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Article

Changes in activities of antioxidant enzymes in radish (*Raphanus sativus*) seedlings in response to allelopathic effect of safflower (*Carthamus tinctorius*)

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ROS (Reactive Oxygen Species) production is a usual plant reaction to environmental stresses such as allelopathy. Plants possess antioxidant enzymes to scavenge cells and resist against the ROS. This study was conducted to evaluate changes in antioxidant enzymes (CAT, GPX, APX) in radish seedlings in response to allelopathic effect of safflower root and shoot residues grown under normal irrigation and drought stress. Safflower allelopathic effect led to an increase in antioxidant enzymes activities. GPX activity increased more than CAT and APX. Radish seedlings exposed to safflower residue grown under drought stress showed more antioxidant enzymes activities. Root residues enhanced the activities of antioxidant enzymes greater than shoot. Seedlings exposed to root residues grown under drought stress had the highest level of antioxidant enzymes activities.

Key Words: ROS. CAT. GPX. APX. Allelopathy.

INTRODUCTION

Some weeds and crop species are able to release biochemicals such as phenol, alkaloids, fatty acids, flavonoids into their rhizosphere, which can enhance, reduce the germination and growth of plants growing in their vicinity (Modhej, Rafatjoo, Behdarvandi, 2013). Allelopathy refers to biotic interactions among plants, microorganisms and algae induced by allelochemicals released into the environment (Cruz-Ortega, Ayala-Cordero, Anaya, 2002; Cruz-Silva *et al*., 2015).

Production of ROS (Reactive Oxygen Species) is a usual plant reaction to environmental stresses such as temperature, salinity, drought, heavy metals, pollutants and allelopathy (Gniazdowska *et al*., 2015). Plants possess antioxidant enzymes to scavenge cells and resist against the ROS. Antioxidant capacity of plants correlates with their stress tolerance (Abedi, Pakniyat, 2010; Adamik, 2015). The cellular system of plants is responsible for controlling concentration of ROS and contains soluble antioxidant compounds such as ascorbic acid, vitamin E, glutathione and a battery of enzymes that can scavenge ROS: superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), thioredoxin (Trx), and the enzymes of Asada-Halliwell-Foyer pathway (Foyer, Noctor, 2005). The activities of antioxidant enzymes are generally grown during stress conditions and correlate with improved cellular protection (Khanna-Chopra, Selote, 2007). Allelopathic effects may lead to an imbalance between antioxidant defense and the amount of ROS, resulting in oxidative stress (Siddique, Ismail, 2013).

Safflower (*Carthamus tinctorius*) is an oilseed crop, which is grown throughout the world for its high quality oil and red and orange pigments extracted from its flowers. In recent years, safflower cultivation has increased due to the adaptability of safflower to varied growth conditions in particular to arid and semi-arid climates (Yousefi Davood *et al.,* 2013).

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Allelopathic potential of safflower has been reported in several studies. Miri (2011) indicated that safflower significantly reduced the germination and root and shoot growth of wild barley and has great potential for management of this weed in wheat production. Farhoudi, Lee (2012) showed that the safflower extracts inhibited the induction of α-amylase in wild mustard seeds. Modhej, Rafatjoo, Behdarvandi (2013) indicated that wild mustard seedling growth and seed germination were negatively affected by safflower allelopathic extract. Furthermore, Bonamigo *et al.* (2013) demonstrated that seedling emergence and early growth stages of canola were negatively affected by safflower aqueous extracts.

This study was conducted to evaluate changes in antioxidant enzymes in radish seedlings in response to allelopathic effect of safflower root and shoot residues grown under drought stress and normal irrigation.

MATERIAL AND METHODS

Plant materials and growth condition

Allelopathic potential of forty safflower genotypes was evaluated in the previous study (Motamedi, Karimmojeni, Ghorbani sini, 2016). The present study was conducted on four safflower genotypes comprising Khorasan (Khorasan330), Egypt (PI 657800), Kerman (CTNIR9) and Australia (PI 262424) from screening forty genotypes. Khorasan (Khorasan330) and Egypt genotype (PI 657800) with the most and Kerman (CTNIR9) and Australia genotype (PI 262424) with the least inhibitory effects on radish seedling growth were used in this study.

A pot experiment with three replications was performed in the growth chamber in research laboratory of agronomy and plant breeding of Isfahan University of Technology. Each plastic pot (the size of 1 kg) was filled with clay soil. Seeds of four genotypes were planted in 24 pots. For each genotype, 6 pots were considered that 3 pots were under normal irrigation and others were under drought stress after seedling establishment. Pots were kept in a growth chamber at 25° C for two months with 14-hour photoperiod. Irrigation was done up to the Field Capacity (FC) level. The amount of water at this level was considered 200 ml for each pot. The first irrigation was done just after cultivation. Next irrigation was done when the soil surface was dried.

Irrigation treatments

Irrigation treatments (normal irrigation and drought stress) were initiated after seedling establishment and just before reproductive phase and continued for a month. Half of pots were under normal irrigation and the others were under drought stress. For normal condition, irrigation was supplied when 30% of the total available water was depleted from the root zone. Drought treatments were irrigated when 60% of the total available water was depleted. To do that, a pot, the size of a kilogram, was watered until reaching the water saturation point and then covered with a plastic layer and weighed after 36 hours. This weight was considered as the Field Capacity (FC). Next irrigation was done when the weight of pot reached to 30% and 60% of the pot weight at FC level for normal irrigation and drought stress, respectively. This way of irrigation continued three times.

Shoot and root residues preparation

Safflower shoots were cut just above the soil surface, air-dried in the shade for 48 hours and ground via a grinder. The safflower roots were retained in the pots and radish seeds were cultivated inside them. New pots, the size of 1 kg, were filled with clay soil and shoot residues (3.14 g) was mixed with 5 cm of the topsoil. Fifteen radish seeds were planted in each pot as test plant. To determine the allelopathic effect of root residues, fifteen radish seeds were also planted in each pot containing safflower root residues. All pots were kept in the growth chamber at 25° C for 2 weeks. On the fifth day after planting, radish seedlings were thinned down to 10 seedlings.

Control condition

For each genotypes 3 pots were considered as control treatment. The pots were irrigated at Field capacity (FC). Fifteen radish seeds were planted in the soil without the presence of safflower residues in theses pots. The pots were also kept in the growth chamber (25° C) for 2 weeks.

Seedlings were thinned down to 10 by the fifth day after planting.

Antioxidant enzyme assay

Two weeks after planting seeds, radish seedlings were harvested and Samples were kept in -80 degree freezer. Then antioxidant enzymes activities of control and treated plants such as catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) were measured.

Enzyme extract preparation

The extraction buffer contained 50 mM potassium phosphate buffer (PH=7), 1% Triton X-100 and 7 mM 2-Mercaptoethanol. All biochemical analyses were performed at 4° C, 0.5 g of fresh leaves of control and treated plants was homogenized to a fine powder with a mortar and pestle under liquid nitrogen. One milliliter of extraction buffer was added to the powder and pipetted into test tubes. Centrifugation was done using Eppendorf 5810 centrifuge (2500 rpm, 20 min, 4° C) and the supernatant was used as the crude extract for the APX and GPX assay. For CAT assay, 0.5 g of fresh leaves was extracted as described above with 4.5 mL of 0.05 mM Tris-HCL buffer (PH=7.5), 3 mM $MgCl_2$ and 1 mM EDTA. The top layer of liquid in test tubes was used to determine the enzymes activities.

Protein assay

The protein concentration was measured by Bradford protein assay (1976) using bovine serum albumin as the standard protein. Based on this method, we mixed 50 ml ethanol (95%), 100 ml orthophosphoric acid, and 100 mg Coomassie brilliant blue, and distilled water was added to bring volume to 1,000 ml. Protein content was assayed using 3 ml of Bradford reagent containing 100 µl of protein extract, mixed and incubated at room temperature for 30 min. Protein content was measured at 595 nm absorbance by spectrophotometer (U-1800, Hitachi, Japan) using bovine serum albumin (BSA) as a standard

Catalase (CAT) measurement

Catalase activity was assayed spectrophotometrically by monitoring the rate of disappearance of H_2O_2 at 240 nm using the method of Maehly, Chance (1959). The reaction mixture consisted of 2.5 mL of 50 mM phosphate buffer (PH=7.4), 0.1 mL of 1% H_2O_2 and 50 µl of enzyme extract diluted to keep measurements within the linear range of the analysis. The decline in H_2O_2 was

$$\text{(U) CAT activity} = \frac{\Delta A \times TV \times D}{\varepsilon \times EV}$$

$$\left[\frac{U}{ml}\right] \text{ CAT volumetric activity} = \frac{CAT\ activity}{Unite\ Volume}$$

$$\left[\frac{U}{mgProtein}\right] \text{ Specific CAT activity} = \frac{CAT\ volumetric\ activity\ \left[\frac{U}{ml}\right]}{Extract\ Protein\ Concentration\ \left[\frac{mg}{ml}\right]}$$

U=A unit of catalase activity is equal to the amount of enzyme that catalysis the H2O2 to O2 and H2O in one min.
ΔA= Differences absorbance in 240 nm in one min
TV=Total bulk of buffer and extract (3 ml)
EV= Extract bulk (0.05 ml)
ε= Extinction coefficient for catalase (39.4 mM-1 cm-1)
D= Dilution factor

Glutathione peroxidase (GPX) measurement

In order to assessment of glutathione peroxidase activity, 50 µl of enzyme extract (pH 7.8) was evaluated in 3 ml final volume of 50 mM Na-phosphate buffer (containing 4.51 µl of H_2O_2 (30%), 3.35 µl Guiacol pH 7.0). Peroxidase activity was measured in 30s interval during 2 min at 470 nm absorbance. The same equation used for specific catalase activity was also used for calculation of specific peroxidase activity, while Ɛ = 26.6 (Teimouri Jervekani et al., 2018).

Ascorbate peroxidase (APX) measurement

The ascorbate peroxide activity of 50 µl of enzyme extract (pH 7.8) was measured in final 3 ml volume of 50 mM Na-phosphate buffer (containing 4.51 µl of H_2O_2 (30%), 100 µl of 5 mM ascorbate, pH 7.0). The absorbance was measured at 290 nm every 30 s for 2 min Based on the equation was used for calculating

the catalase activity, the ascorbate peroxide activity was also calculated while Ɛ = 2.8 (Teimouri Jervekani et al., 2018).

Statistical analysis

A factorial experiment with three factors was conducted in the form of a completely randomized design. First factor was the type of safflower genotype with four levels. Second factor was irrigation treatment with two levels of normal irrigation and drought stress. Third factor was the type of organ used as residues with two levels of root and shoot residues. In all experiments, three replicates were performed for each sample. All collected data were analyzed by SAS Ver.9.1 and mean comparisons were performed using least significant difference (LSD) test ($P < 0.01$).

RESULTS

In this experiment, radish has been used merely as a test plant for the comparison of safflower genotypes in terms of allelopathic potential, since radish is a susceptible plant to allelochemicals and rapidly reacts to these substances. The results showed that type of safflower genotype, irrigation level and the type of safflower organ had significant effects on all studied antioxidant enzymes in radish leaves at 1% probability level. However, the interaction effects had no significant impact on antioxidant enzymes (Table I).

TABLE I - Variance analysis of allelopathic effect of safflower root and shoot residue on antioxidant enzymes activities of radish seedlings

Source of variation	Degree of freedom	CAT	GPX	APX
Safflower variety	3	0.1697**	29.6152**	0.1043**
Irrigation Level	1	0.0816**	5.7228**	0.0857**
Safflower organ type	1	0.1699**	16.0006**	0.243**
Safflower variety × Irrigation Level Safflower variety ×	3	0.0002 ns	0.0589 ns	0.0027 ns
Safflower organ type	3	0.0025 ns	0.1166 ns	0.0049 ns
Safflower organ type × Irrigation Level	1	0.0015 ns	0.0382 ns	0.0065 ns
Safflower organ type × Irrigation Level × Safflower variety	3	0.0024 ns	0.0294 ns	0.0003 ns
Experimental error	32	0.0016	0.2050	0.0019
CV%		11.4242	12.4289	14.7209

**: significant at 5% probability level. **: significant at the 1% probability level. ns: not significant*

Allelopathic effect of safflower shoot residues on antioxidant enzymes activities in radish seedlings

Normal irrigation

Mean comparison results indicated that while shoot residues of Khorasan led to the most activities of CAT, GPX and APX in radish seedlings shoot residues of Kerman led to the least enzymes activities (Table II).

TABLE II - Mean comparison for antioxidant enzymes activities of radish seedlings affected by allelopathic effect of safflower shoot residue grown under normal irrigation and drought stress

Genotype type	CAT (unit mg^{-1} pro min^{-1})		GPX (unit mg^{-1} pro min^{-1})		APX (unit mg^{-1} pro min^{-1})	
	Normal	Drought	Normal	Drought	Normal	Drought
Khorasan (Khorasan330)	0.5874 [a]	0.6891 [a]	4.1381 [a]	4.8998 [a]	0.3894 [a]	0.5260 [a]
Egypt (PI 657800)	0.4955[b]	0.5533[b]	3.9734 [a]	4.9156[a]	0.3801[ab]	0.4923[a]
Kerman (CTNIR9)	0.3246 [c]	0.4324 [c]	1.1755 [b]	1.7807 [b]	0.2537 [c]	0.3482 [b]
Australia (PI 262424)	0.3797 [c]	[c] 0.488	1.4814 [b]	2.1605 [b]	0.3013 [b]	0.3897 [b]
Control (Absence of residue)	0.2287[d]	0.2287[d]	1.1056[c]	1.1056[c]	0.2795[c]	0.2795[c]
LSD	0.0646	0.0625	0.7965	0.5975	0.1005	0.0793

In each column, means which have similar letters do not have significant difference *based on LSD test. Values are mean.*

Drought stress

While the highest activities of CAT and APX were observed in seedlings affected by Khorasan genotype, Egypt led to the most activity of GPX. The least activities of CAT, GPX and APX were found in seedlings affected by Kerman (Table II).

Allelopathic effect of safflower root residues on antioxidant enzymes activities in radish seedlings

Normal irrigation

Mean comparisons showed that the most and the least activities of CAT and GPX were observed in radish seedlings affected by root residues of Khorasan and Kerman, respectively. Khorasan was classified in the same group as Egypt and Kerman was categorized in the same group as Australia. The highest and the lowest activities of APX were observed in radish seedlings affected by root residues of Khorasan and Australia, respectively (Table III).

TABLE III - Mean comparison for antioxidant activities of radish seedlings affected by allelopathic potential of safflower root residue grown under normal irrigation and drought stress

Genotype type	CAT (unit mg^{-1} pro min^{-1})		GPX (unit mg^{-1} pro min^{-1})		APX (unit mg^{-1} pro min^{-1})	
	Normal	**Drought**	**Normal**	**Drought**	**Normal**	**Drought**
Khorasan (Khorasan330)	0.7056 [a]	0.7889 [a]	5.338 [a]	5.8446 [a]	0.5999 [a]	0.7094 [a]
Egypt (PI 657800)	0.6293 [a]	0.7429 [a]	4.9431 [a]	5.944 [a]	0.5444[ab]	0.6193[b]
Kerman (CTNIR9)	0.4508 [b]	0.498 [b]	2.5993 [b]	3.0322 [b]	0.4509[bc]	0.4121 [d]
Australia (PI 262424)	0.5231 [b]	0.5634 [b]	2.7328 [b]	3.3279 [b]	0.392 [c]	0.4904 [c]

TABLE III - Mean comparison for antioxidant activities of radish seedlings affected by allelopathic potential of safflower root residue grown under normal irrigation and drought stress

Genotype type	CAT (unit mg^{-1} pro min^{-1})		GPX (unit mg^{-1} pro min^{-1})		APX (unit mg^{-1} pro min^{-1})	
	Normal	Drought	Normal	Drought	Normal	Drought
Control (Absence of residue)	0.2287^{c}	0.2287^{c}	1.1056^{c}	1.1056^{c}	0.2795^{d}	0.2795^{e}
LSD	0.0995	0.0771	0.8273	1.169	0.0936	0.0524

In each column, means which have similar letters do not have significant difference based on LSD test. Values are mean.

Drought stress

According to mean comparison results (Table III), the most activity of CAT was found in radish seedlings affected by root residues of Khorasan, which was 0.7889 unit mg^{-1} pro min^{-1}. Khorasan was categorized in the same statistical group as Egypt. The least activity of CAT was observed for Kerman, which was classified in the same group as Australia.

Maximum and minimum activities of GPX were observed when radish seedlings were affected by Egypt and Kerman, respectively. No significant difference was found between Egypt and Khorasan. Also Kerman was classified in the same group as Australia (Table III).

The most activity of APX was found in seedlings affected by root residues of Khorasan (0.7094) and the least activity of APX was observed in seedlings affected by Kerman (0.4121) (Table III).

Correlation between growth and antioxidant enzymes activity in radish seedlings

The correlation coefficients between antioxidant enzymes and growth traits of radish seedlings were calculated based on Pearson correlation (Table IV). The results revealed positive correlation between CAT and GPX enzymes and CAT and APX enzymes as 0.92 and 0.83 respectively, and both correlations were significant at 1% level. This means that the allelopathic stress of Khorasan residues leads to increase of antioxidant enzymes concentration. However, there is negative correlation between antioxidant enzymes with seedling growth traits as the correlation coefficients between CAT, GPX and APX with shoot length were as -0.82, -0.93 and -0.81 and between these enzymes with wet weight of seedling were -0.84, -0.94 and -0.81, respectively.

TABLE IV - Pearson correlations among growth and antioxidant enzymes activity of radish seedlings affected by allelopathic potential of Khorasan's root residue grown drought stress

	CAT Concentration	GPX Concentration	APX Concentration	Shoot lenght	Wet Weight
CAT Concentration	1.00000				
GPX Concentration	0.92180**	1.00000			
APX Concentration	0.83181**	0.84716**	1.00000		
Shoot lenght	-0.82953**	-0.93620**	-0.81008**	1.00000	
Wet Weight	-0.84956**	-0.94896**	-0.81884**	0.99446**	1.00000

DISCUSSION

Generally, the results of this experiment showed that drought stress leads to increase in allelopathic potential of plant residues especially in case of root residues. This finding is in agreement with the results of similar researches. Tangma *et al*. (2001) found that the allelopathic potential of plant grown in arid soils is more than those grown in well irrigated soil. They reported that Mexican sunflower grown under drought stress contained a greater amount of alleloathic substances than in the absence of water stress.

Also, the results showed that the activity of radish seedling antioxidant enzymes (catalase, glutathione peroxidase, ascorbate peroxidase) was increased when the recipient plant was exposed to allelochemicals. Yu *et al* (2003) reported the significant increase in activity of peroxidase and superoxide dismutase in cucumber root due to the action of aqueous extract of cucumber and allelochemicals such as benzoic acid. In a study on allelopathic potential of aqueous extracts from aerial parts of Sorghum (*Sorghum bicolor)* and Russian knapweed (*Acroptilon repens*) against Fat hen (*Chenopodium album*), Wheat (*Triticum aestivum*) and Sugar beet (*Beta vulgaris*), Hatami Hampa *et al*. (2018) found that the activity of antioxidant enzymes in treated plants was significantly increased parallel to increasing the concentration of aqueous extract of Sorghum and Russian knapweed. Oracz *et al*. (2007) treated the Mustard (*sinapis arvensis*) seed with Sunflower (*Helianthus annuus*) extract and found that the malondialdehyde content and antioxidant activity of superoxide dismutase, glutathione reductase and catalase of treated plants were increased. As a result, allelochemicals can cause oxidative stress in target tissue and activate an antioxidant mechanism (Li, Hu,, 2005; Niakan, Saberi, 2009).

One of the biochemical changes in plants due to harmful stress conditions is the production of active oxygen forms. The evidence also clarifies that severe allelopathic stress induces oxidative stress (Gill, Tuteja, 2010). Oxidative stress causes damage to DNA and proteins, induces lipid peroxidation, and finally leads to cell death (Ding *et al*., 2007). The activity of antioxidant enzymes can reduce the cellular damage of allelochemicals and provide secondary protection against oxidative stress. The high activity of antioxidant enzymes in some radish seedlings probably indicates that these seedlings, due to these enzymes, have been able to scavenge harmful oxidants and to prevent severe damage to the membrane. Thus resistant seedlings maintain their growth naturally (Oracz *et al*., 2007). The increase in antioxidant enzyme activity may be attributed to the increased production of active oxygen species as substrate that leads to increased expression of genes encoding antioxidant enzymes (Abili, Zare, 2014).

In this study, the control plants showed the minimum CAT, APX and GPX activities as compared to other treatments. These results revealed an increase in CAT, GPX and APX activities in radish leaves in response to shoot and root residues which were grown under drought stress and normal irrigation. GPX activity increased significantly in response to residues in comparison to CAT and APX. The increase in expression of CAT, GPX and APX could be part of radish defense mechanism to maintain cell membrane versus the oxidative damage caused by the allelopathic effect of safflower residues. Therefore, the positive correlation between antioxidant enzymes in case of increasing allelochemicals concentration is a defense mechanism for removing ROIs (Table IV). This was in line with the findings of Oracz *et al*. (2007) in induction of oxidative stress by sunflower phytotoxins in germinating mustard seeds. The negative correlation between antioxidant enzymes with the height and wet weight of seedlings clarifies that parallel to increasing of allelopathic potential of residues, the plant serves more energy for antioxidant enzymes production in order to reduce damage. As a result lower energy will be provided for plant growth (Sunaina, Singh, 2014).

The increase of individual enzymatic activities during alleopathic stress was dependent on safflower genotype. In other words, radish seedlings responded differently to the residues of different safflower genotypes grown under both normal irrigation and drought stress in terms of the activities of CAT, APX and GPX content. The maximum increase in CAT, APX and GPX activities was observed in radish seedlings exposed to Khorasan residues while the minimum increase was detected in radish exposed to Kerman residues for almost all treatments.

The antioxidant enzymes activities of radish seedlings exposed to safflower root residues enhanced over shoot. Thus, it may be concluded that allelochemicals distributed differently in different parts of safflower and roots contained more allelochemicals compared to shoots. Similarly, Wu *et al*. (2000) found different allelochemical distribution in different parts of wheat. They reported that roots contained more allelochemicals compared to shoots. Results of the current study are broadly in agreement with those of Oueslati (2003), Sodaeizadeh *et al*. (2009), Fernandez *et al*. (2009) and Aryakia *et al*. (2015) who indicated that different plant parts contained different allelopathic effects.

CONCLUSION

Regarding that some crops are susceptible to safflower, in order to reduce the inhibitory effects of safflower residues on growth and biomass of sensitive crops, it is necessary that sufficient time interval be regarded between safflower harvest and the next planting in crop rotation. Also, with regard to the allelopathic effect of safflower residues in control of weeds, it is recommended that in the fallow period following safflower culture, the safflower residues be remained in order to inhibit growth of weed seedlings and reduce the need for herbicide application in sustainable agriculture.

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DECLARATION OF INTEREST STATEMENT

Marzieh Motamedi, Ph.D student carried out work, collected samples and data, performed laboratory and chemical analyses of the samples, and prepared the first draft of the manuscript. Hassan Karimmojeni, Ph.D., Associated Professor, Adviser, helped in designing the experiment and more vigorously reviewing and improving the manuscript, Fatemeh Ghorbani Sini, M.Sc. graduated, Co-Author helped in doing the experiment and reviewed the manuscript for language skills. Mohammad Mahdi Majidi, Professor of Genetic and Plant Breeding, helped in data analysis.

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