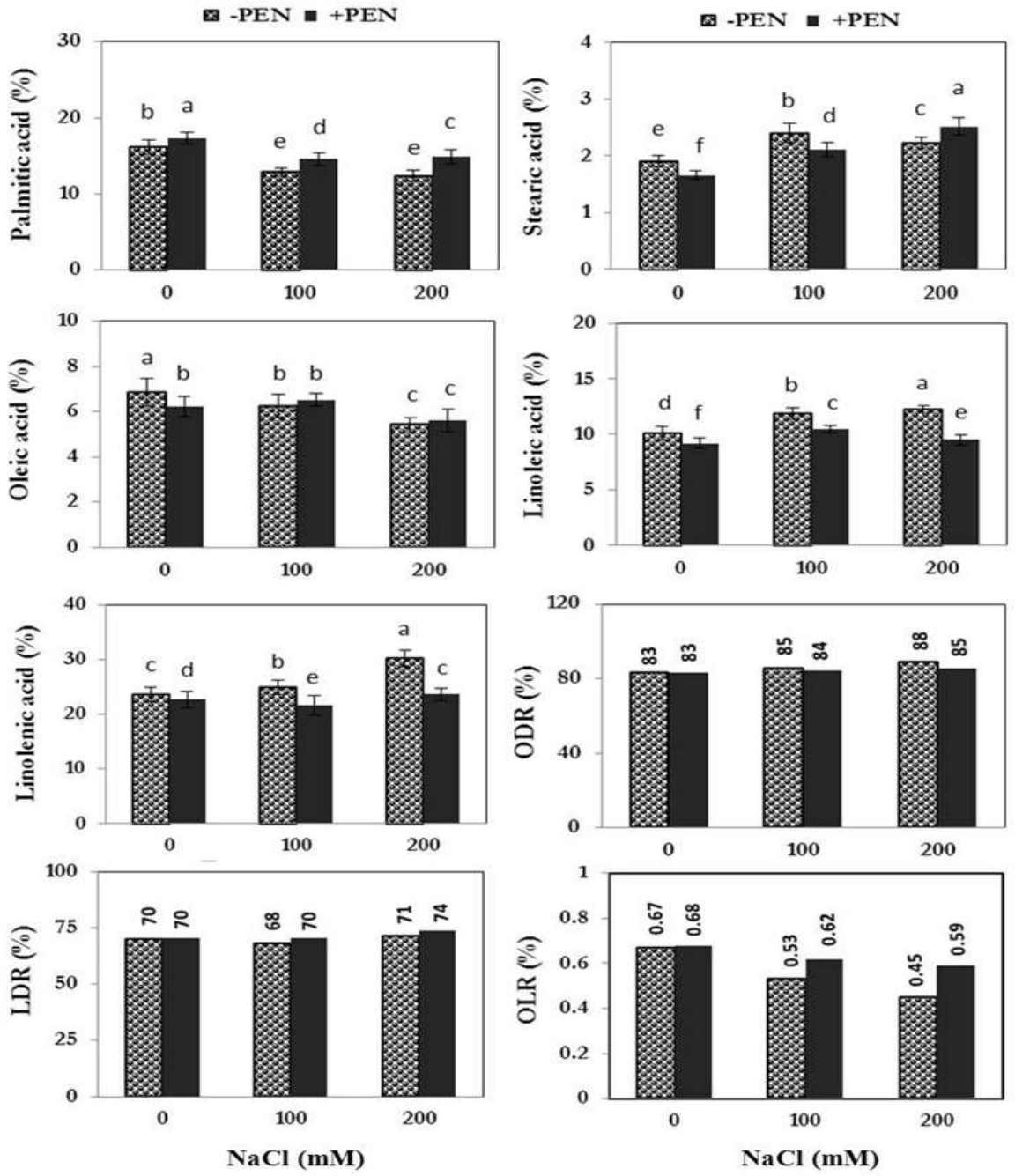

Fig. III. Effects of salinity and penconazole (PEN) on the content of fatty acids composition in safflower 21 days after the start of treatments; the groups are -PEN (plants with no PEN treatment) and +PEN (plants sprayed with 15 mg/L PEN once a week). Columns indicate mean ± SE. ODR: oleic desaturation ratio, LDR: linoleic desaturation ratio, OLR: oleic acid/linoleic acid ratio

stressful environments. This analysis allowed us to obtain the key information from a multivariate table. Therefore, according to the results, the loadings in F1 and F2 were compared (Fig. IV) to investigate the contributors of the measured parameters. It is suggested that parameters with vectors in the same directions had a positive correlation with each other, and a negative correlation with other measured parameters, which indicate the effects of salinity on these parameters might be different from that on the other parameters. In addition, it was observed that PEN had a positive effect on the most measured parameters in unstressed and salt-stressed safflower plants under 100 and 200 mM NaCl treatments.

Conclusion

Taken together, our data revealed that PEN, a triazolic fungicide, helps safflower plants to cope with saline conditions. This is supported by the desaturation of fatty acids in cell membrane. In addition, the results indicated that PEN reduces the negative effects of salt stress by enhancement of key plant hormones observed in such conditions, and therefore, it could be used for improvement of plant growth and productivity in stress conditions. Further, the results show the existence of a crosstalk between plant hormones, showing another junction in the complex mechanism of hormone interactions. The data obtained from this work provide new insights to discovering the important mechanisms responsible in salt tolerance of safflower. In summary, it is suggested that PEN can reduce negative effects of salt in safflower, due to availability and low price, which are important in crop production.

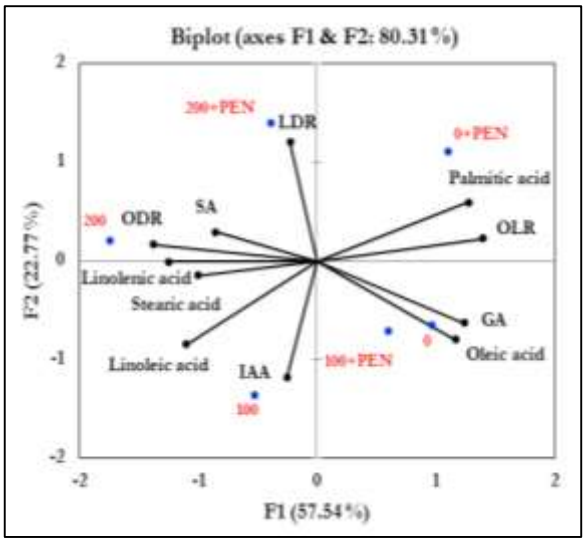

Fig. IV. Loading plots of principal components 1 and 2 of the PCA.; results obtained from the biochemical data of safflower subjected to salinity and penconazole (PEN); SA: salicylic acid, IAA: indol-3-acetic acid, GA: gibberellic acid, ODR: oleic desaturation ratio, LDR: linoleic desaturation ratio, OLR: oleic acid/linoleic acid ratio

References

Aharoni, N., A. Blumenfeld and A.E. Richmond. 1977. 'Hormonal activity in detached lettuce leaves as affected by leaf water content'. *Plant Physiol.* 59(6):1169-1173.

Ali, A. and **D. J. Yun.** 2017. 'Salt stress tolerance; what do we learn from halophytes?' J. *Plant Biol*. 60: 431-439.

Alonso-Ramírez, A., D. Rodríguez, D. Reyes, J.A. Jiménez, G. Nicolás, M. López-Climent, A. Gómez-Cadenas and **C. Nicolás.** 2009. 'Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in Arabidopsis seeds'. *Plant Physiol.* 150(3):1335-1344.

Amzallag, G., H. Lerner and **A. Poljakoff-Mayber.** 1992.' Interaction between mineral nutrients, cytokinin and gibberellic acid during growth of Sorghum at high NaCl salinity'. *J. Exp. Bot.* 43(1):81-87.

Azachi, M., A. Sadka, M. Fisher, P. Goldshlag, I. Gokhman and **A. Zamir.** 2002. 'Salt induction of fatty acid elongase and membrane lipid modifications in the extreme halotolerant alga *Dunaliella salina*.' *Plant Physiol.* 129(3):1320-1329.

Azimian, F. and **P. Roshandel.** 2016. 'Physiological and phytochemical changes induced by seed pretreatment with hydrogen peroxide in *Artemisia sieberi* under salt stress'. *Plant Physiology* 7(1):1875-1887.

Bassil, E.S. and **S.R. Kaffka.** 2002. 'Response of safflower (*Carthamus tinctorius* L.) to saline soils and irrigation: I. consumptive water use. Agric'. *Water Manag*. 54(1):67-80.

Berríos, J., A. Illanes and **G. Aroca.** 2004. 'Spectrophotometric method for determining gibberellic acid in fermentation broths'. *Biotechnol. Lett.* 26(1):67-70.

Bligh, E.G. and **W.J. Dyer.** 1959.' A rapid method of total lipid extraction and purification'. *Can. J. Biochem. Physiol*. 37(8):911-917.

Chavoushi, M., F. Najafi, A. Salimi and **S. Angaji.** 2019. 'Improvement in drought stress tolerance of safflower during vegetative growth by exogenous application of salicylic acid and sodium nitroprusside'. *Ind. Crop Prod.* 134, 168-176.

Du, H., H. Liu and **L. Xiong.** 2013. 'Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice'. *Front. Plant Sci.* 4:397.

Gengmao, Z., H. Yu, S. Xing, L. Shihui, S. Quanmei and **W. Changhai.** 2015. 'Salinity stress

increases secondary metabolites and enzyme activity in safflower'. *Ind. Crop Prod*. 64, 175-181.

Gigon, A., A-R. Matos, D. Laffray, Y. Zuily-Fodil and **A-T. Pham-Thi.** 2004. 'Effect of drought stress on lipid metabolism in the leaves of *Arabidopsis thaliana* (ecotype Columbia)'. *Ann. Bot.* 94(3):345-351.

Hamayun, M., S.A. Khan, A.L. Khan, J-H. Shin, B. Ahmad, D-H. Shin and **I-J. Lee.** 2010. 'Exogenous gibberellic acid reprograms soybean to higher growth and salt stress tolerance'. *J. Agric. Food Chem*. 58(12):7226-7232.

Hassanpour, H., R.A. Khavari-Nejad, V. Niknam, F. Najafi and **K. Razavi.** 2012. 'Effects of penconazole and water deficit stress on physiological and antioxidative responses in pennyroyal (*Mentha pulegium* L.).' *Acta physiol. plant*. 34:1537-1549.

Hassanpour, H., R.A. Khavari-Nejad, V. Niknam, F. Najafi and **K. Razavi.** 2013. 'Penconazole induced changes in photosynthesis, ion acquisition and protein profile of *Mentha pulegium* L. under drought stress'. *Physiol. Mol. Biol. Plants*, 19(4):489–498.

Hayat, Q., S. Hayat, M. Irfan and **A. Ahmad.** 2010. 'Effect of exogenous salicylic acid under changing environment: a review'. *Environ. Exp. Bot.* 68(1):14-25.

Hoagland, D.R. and **D.I. Arnon.** 1950. 'The water-culture method for growing plants without soil.' *Circ. Calif. Agric. Exp. Stn.* 347 (2nd edit).

Jayakannan, M., J. Bose, O. Babourina, Z. Rengel and **S. Shabala.** 2013. 'Salicylic acid improves salinity tolerance in *Arabidopsis* by restoring membrane potential and preventing salt-induced K^+ loss via a GORK channel'. *J. Exp. Bot.* 64(8):2255-2268.

Leul, M. and **W. Zhou.** 1999. 'Alleviation of waterlogging damage in winter rape by uniconazole application: effects on enzyme activity, lipid peroxidation, and membrane integrity'. *J. plant growth regul.* 18(1):9-14.

López-Pérez, L., M. del Carmen Martínez-Ballesta, C. Maurel *and* M. Carvajal. 2009. 'Changes in plasma membrane lipids, aquaporins and proton pump of broccoli roots, as an adaptation mechanism to salinity'. *Phytochem.* 70(4):492-500.

Malik, C.P. and **M.B. Singh.** 1980. 'Plant enzymology and histoenzymology.' *New Delhi: Kalyani Publisher*. 51-53.

Merati, M.J., H. Hassanpour, V. Niknam and **M. Mirmasoumi.** 2014. 'Exogenous application of penconazole regulates plant growth and antioxidative responses in salt-stressed *Mentha pulegium* L.' *J. Plant Interac.* 9:791-801.

Mondal, N., K. Bhat and **P. Srivastava.** 2010. 'Variation in fatty acid composition in Indian germplasm of sesame'. *J. Am. Oil Chem' Soc*. 87(11):1263-1269.

Naqvi, S., R. Ansari *and* M. Hanif. 1986. 'Auxin physiology in *Zea mays* L. under saline growth conditions'. *Prospects for Biosaline Research*. 307.

Peiretti, P.G. 2009. 'Effects of growth stage on chemical composition, organic matter digestibility, gross energy and fatty acid content of safflower (*Carthamus tinctorius* L.).' Livestock Research for Rural Development, 21(12): Paper 206.

Peiretti, P.G., F. Gai, M. Karamać and **R. Amarowicz.** 2017. 'Antioxidant activities and phenolic composition of safflower (*Carthamus tinctorius* L.) plant during their growth cycle.' In: "Agricultural Research Updates" (Eds: Gorawala P. and Mandhatri S.), Nova Science Publishers, Inc., NY, Volume 19, Chapter 2, pp. 23–39.

Prakash, L. and **G. Prathapasenan.** 1990. 'NaCl- and gibberellic acid-induced changes in the content of auxin and the activities of cellulase and pectin lyase during leaf growth in rice (*Oryza sativa*)'. *Ann. Bot.* 65(3):251-257.

Rubin, N., J.G. Carman and **F.B. Salisbury.** 2002. 'Water stress, COs and photoperiod influence hormone levels in wheat'. *J. Plant Physiol*. 159 (3):307.

Samadi, S. G. Habibi and **A. Vaziri.** 2019. 'Effects of exogenous salicylic acid on antioxidative responses, phenolic metabolism and photochemical activity of strawberry under salt stress'. I*ranian Journal of Plant Physiology* 9(2):2685-2694.

Shaki, F., H. Ebrahimzadeh Maboud and **V. Niknam.** 2017. 'Central role of salicylic acid in resistance of safflower (*Carthamus tinctorius*

L.) against salinity'. *J. Plant Interac*. 12(1):413-419.

Wen, D., C. Li, H. Di, Y. Liao and **H. Liu.** 2005.' A universal HPLC method for the determination of phenolic acids in compound herbal medicines'. *J. Agri. Food Chem*. 53(17):6624-6629.