Increasing the tolerance of canola plant using nitric oxide under lead and drought stress

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Abstract

Lead and drought stress have negative effects on plant growth and decrease crop production. In recent years, many studies have been reported on the role of nitric oxide (NO) in reducing these effects. The present study was conducted on *Brassica napus* L. to investigate the interaction of lead, drought, and their combination with NO using Hyola 401 cultivars. One hundred (100) µmol / L lead with Pb $(NO_3)_2$, drought with 0.5% polyethylene glycol (PEG) 6000 (-0.003 MPa), and NO with 100 µmol / L sodium nitroprusside (SNP) were used. Lead and its combined treatment significantly decreased growth while drought stress affected only shoots. NO treatment reduced negative effects on plant growth. Adding NO in the lead treated plants under drought stress caused an increase in soluble sugar contents. Plant proline significantly increased by application of NO in control, lead treatment, and simultaneous lead and drought treatment. A significant increase in the activity of the enzymes assayed (peroxidase and catalase) was observed in the plant exposed to lead and its combination with drought stress. The use of NO caused a significant decrease in the activity of these enzymes. The antioxidant role of NO may be the reason for this decrease in activities.

Keywords: *Brassica napus* L., catalase, peroxidase, polyethylene glycol, proline, soluble sugar

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Introduction

An important agricultural product in Iran is Canola (*Brassica napus* L.) as the presence of oilseeds contributes to the supply of edible oil. Lead is the second most dangerous heavy metal after arsenic (Balali-Mood et al., 2021). Although plants do not need Pb, it is absorbed by the plant and has adverse effects on its performance. The presence of lead in plant tissues increases the production of reactive oxygen species (ROS) in them, causing lipid peroxidation, damaging DNA, and preventing the production of ATP (Kumar and Prasad, 2018). Similar to lead stress, drought stress also leads to production of a lot of ROS with its damaging effects on the plant (Zulfiqar et al., 2019). One of the most critical environmental stresses is the drought that affects the physiology, biochemistry, and morphology of the plant and has devastating effects on crops. Preventing cell growth at the time of elongation and constant stopping of proton pump activity as a result of the toxicity of lead is stated as the reason for reducing plant growth (Rani et al., 2024). Significantly, sensitivity to lead stress increases in drought stress (Samuilov et al., 2016).Significant changes, such as gene

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expression and proteomics, are caused by the activation of the transduction pathways by soluble sugars (signal molecules), in particular, fructose, sucrose, and glucose, which play a significant role in maintaining the structure and metabolism within the plant (Samuilov et al., 2016). The three leading roles of proline amino acid as an effective Osmolyte include acting as a signal molecule, an antioxidative defense molecule, and a metal chelator (Ghosh et al., 2022). Out of the three osmolytes studied, proline was the most effective in reducing drought stress (Bhuiyan et al., 2019).

In-plant cells, antioxidant enzymes such as catalase (CAT) and peroxidase (POD) are identified as a defensive team aimed at protecting cells from oxidative damage (Fujita and Hasanuzzaman, 2022). Plants with antioxidant enzymes remove ROS, including superoxide, hydrogen peroxide, and singlet oxygen, resulting in better plant growth. Reports indicate an increase in oxidative stress indices and the activity of antioxidant enzymes in canola seeds(Naghisharifi et al., 2024)

Recently, the role of nitric oxide (NO) regulator has been highlighted in many types of stress (Zhou et al., 2021). Under conditions of localization and concentration, NO can play a dual (positive or negative) role and protects the plant against the destructive effects of ROS and is involved in reducing stress and regulating gene expression (Goel et al., 2021). The levels of intracellular ROS and NO may affect the activities of antioxidant enzymes (Sadhu et al., 2019).

Lead and drought are environmental factors that have caused some problems in the cultivation of canola oilseeds. This study was conducted to study the effect of NO treatment on growth parameters (fresh and dry weight), soluble sugar changes, proline content, and CAT and POD activities in root and aerial parts of Hyola 401 cultivars of canola under drought stress, its combination with lead.

Material and Methods

Plant condition

Canola seeds of Hayola 401 cultivar obtained from Golestan University in northern Iran were disinfected with sodium hypochlorite liquid (0.5%) and washed twice with distilled water. Afterwards, they were transported inside specially prepared Petri dishes containing filter paper (two wet layers). Seeds were germinated in an incubator at 25 °C in no light condition. After one week, the obtained seedlings were put into containers containing 0.5% Hoagland nutrient solution and were kept in a growth chamber, exposed to 10 hours of darkness and 14 hours of light at 70% air humidity in the darkness of the study environment at 20 ° C and daylight with 55% humidity and 25 ° C. Six days after being exposed, Hoagland nutrition was changed, as control, 100 µmol/L sodium nitroprusside (SNP) as NO donor, and lead nitrate ($Pb(NO_3)_2$). Also, polyethylene glycol (PEG-6000) 0.5% (-0.003 MPa) was used as a drought generator, added to containers (Michel and Kaufmann, 1973). The root and shoot samples were taken to the laboratory one week later.

Analysis of Growth Parameters

After washing with water, different parts of the fresh seedlings were weighed. The plant materials were kept at 90 °C for 24 hours before dry weights were measured.

Soluble sugars assay

Soluble sugars were measured by the phenol sulfuric acid method (Kochert, 1978). After drying, 10 ml of 70% ethanol was added to 0.02 g of the samples and kept in a refrigerator for seven days. After centrifugation at 2000g for 15 minutes at 25 °C, one ml of the top solution was extracted, and its volume was increased to 2 ml by adding distilled water. One ml of 5% phenol and 5 ml of concentrated sulfuric acid was added to the above solution. A yellow solution was obtained, which gradually shifted to a light brown. The solution was kept for half an hour at 25 °C, and finally, the optical absorption of the solution was read by a spectrophotometer at 485 nanometers. The glucose standard curve was used to calculate the concentration of soluble sugars in the samples (mg g^{-1} DW).

Proline assay

Bates et al., (Ls, 1973) was followed to assay proline contents of the samples. One gram of the dry samples was ground in 10 ml of 3% sulfosalicylic acid solution and homogenized. After

centrifugation at 2000 g for 10 minutes, the residue was removed. Two ml of ninhydrin and two ml of glacial acetic acid was added to the extract and incubated for 60 minutes at 100 °C and placed in an ice chamber for 30 minutes to stop the reaction. Toluene (4 mL) was added and shaken well, and the absorbance of upper colored layer (containing toluene and proline) was read using the spectrophotometer and quartz cuvette at 520 nanometers. The concentration of samples (µmol g^{-1} DW) was calculated using the standard proline curve.

Antioxidant Enzymes Assay

About 4 grams of sodium tetraborate, 50 grams of polyethylene glycol 2000, 2 grams of ascorbic acid, 1.5 grams of Tris, and 2 grams of EDTA-Na_2 were mixed, and pure water was added to prepare 100 mL solution for extraction. One g of the plant samples (shoot and root) was ground in 4 ml of extract solution for 30 minutes, and the solution was kept at 4 °C overnight for enzyme extraction. The solution was centrifuged at 4000 g for 30 minutes, and the resulting extraction of supernatant was kept at 4 °C.

POD level was expressed as OD g^{-1} FW min^{-1} (Koroi, 1989). About 0.5 mL of 3% hydrogen peroxide, 0.2 mL of 0.01 mol/L benzidine soluble in 50% ethanol, and 1 ml of 0.2 mol/l acetate buffer (pH = 5) were used and mixed thoroughly. Enzyme extract (0.1 ml) was added to the obtained compound, and the absorbance changes were read at 530 nanometers against the control.

CAT activity assay was carried out following the method described by Chance and Maehly (1955)with some modifications. One ml of the enzyme extraction (diluted two times) was added to 5 ml of 300 µmol/l phosphate buffer (pH = 6.8) and 100 µmol/l hydrogen peroxide solution, and the resulting solution was kept at 25 °C for 60 seconds. Next, 10 ml of sulfuric acid (2%) was titrated with potassium permanganate (0.01 N), and a pale purple color was observed, preventing further reaction. The number of enzymes that decomposed one µmol of hydrogen peroxide in 60 seconds was considered as a unit of CAT activity.

Statistical Analysis

The obtained data was submitted to SPSS software for analysis using Duncan's test at p≤0.05. The graphs were plotted using Excel.

Results

Growth parameters

Lead and its combined treatment with drought stress significantly decreased root and shoot fresh weights in comparison with control (P≤0.05), but drought stress decreased only fresh shoot weight (Fig. I and Table 1). All stress levels significantly reduced shoot dry weight (P≤0.05) as compared with control (Fig. I and Table 1). Root dry weight significantly decreased under lead stress and its combination with drought in comparison with control. The combined treatment of lead and drought showed a significant decrease in weight indexes, whereas NO treatment did not result in differences from control. Application of NO in the lead and combined treatment of lead and drought stress significantly increased shoot and root fresh weights, but had no significant effect on drought stress. The maximum dry weight was observed in NO treatment, only in aerial parts with a significant difference from control. Furthermore, application of lead and NO increased root dried weight. Finally, NO treatment increased shoot and root dry weight under combination stress condition.

Soluble sugars

Drought stress and its combined treatment significantly (P≤0.05) increased soluble sugar contents in shoots (Fig. II. A and Table 1). A significant increase in the root sugar content was observed only under the simultaneous lead and drought stress (Fig. II. B and Table 1). The lowest amount of shoot soluble sugar was observed in the nitric oxide treatment under drought stress, showing a significant decrease compared to drought stress alone. Application of NO in lead and its combined treatment with drought stress increased soluble sugar contents. We further observed that the application of NO alone significantly increased root soluble sugar contents

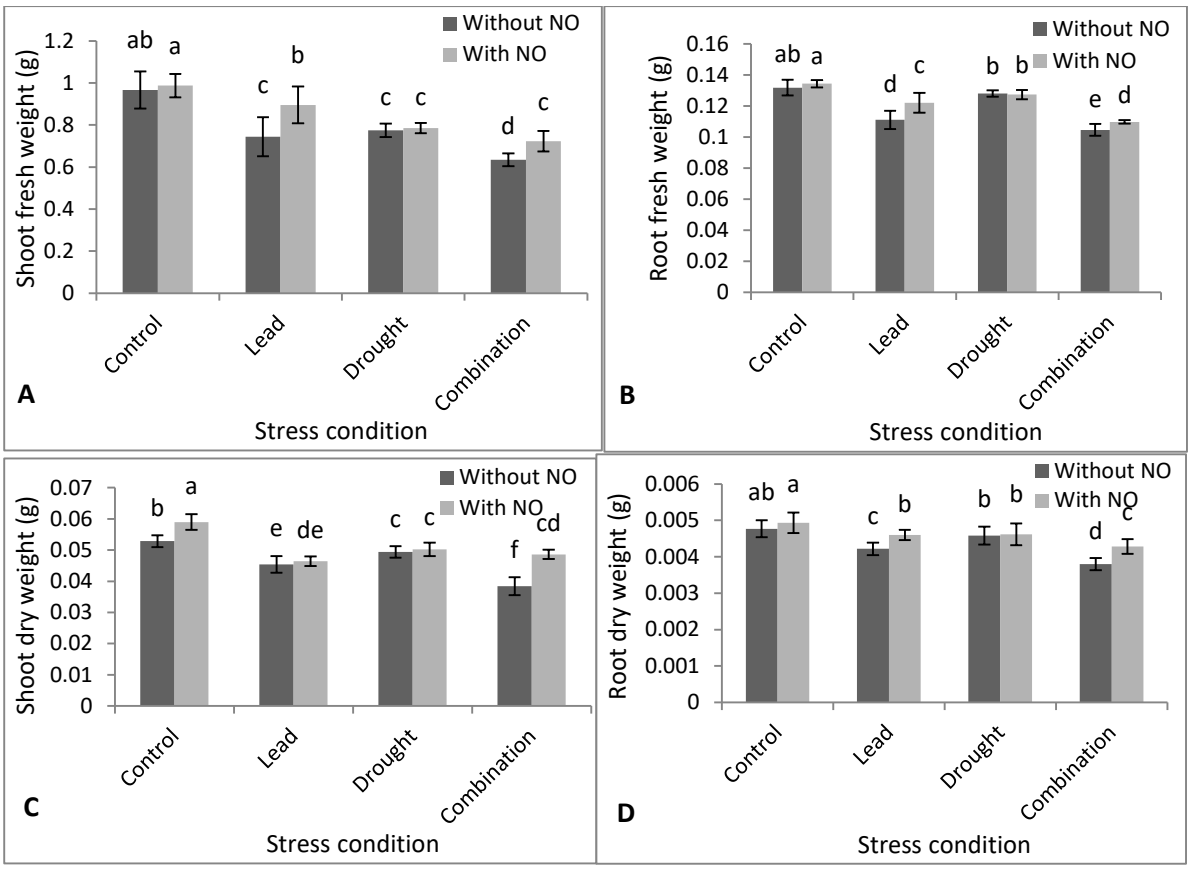

Fig. I. A comparison of the mean interaction effects of NO, lead, drought, and combination of lead and drought on the fresh and dry weight of shoot (a, and c) and root (b, and d) in canola; means with common letters are not significantly different (P<0.05) according to Duncan's multiple range tests.

Table 1
Variance analyses of the shoot and root parameters

		Fresh weight		Dry weight		Soluble sugars		Proline		POD		CAT	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Lead	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.15**	0.00**	0.00**	0.00**	0.00*	0.00*	0.01ns	10.32**	0.00ns	0.00**	347.23**	806.55**
	CV	0.17	0.10	0.09	0.08	0.02	0.02	0.03	0.21	0.05	0.15	0.10	0.32
Drought	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.11**	0.00ns	0.00**	0.00ns	0.00**	0.00ns	0.07ns	4.94*	0.00ns	0.00ns	0.04ns	0.23ns
	CV	0.14	0.03	0.05	0.05	0.04	0.02	0.05	0.21	0.05	0.08	0.03	0.05
Lead & Drought	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.33**	0.00**	0.00**	0.00**	0.00**	0.00**	0.18ns	12.99**	0.00ns	0.00**	489.09**	865.13**
	CV	0.23	0.13	0.17	0.13	0.05	0.04	0.04	0.23	0.06	0.22	0.10	0.32
Nitric oxide	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.00ns	0.00ns	0.00**	0.00ns	0.00ns	0.00ns	2.25**	9.54**	0.00ns	0.00**	4.44ns	0.21ns
	CV	0.07	0.03	0.07	0.05	0.02	0.02	0.07	0.20	0.05	0.11	0.06	0.04
Nitric oxide & Lead	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.02ns	0.00*	0.00**	0.00ns	0.00**	0.00**	0.48*	17.89**	0.00*	0.00ns	1.91ns	831.83**
	CV	0.10	0.06	0.08	0.04	0.04	0.03	0.05	0.25	0.07	0.06	0.04	0.32
Nitric oxide & Drought	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.10**	0.00ns	0.00*	0.00ns	0.00*	0.00*	0.12ns	10.22**	0.00ns	0.00ns	67.21**	0.01ns
	CV	0.13	0.04	0.05	0.06	0.02	0.04	0.04	0.21	0.05	0.07	0.05	0.05
Nitric oxide & Combination	df	1	1	1	1	1	1	1	1	1	1	1	1
	Mean squares	0.18**	0.00**	0.00**	0.00**	0.00**	0.00**	1.61**	29.56**	0.00ns	0.00**	371.85**	848.57**
	CV	0.17	0.10	0.05	0.07	0.09	0.04	0.07	0.30	0.06	0.18	0.09	0.32

ns, *, and ** show no significant difference, significant at 5%, and significant at 1% probability levels, respectively.

under drought while it did not show increase under other stress conditions.

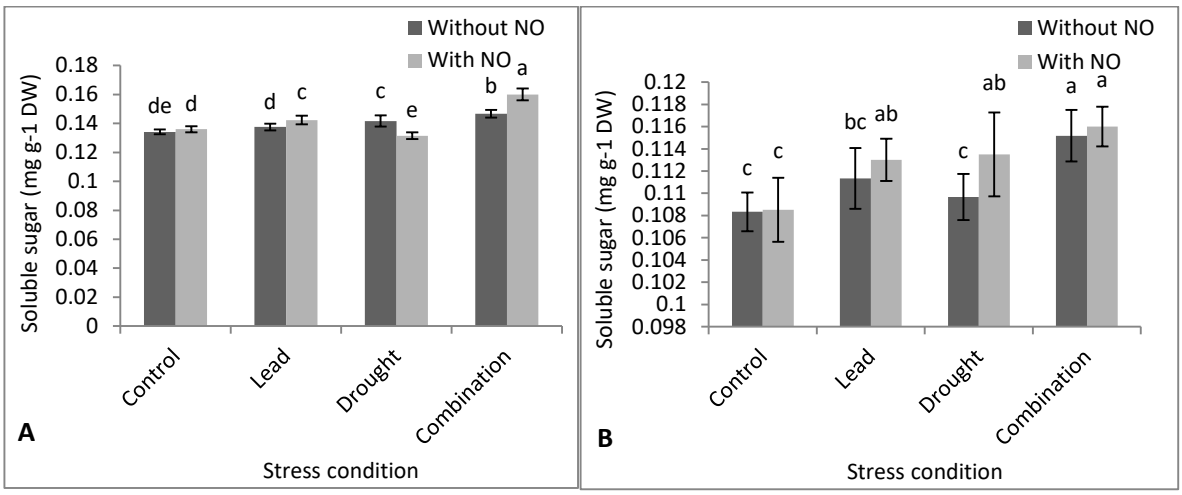

Fig. II. A comparison of the mean interaction effects of NO, lead, drought, and combination on soluble sugars of the shoots (a) and roots (b) in canola; means with common letters are not significantly different (P<0.05) according to Duncan's multiple range tests.

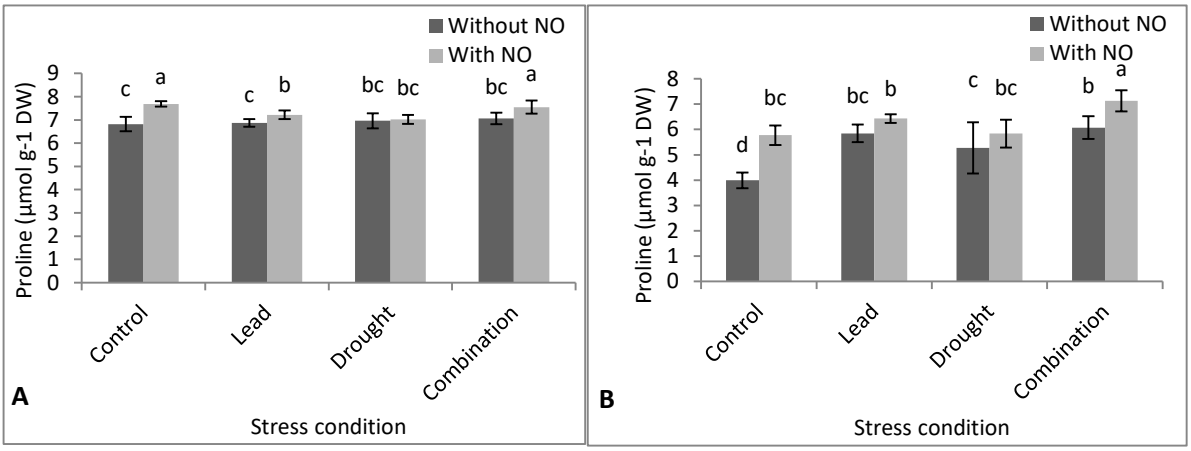

Fig. III. comparison of the mean interaction effects of NO, lead, drought, and combination on the proline contents of shoots (a) and roots (b) in canola; means with common letters are not significantly different (P<0.05) according to Duncan's multiple range tests.

Proline

The proline content of shoots did not increase significantly (P≤0.05) under any of the stress conditions compared to the control (Fig. III. A and Table 1). All stress conditions showed a significant increase in root proline content compared to the control (Fig. III. B and Table 1). Significant increasing effects of NO were observed in control, lead, and lead + drought treatments on shoot proline content, but no significant increase was found under drought stress. The addition of NO in the control and the stress condition of drought + lead showed a significant increase in root proline content.

Antioxidant enzymes

Aerial POD enzyme activity did not show a significant increase (P≤0.05) at all stress conditions compared to control (Fig. IV and Table 1). POD activity in the roots of plants under lead and lead + drought stress increased compared to the control but did not increase significantly under drought stress (Fig. IV and Table 1). Application of NO in all treatments significantly decreased root POD activity. Application of NO significantly decreased shoot POD enzyme activity under lead and simultaneous lead and drought stress conditions while in other treatments, no significant change was observed.

A comparison of the mean CAT activity data showed a significant increase (P≤0.05) in lead and

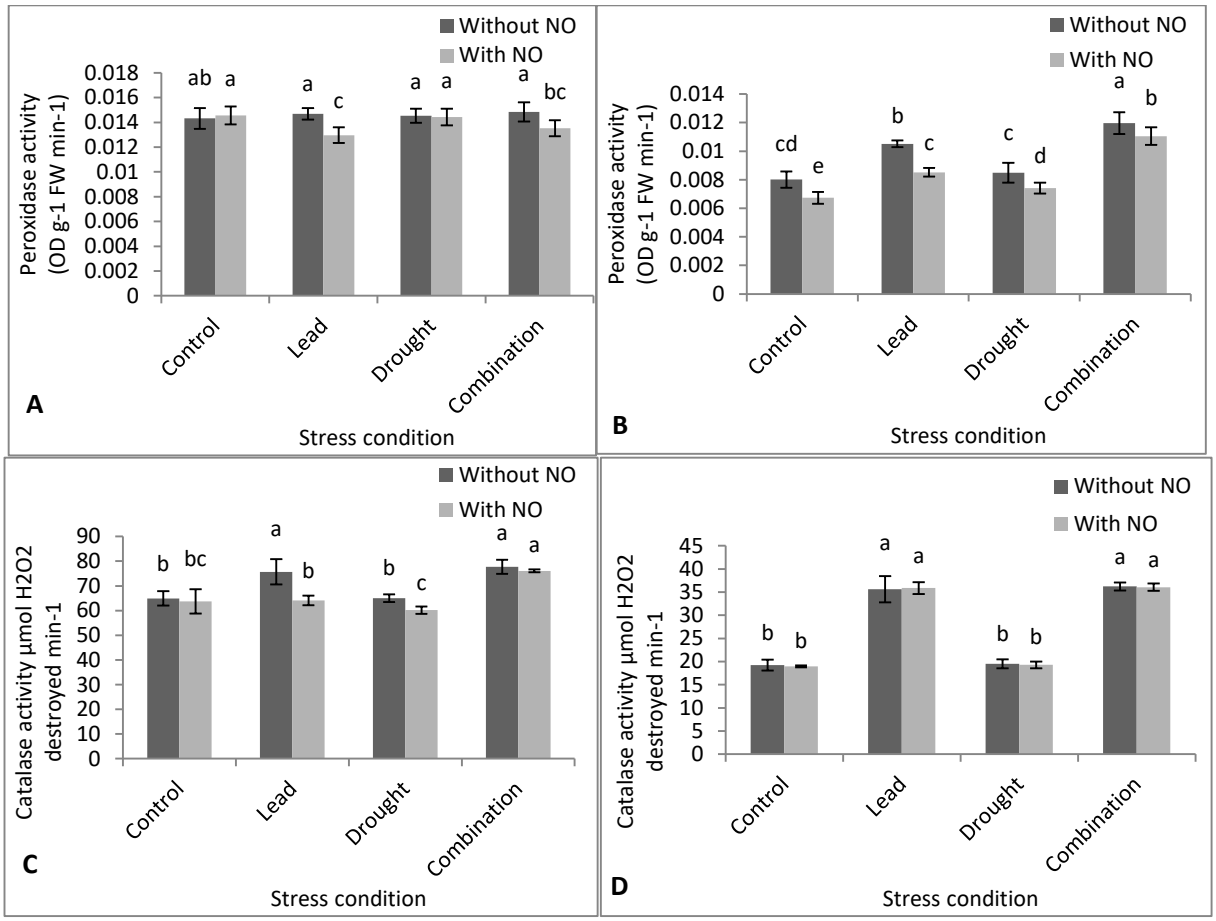

Fig. IV. Comparison of the mean interaction effects of NO, lead, drought, and combination on the antioxidant enzymes of the shoots (a, and c) and roots (b, and d) of canola; means with common letters are not significantly different (P<0.05) according to Duncan's multiple range tests.

combination of lead and drought stress, but no significant increase was observed in this attribute under drought stress alone (Fig. IV and Table 1). NO treatment caused a significant decrease only in aerial parts of the plants under lead and drought conditions while the application of NO in other stress conditions of the study did not cause affect roots and shoots.

Discussion

Heavy metals increase the effect of water dehydration stress and cause more damage to the plants exposed to drought stress (Islam and Sandhi, 2023). Low levels of SNP may reduce lead toxicity in stressed plants, but high concentrations of SNP did not reduce lead toxicity (Bai et al., 2015).

Decrease in plant growth was reported with increasing lead concentration, which accumulated in leaves, stems, and the roots (Zhou et al., 2018). Growth traits have been reported to be inhibited by water deficits caused by irrigation regimes (Noori et al., 2022). The protective or toxic effect for plants is entirely dependent on NO concentration. Low concentration can prevent water loss and enhance seedling growth while the high concentration of SNP has a different effect (Zhou et al., 2023). This growth seems to be due to the increased resistance of the plant under nitric oxide treatment.

Relative accumulation has reported due to heavy-metal contamination (Saad-Allah and Elhaak, 2017). NO may enhance the plant's resistance to drought stress by increasing the amount of sugar and proline (AlKahtani et al., 2021). The use of NO in stresses by increasing the osmotic pressure enhanced the adaptability of canola plants to drought conditions in the present study.Proline supplementation in olive has been reported to reduce lead toxicity in roots and leaves through

reducing lead concentration and stress-induced oxidative damage (Zouari et al., 2018). There is a direct relationship between proline accumulation and the plants' ability to tolerate drought stress (Zaman et al., 2024). Increased proline content has also been reported in plants under salinity stress (Tavakoli et al., 2016). Another study has shown that using NO treatment under drought stress mitigates the adverse effects of drought in plants by increasing the proline content of the seedlings (Jday et al., 2016). Findings of the present study showed that all stress levels led to oxidative damage, and NO treatments reduced the damage through increasing proline contents of the canola plants.

Excessive heavy metals activate the antioxidant defense mechanism in plants (Khelfaoui et al., 2024). Exposure to toxic levels of lead significantly increased oxidative stress markers, including malondialdehyde, hydrogen peroxide, and electrolyte leakage, as well as elevated enzymatic and non-enzymatic antioxidants, their gene expression, and the pattern of organic acid exudation in the roots of canola (Chen et al., 2024). Plants experiencing drought stress induced by PEG demonstrated notable elevations in the levels of superoxide dismutase (SOD), POD, and CAT enzymes (Nazari and Smith, 2023). NO reduced oxidative damage under drought stress (Kaya et al., 2024). CAT and POD activity decreased with increasing SNP at all levels of PEG (Ghassemi-Golezani et al., 2018). Drought stress through excessive production of ROS can increase the oxidative damage, and the suitable level of NO can increase the plants' tolerance to water deficit conditions (Hussain et al., 2019).

Conclusions

All levels of drought stress reduced canola weight. In combined treatment of drought and lead stress, weights of roots and shoots decreased which was then significantly increased following NO application, enhancing plant resistance. The sugar contents of the plants significantly increased under combined treatments of drought and lead while they increased in shoots under the drought stress condition alone. Addition of NO under drought stress and lead stress and its combined treatment with drought significantly increased the soluble sugar contents by enhancing the adaptability of the seedling. It was observed that, in contrast to the shoot, root proline content significantly increased in all stress conditions of the study compared to control. NO in control treatment and combination of drought and lead stress significantly enhanced shoot and root proline contents, but under lead stress, it only increased the amount of proline in shoots. Increasing proline can reduce damage to the seedling and lead to resistance to oxidative damage. POD levels of roots under all stress conditions were higher than that of control. The use of lead and NO significantly decreased the activity of POD in shoots and roots. Under drought stress, treatment with NO caused a significant decrease in root POD activity, but no significant changes were observed in shoots. Application of NO under combination stress caused an essential reduction in the POD enzyme levels in roots and shoots. CAT enzyme level significantly increased under lead stress while NO treatment only decreased CAT in shoots. NO may be involved in removing hydrogen peroxide in drought stress conditions. Decreased CAT and POD enzyme levels can be attributed to the antioxidant activity of NO.

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