Investigating the Impact of Methanol and Salicylic Acid Foliar Application under Deficit Irrigation Conditions on Enzymatic Traits of *Brasicanapus* L.

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Abstract
This study was conducted to investigate the impact of foliar application of salicylic acid and methanol on enzymatic traits of Brasicanapus L. Hyola 401 under the field experimental drought stress as a factorial split plot in a randomized complete block design with three replicates during the two crop years of 2017 and 2018 in Safi Abad agricultural and natural resources research and education center of Dezful. In order to do this experiment in the main plot, drought stress treatment including three levels (70, 140 and 210mm evaporation) and in the sub-plot salicylic acid foliar application treatments in three levels (control, 100 and 20 µmol) and Methanol were administered at three levels (control, 10 and 20 volume percent). The results of variance analysis showed that the effect of drought stress on the activity of enzymes of catalase, guaiacol peroxidase and ascorbate peroxidase and also, on the amount of soluble carbohydrates and proline was significant at 1% probability level (P <0.01). Foliar application of salicylic acid and methanol was also significant on the measured traits at 1% probability level (P <0.01). The results of the mean comparison indicate the existence of interactions between the three factors studied in this experiment in the traits of catalase, guaiacol peroxidase and proline. The overall obtained results of this study showed that drought stress increased the activity of antioxidant enzymes as well as the soluble carbohydrates content and proline.
Keywords: Deficit irrigation, Foliar application, Methanol, Salicylic acid and Brasicanapus L.

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Introduction

Brasicanapus L. is a valuable oilseed that has the highest yield among oilseeds in the world in recent decades and today ranks third in vegetable oil production after Glycine max L. and Elaeisoleiferal L. (Arvin et al., 2018). In Brasicanapus L., flowering and pod filling stages are the most sensitive stages affected by drought stress (Sinaki et al., 2007). The results of studies have shown that drought stress at the reproductive stage reduces most of the traits depending on Brasicanapus L. yield (Ma et al., 2006). In tropical and subtropical regions, drought stress is one of major problems in agricultural production and poses a serious threat to the successful production of crops in the world. Drought stress causes changes in the synthesis pathways of secondary compounds and metabolites due to causing secondary stresses such as oxidative stress, in addition to decrease in plant growth and changes in plant anatomical structures. In the dry state, the photorespiration increases due to limited absorption and stabilization of carbon dioxide and increased oxygenation activity of Rubisco enzyme, which can also increase H_2O_2 production.The H_2O_2 of the exterior of the chloroplasts is purified by the enzyme catalase within the leaf cells. Some researchers believe that increasing CO_2 concentration can counteract the effects of drought stress. Therefore, the usage of substances that can increase the CO_2 concentration in the plant can improve the crop yield in drought conditions (Zbiec et al., 2003). Methanol is one of the simplest natural products from plants that is produced by plants, particularly during leaf growth and due to the demethylation of cell wall pectins. After producing this volatile organic compound inside the plants, a part of it leaves the leaves and enters the boundary layer and then the atmosphere, and another part of it is converted to formaldehyde, then to formic acid, and finally to carbon dioxide. This produced carbon dioxide can affect the assimilation of CO_2 in plants. Some researchers have reported that phytohormones play an important role in the regulation of ROS production and proline synthesis. Salicylic acid also has a special role in the regulation of the response to drought stress. Recent studies attempt to explain a complex network of hormonal interactions and signaling pathways in experiments on mutant plants (Ramirez et al., 2006). There is abundant evidence that the complex relationship between salicylic acid and the hormone ABA regulates the plant response to stress conditions. Some researchers reported that ROS interferes with salicylic acid biosynthesis by disrupting the Ca^{2+} signaling pathway. It also disrupts the proline synthesis pathway through the NDR1-dependent signaling pathway (Lee et al., 2019). Salicylic acid also induces proline synthesis in a hormonal response, although its pathway is still unknown. Given the role of antioxidant enzymes and proline in reducing oxidative stress damage and yield due to drought stress in tropical and subtropical regions, the present study aimed to investigate the impact of methanol and salicylic acid foliar application on antioxidant enzymes of Brasicanapus L. Hyola 401 under water deficit tension.

Materials and methods

In order to study the impact of water deficit tension and foliar application of methanol and salicylic acid on antioxidant enzymes and proline synthesis in Brasicanapus L. Hyola 401, an experiment was conducted during the two crop years of 2017 and 2018 in Safi Abad agricultural and natural resources research and education center of Dezful. The experiment was conducted as a factorial split plot in a randomized complete block design with three replicates. The treatments studied in the experiment included water deficit tension at four levels (70, 140, 210 mm evaporation) as the main plot and foliar application of salicylic acid at three levels (0, 100 and 200 μmol) with methanol at three levels (control, 10 and 20 volume percent) as subplots. The hybrid tested was Brasicanapus L. Hyola 401. The most suitable date was considered for planting Brasicanapus L. in Dezful. After planting, while recording the growth stage, the experiment of performing foliar application treatments was conducted in 4-8 leaf stage. Irrigation was done in the form of leakage. The final harvest was done after physiological examination.

After preparing the samples, the required amount of extraction was extracted from the samples and the activities of CAT (Catalase) and guaiacol peroxidase (GPX) enzymes were measured at 25°C by Chance and Maehly (1955) methods. Ascorbate peroxidase was extracted using 250 mM phosphate buffer (pH 7) and Nakano and Asad method (Nakano and Asad, 1981). Ascorbate peroxidase activity was determined based on the oxidation rate of ascorbate at 290 nm using a extinction coefficient of 2/8 Mm^{-1}/cm^{-1}. Sheligl (1986) method was used to measure the amount of carbohydrates. Proline was also determined by Bates et al. method (1973). Data was analyzed using SAS9.2 software through analysis of variance and mean comparison. The mean comparison of simple effects of treatments was done based on LSD test and the interaction effects of treatments were done based on Duncan mean comparison test.

Results and Discussion

Catalase

The results of variance analysis showed that the simple effects of water deficit tension, foliar application of methanol and salicylic acid as well as the interaction effects of water deficit tension×methanol foliar application and methanol foliar application×salicylic acid foliar application along with the interaction effect of water deficit tension treatments×salicylic acid foliar application×methanol foliar application were significant on catalase activity at 1% probability level ($P \leq 0.01$) (Table 1). According to the mean comparison results, the highest activity of catalase in the treatment under water deficit tension was 210 mm and the level of methanol and salicylic acid spraying was zero and the lowest activity of catalase under the interaction of water deficit tension was 70 mm and 200 μmol foliar application of salicylic acid and 20% methanol were observed (Table 2). The results of this study on catalase show that with increasing the water deficit tension, the activity of catalase increases due to increase of ROS production, which foliar application of salicylic acid and methanol leads to a decrease in the activity of this enzyme. This seems to be due to the reduction in ROS production. The reports have been shown that Salicylic acid controls the activity of antioxidant enzymes through transient accumulation of ABA (Ahmed et al., 2002). According to Guan et al. (2000), H2O2 production increases following the increase in ABA amount. Following the increase in H2O2, this molecule acts as a messenger and leads to the protective reactions in plants against the drought stress (Wang et al., 2007). The results of this study are also consistent with the findings of Lee et al. (2019). On the other hand, methanol foliar application increases the concentration of intracellular carbon dioxide, which will decrease the intensity of photorespiration (Ramberg et al., 2002). Therefore, by decreasing the photorespiration, the hydrogen peroxide produced in peroxisomes is decreased (Simova-Stoilova et al., 2008), which as a result, leads to the decrease of the amount of hydrogen peroxide and thus the activity of catalase enzyme. Given the decrease in catalase activity due to methanol foliar application, it seems that methanol has a role in decreasing catalase activity by decreasing the amount of hydrogen peroxide.

Guaiacol peroxidase

The results of variance analysis showed that the simple effects of water deficit tension, foliar application of methanol and salicylic acid as well as the interaction effects of water deficit tension×methanol foliar application, water deficit tension×salicylic acid foliar application and methanol foliar application×salicylic acid foliar application along with the interaction effect of water deficit tension treatments×salicylic acid foliar application×methanol foliar application were significant on guaiacol peroxidase enzyme activity at 1% probability level (P≤0.01) (Table 1).The results of mean comparing oftreatments interaction by Duncan method showed that the highest activity of guaiacol peroxidase under water deficit tension was 210 mm and no foliar application was observed and the lowest activity of guaiacol peroxidase under water deficit tension was 70 mm, andmethanol foliar application was 20% and no salicylic acid foliar application was observed (Table 2). Guaiacol peroxidase is one of the most important enzymes in peroxidase groups that oxidizes guaiacol as a reducing substrate. The reaction of guaiacol with hydrogen peroxide leads to a production of a compound called tetragyacaoquinone (Amiri et al., 2011). Increased guaiacol peroxidase can be an important factor in decomposition of hydrogen peroxide, particularly when catalase is inactivated (Abedi and Pakniyath, 2012). Salicylic acid can increase the hydrogen peroxide of plant tissue and thus, induce the expression of encoding genes of antioxidant enzymes and increase the plant resistance to abiotic stresses (Szepesi et al., 2008). Methanol also leads to more carboxylation and less oxygenation in the plant by rapidly metabolizing to carbon dioxide and increasing the carbon dioxide. Also, photorespiration decrease has also been reported due to methanol application in soybean and spinach chloroplasts (Gout et al., 2000).

Ascorbate peroxidase

Based on the results of variance analysis, simple effects of water deficit tension, foliar application of methanol and salicylic acid as well as interactions of year×methanol foliar application; water deficit tension×Methanol foliar application, Methanol foliar application×Salicylic acid foliar application×year, water deficit tension×Methanol foliar application×Salicylic acid foliar application and water deficit tension stress×Methanol foliar application×Foliar application Salicylic acid×year at 1% level (P≤0.01) and interactions of water deficit tension×salicylic acid foliar application, water deficit tension×salicylic acid foliar application×year at level of 5% (P≤0.05) were significant (Table 1). According to Duncan's mean comparison results, the highest level of ascorbate peroxidase activity was 210 mm under water deficit tension and no methanol and salicylic acid foliar applications were observed, and the lowest enzyme activity was 70 mm under water deficit tension, and methanol foliar application was 20% and 200 µmol salicylic acid was observed (Table 2). The results of this study are contrary to the findings of Shuriabi et al. (2011) who reported that the interaction of drought stress and salicylic acid foliar application had no significant effect on ascorbate peroxidase activity. The results of this study were consistent with the results of Tabatabai study (2014), who reported that salicylic acid treatment with its antioxidant trat may reduce the stress and thus, decrease the activity of catalase and ascorbate peroxidase. One of the important roles of methanol foliar application is preventing the reduction of the effect of stress induced on the plant by photorespiration (Downie et al., 2004). Ascorbate peroxidase metabolizes H2O2 through the ascorbate-glutathione cycle that seems the methanol foliar application provide the energy needed for this process by providing the carbon dioxide needed for the photorespiration cycle.

Proline

The results of variance analysis showed that the simple effects of water deficit tension, foliar application of methanol and salicylic acid as well as the interaction effects of water deficit tension×methanol foliar application, water deficit tension×salicylic acid foliar application, methanol foliar application×salicylic acid foliar application and water deficit tension×salicylic acid foliar application×methanol foliar application were significant at 1% probability level ($P \leq 0.01$) (Table 1). As the results of the mean comparison show, the highest amount of proline under the water deficit tension was at the 210 mm level and foliar application of 20% methanol and 200 μmol of salicylic acid were observed and the lowest level of proline under the water deficit tension was at the 70 mm level and no foliar application of salicylic acid and methanol were observed (Table 3). According to studies by other researchers, foliar application of methanol on leaves is changed to formate (methanoic acid) by methanol oxidase and at a loss of $2H^+$. Formate is subsequently changed to carbon dioxide and H^+ by the formate dehydrogenase enzyme (Nonomura and Benson, 1992). Also, pyrrolen-5 carboxylate synthetase (P5CS) has the highest activity in acidic conditions (Yordanov et al., 2003). As a result, methanol seems to increase the activity of the synthesized pyrrole-5-carboxylate enzyme with pH decrease in the plant that finally, causes the accumulation of proline in the leaves. A review of studies on the effect of salicylic acid on proline showed that ROS plays a key role as an intercellular signal in proline production (Rejeb et al., 2014). In a study by Lee et al. (2019), proline accumulation due to drought stress and salicylic acid application is coincided with NADPH oxidase gene expression, and it was found that ROS (especially H2O2) signal for NADPH oxidase acts as a signal (Chung et al ., 2008; Rejeb et al., 2014). In some studies, the increase in proline levels due to the salicylic acid application in drought stress has been attributed to increased levels of ABA and JA (Lee et al., 2019). In a similar study, Hayat and Ahmad (2007) reported that salicylic acid controls the activity of antioxidant enzymes through transient accumulation of ABA. Momeni et al. (2016) in a study investigating the role of salicylic acid on proline reported that foliar application of salicylic acid leads to osmotic stress that in turn produces osmolytes like proline and soluble sugars. According to other researchers, proline in plant cells acts as a means of maintaining the osmotic balance between the cytoplasm and the vacuole, and the reason of proline storage may be due to the reduced consumption of proline to make protein during the draught stress. In similar results, Mohammadi et al. (2015) showed that foliar application of salicylic acid on Brasicanapus L. has increased the proline content in Brasicanapus L. at the 31.79% level. The findings of this study are similar to the results obtained in the studies of Keshavarz et al. (2011), Chegeni et al. (2015), Yousefi Rad et al. (2015) and Mordshahi et al. (2004).

Soluble carbohydrates

The results of variance analysis showed that the effect of water deficit tension, the treatment effect of foliar application of methanol and the effect of foliar application of salicylic acid was significant at 1% probability level ($P \leq 0.01$), but the interactions of treatments on soluble carbohydrates were not significant (Table 1). According to the results of mean comparing of LSD, the most amount of soluble carbohydrates was observed among the levels of water deficit tension at the 210 mm level of dehydration stress and the lowest amount of soluble carbohydrates was observed at the 70 mm level. Also, among the salicylic acid foliar application levels, the highest amount of soluble carbohydrates was observed in non-foliar application and the lowest amount was observed at the foliar application level of 200 µmol salicylic acid. The highest amount of soluble carbohydrates among methanol foliar application levels was observed in non-foliar application and the lowest amount of it was observed at 20% foliar application level (Table 3).Increased amount of carbohydrates under drought stress can act as a metabolic signal to drought and salinity stress and play a role in developing the defense responses (Radmirez et al., 2006). The increase in soluble carbohydrates during the stress can be attributed to stunt, synthesis of compounds in non-photosynthetic pathway, as well as degradation of insoluble sugars that increases the soluble sugars (Hayat and Ahmad, 2005).Methanol foliar application increases the carbon dioxide concentration that can increase the carbon dioxide stabilization in the plant. Rapidly metabolized methanol to carbon dioxide and increased carbon dioxide leads to more carboxylation and less oxygenation in the plant. Therefore, the amount of soluble carbohydrates increases with increasing photosynthesis due to methanol application (Zbiec et al., 2003). Most researchers have considered changes in carbohydrates during the stress as one of the important factors in plants' survival in these conditions, which result in reduced phloem loading, reduced assimilation capacity or reduced rate of the utilization in sink organs. The researchers reported that the increase of sugars concentration during the drought stress was useful in osmotic protection of membranes and proteins, as well as sweeping oxygen free radicals. These results are similar to the results obtained in the studies of Mir et al. (2015) and Heidari et al. (2015) and also are contradictory to the results obtained in the studies of Keshavarz et al. (2011) and Momeni et al. (2016).

Table 1: Combined analysis of variance of canola antioxidant enzymes of Yola 401 cultivars under the effect of year, dehydration, methanol and salicylic acid foliar application

Sourses of Changes	Freedom Degree	Mean of squares(MS)				
		Catalase	Guaiacol peroxidase	Ascorbate peroxidase	Soluble carbohydrates	Proline
Year (Y)	1	0 ns	0.00000016ns	0.00045ns	0.00000593*	0.00002434ns
Block (R)	2	0.00000039**	0.00000006ns	0.00106728*	0.0000016ns	0.00061418**
Y* R	2	0.00000008ns	0.00000001ns	0.00016852ns	0.00000138ns	0.00003878ns
Dehydration (C)	3	0.00022827**	0.07903444**	0.74209136**	0.00163327**	1.27280247**
Y* C	3	0ns	0.00000001ns	0.00020741ns	0.00000264ns	0.00002985ns
R* C	12	0.00000011ns	0.0000001ns	0.00014105ns	0.00000105ns	0.00016941ns
Methanol foliar application (D)	2	0.00002749**	0.00823522**	0.1551173**	0.00059262**	0.1451172**
Y* D	2	0.00000001ns	0ns	0.00226852**	0.00000106ns	0.00001325ns
C* D	6	0.00000192**	0.01106059**	0.00758673**	0.00000338ns	0.02035013**
Y* C* D	6	0ns	0.00000016ns	0.00275648**	0.00000038ns	0.0000065ns
Error	32	0.00000009ns	0.00000005ns	0.00025463ns	0.00000153ns	0.00076178*
Foliar application of salicylic acid (F)	1	0.00003962**	0.0001176**	0.0451284**	0.00042801**	0.04452064**

Y* F	1	0.00000001ns	0.00000004ns	0.00031852ns	0.00000271ns	0.00002056ns
C* F	3	0.00000144**	0.00001109**	0.00091451*	0.00000127ns	0.00077889**
Y* C* F	3	0.00000003ns	0.00000005ns	0.00087315*	0.00000058ns	0.00003615ns
D* F	2	0.00000119**	0.0000168**	0.00021821ns	0.00000178ns	0.00205805**
Y* D* F	2	0ns	0.0000004ns	0.00094537**	0.00000134ns	0.00005313ns
C* D* F	6	0.00000098**	0.00001702**	0.00091682**	0.0000009ns	0.00399449**
Y* C* D* F	6	0.000000003ns	0.00000006ns	0.00119306**	0.00000049ns	0.00004688ns
Total error	48	0.00000008	0.00000007	0.00026	0.00000144	0.00001748
Coefficient of variation		0.19	0.18	1.36	4.26	0.26
ns, * and ** mean insignificant and significant at 5 and 1% probability levels, respectively.						

Table 2: Comparison of the mean interaction of dehydration stress treatments, methanol foliar application and salicylic acid foliar application on catalase, guaiacol peroxidase and ascorbate peroxidase enzymes and proline content

	Salicylic acid foliar application levels (Micromoles)	Methanol foliar application levels (volume percentage)	Catalase	Guaiacol peroxidase	Ascorbate peroxidase	Proline
Dehydration stress levels 70 (mm evaporation)	0	0	0/146hi	0/0494833r	hi1/13	1/4328t
		12	0/144mn	0/04825s	jkl1/1	1/457267s
		20	0/14215q	0/0473833t	mn1/06	1/463767r
	100	0	0/14445l	1/1147p	kl1/09167	1/45635s
		12	0/1436833no	1/1135167q	lm1/075	1/4679qr
		20	0/1437625mno	0/121575o	jkl1/095	1/500563p
	200	0	0/1435167o	1/1254333l	nm1/055	1/469933q
		12	0/1429p	0/1243m	1/035n	1/496783p
		20	0/1417r	0/1224333n	0/99333o	1/511033o
Dehydration stress levels 140 (mm evaporation)	0	0	0/1468833e	0/1536833h	2/26d	1/514533o
		12	0/1460167hi	0/1524667i	1/235de	1/536533m
		20	0/1456j	0/151525j	1/225e	1/ 54715l
	100	0	0/1463167gh	0/1516167j	1/225e	1/527283n
		12	0/1457333ij	0/1516167j	1/16667fg	1/56735k
		20	0/1449333k	0/1516167j	1/145gh	1/59795j
	200	0	0/1449167k	0/1523i	1/14667gh	1/6215i
		12	0/1440333m	0/1516833j	1/12167hij	1/62755h
		20	0/1436o	0/1425833k	1/11ijk	1/657717f
Dehydration stress levels 210 (mm evaporation)	0	0	0/149a	0/17215a	1/38833a	1/644933g
		12	0/1478167c	0/1708b	1/355b	1/66365e
		20	0/1473d	0/1704667c	1/32811b	1/6736d
	100	0	0/1483167b	0/1693667d	1/34833b	1/657933f
		12	0/1477667c	0/1681833e	1/33167b	1/667567e
		20	0/1466983ef	0/1673833f	1/29167c	1/696433c
	200	0	0/1477c	0/1673667f	1/25333d	1/700433c
		12	0/1473167d	0/1650333g	1/235de	1/733617b
		20	0/14655fg	0/1652833g	1/17333f	a1/7583

Table 3: Comparison of the mean of simple effect of dehydration stress, methanol foliar application and Foliar application of salicylic acid on soluble carbohydrates

Treatment	Treatment levels	Soluble carbohydrates
Dehydration	Mm evaporation 70	c0/0217963
	Mm evaporation140	b0/0306296

	Mm evaporation219	a0/0318889
Foliar application of salicylic acid	**0 Micromol**	a0/0318333
	100 Micromol	b0/0269815
	200 Micromol	c0/0255000
Methanol foliar application	**1 Volume percentage**	0/0308889a
	10 Volume percentage	0/0281667b
	20 Volume percentage	0/0252593c

Conclusion

According to the results of the present study, the highest activity of catalase in the treatment under dehydration stress was 210 mm and the level of methanol and salicylic acid was zero and the lowest activity of catalase under the interaction of low stress treatment. Aqueous 70 mm and foliar application of 200 µmol salicylic acid and 20% methanol were observed.Also, the highest activity of guaiacol peroxidase was observed at the level of 210 mm from dehydration stress and lack of foliar application. The lowest activity of guaiacol peroxidase was observed at the level of 70 mm of dehydration stress and foliar application of 20% methanol and non-foliar application of salicylic acid. According to the results of this study, the highest level of ascorbate peroxidase activity was observed at the level of 210 mm of dehydration stress and non-foliar application of methanol and salicylic acid. The lowest enzyme activity was observed at the level of 70 mm of dehydration stress and foliar application of 20% methanol and 200 µmol salicylic acid. The highest amount of proline was observed at the level of 210 mm of dehydration and foliar application of 20% methanol and 200 µmol of salicylic acid and the lowest level of proline was observed at the level of 70 mm of drought stress and non- foliar application of salicylic acid and methanol. Also, the highest amount of soluble carbohydrates was observed among the levels of dehydration stress at the level of 210 mm of dehydration stress and the lowest amount of soluble carbohydrates was observed at the level of 70 mm. Among the salicylic acid foliar application levels, the highest amount of soluble carbohydrates was obtained in non-foliar application and the lowest amount was observed at the foliar application level of 200 micromoles of sialicylic acid. The highest amount of soluble carbohydrates among methanol foliar application levels was in non-foliar application and the lowest amount was at 20% foliar application level. Salicylic acid seems to be effective on the activity of antioxidant enzymes on the one hand with a temporary increase in ABA and on the other hand with its antioxidant role. Methanol is also prepared by providing carbon dioxide and increasing the concentration of carbon dioxide needed for light respiration, and as a result, the consumption of high-energy molecules and the production of ROS can affect the activity of antioxidant enzymes.

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