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SEYED AHMAD KALANTAR AHMADI

ALI EBADI

SODABEH JAHANBAKHSH

JAHANFAR DANESHIAN

SEYED ATAOLAH SIADAT

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Changes in enzymatic and nonenzymatic antioxidant defense mechanisms of canola seedlings at different drought stress and nitrogen levels

Seyed Ahmad KALANTAR AHMADI[1,*], Ali EBADI[1], Sodabeh JAHANBAKHSH[1], Jahanfar DANESHIAN[2], Seyed Ataolah SIADAT[3]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Mohaghegh Ardabili, Iran
[2]Seed and Plant Improvement Institute, Iran
[3]Department of Agronomy and Plant Breeding, Ramin Agriculture and Natural Resources University, Khuzestan, Iran

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Abstract: To evaluate the responses of canola seedlings to different levels of drought stress and nitrogen at different growth stages, a factorial experiment was conducted in a completely randomized design with three replications at the experimental greenhouse of Mohaghegh Ardabili University in 2013. Treatments included three levels of drought stress (30%, 50%, and 70% of FC) and five levels of nitrogen (control, based on soil test results; 25% less than the recommended level; 50% less than the recommended level; 25% more than the recommended level; and 50% more than the recommended level). The recommended level of nitrogen, based on soil test results, was 0.09 g of nitrogen per kilogram of soil. Results showed that drought stress, nitrogen, and their interaction significantly affected the enzymatic activity of antioxidant catalase (CAT), polyphenol oxidase (PPO), and peroxidase (PO) at various stages of growth. Proline was also affected by drought stress and nitrogen. Maximum CAT activity (794.04 OD mg protein min^{-1}) was observed at the 4–6 leaf stage under mild drought stress conditions (50% of FC) and a nitrogen application rate 50% less than the recommended level. Increased rates of nitrogen enhanced the PPO activity at the 4–6 and 6–8 leaf stages. PO had a negative response to increased rates of nitrogen application. The highest rate of increase in proline was at the 8–10 leaf stage: 205% and 207% higher under mild drought stress (50% of FC) and severe drought stress (30% of FC) conditions, respectively, compared to favorable moisture conditions (70% of FC). Increased nitrogen application led to an increase in proline production at all stages of sampling (4–6, 6–8, and 8–10 leaf stages).

Key words: Catalase, peroxidase, proline, water deficit

1. Introduction

Drought stress limits plant growth and production in arid and semiarid regions more than any other environmental factor (Zhang et al., 2007). Under normal growth conditions plants are inevitably influenced by various environmental stresses that may lead to increased production of reactive oxygen species (ROS) (Smirnoff, 1993). Drought is a major environmental stress which generates harmful compounds and removes balance in the formation of oxygen species (superoxide, hydrogen peroxide, hydrogen radical, and singlet oxygen) (Arora et al., 2005). Reducing the impact of drought stress on metabolic adjustment and ensuring that plant growth takes place in an appropriate manner is achieved through the development of plant adaptation responses.

The role of antioxidant defense enzymes such as superoxide dismutase, peroxidase, and catalase is to reduce the concentration of superoxide and hydrogen peroxide. Malondialdehyde (MDA) level is also a scale for peroxidation of lipids and results from the accumulation of ROS. Therefore, the antioxidant enzyme activities and MDA levels determine the degree of their toxicity towards plants (Saneoka et al., 2004; Zhao et al., 2005). The water deficit in leaf tissues influences many physiological processes and ultimately reduces the yield (Pidgeon et al., 2001; Tognetti et al., 2003). Proline concentration increases in response to water deficit (Hanson et al., 1977; Hasegawa et al., 1994; Yeo, 1998), and many reports suggest a positive correlation between proline accumulation and increased tolerance to drought and salinity stresses (Rensburg and Kruger, 1994; Kishor et al., 1995). Other empirical evidence suggests that proline accumulation is more an indication of stress damage than an indication of stress tolerance (Liu and Zhu, 1997). Ion accumulation under drought stress conditions is higher than under favorable conditions (Toker et al., 2009).

Nitrogen is one of the nutrients plants need in great amounts, but its use efficiency is generally low in dry areas,

* Correspondence: kalantar@uma.ac.ir

and this is one of the factors that limit yield increase and quality improvement (Li, 2007). Application of nitrogen not only provides one of the nutrients necessary for plant growth, it also improves drought tolerance and increases yield (Zaman and Das, 1991; Boutraa and Sanders, 2001; Xu et al., 2005). Moreover, nitrogen plays an important role in the activity of antioxidant defense enzymes and lipid peroxidation metabolism in crops under stress conditions (Zhang and Liu, 2001; Sun et al., 2001; Saneoka et al., 2004). Optimal nitrogen nutrition is the basis of plant growth and production and is necessary for the biosynthesis of amino acids, proteins, and enzymes (Sinclair and Vadez, 2002). Its lack may be an abiotic stress that reduces yield (Zhang et al., 2007). Higher nitrogen application rates increased the accumulation of dry matter (Duan et al., 2014). Aires et al. (2006) stated that nitrogen is one of the most important factors affecting plant growth as well as the biosynthesis of secondary metabolites. Increased production and improved drought tolerance in crops with increasing nitrogen supplies under stress conditions have been reported by several researchers (Boutraa and Sanders, 2001; Sun et al., 2001; Sinclair and Vadez, 2002; Zhang et al., 2007). Mohammadian et al. (2005) declared that the effects of mild drought stress on root dry weight were greater than on shoot dry weight in sugar beet. Ibrahim et al. (2011) also reported that increasing the rate of nitrogen application (270 kg ha^{-1}), compared to control treatment (no application of nitrogen), reduced antioxidant enzyme activities. Water stress and nitrogen deficit increased H_2O_2 production and MDA concentration in *Arabidopsis* (Shin et al., 2005) and decreased antioxidant enzyme activities in corn leaves (Sun et al., 2001). Increased nitrogen could also reduce lipids peroxidation by increasing the activity of antioxidant enzymes and decreasing MDA concentrations in order to maintain the photosynthetic processes in leaves under drought stress conditions (Jiang et al., 2005). Mistra and Gupta stated that antioxidant enzyme activities were influenced by various nitrogen sources, and plants fed with ammonium had higher catalase and glutathione S-transferase activities, while the activity of peroxidase and superoxide dismutase was higher in plants fed with nitrate.

Considering the above-mentioned studies, the objective of this experiment was to evaluate the effects of drought stress and different levels of nitrogen on canola seedlings in terms of some drought tolerance mechanisms.

2. Materials and methods

This experiment was conducted as a factorial in a completely randomized design with three replications at the experimental greenhouse of Mohaghegh Ardabili University in 2013. Treatments included three levels of drought stress (30%, 50%, and 70% of field capacity (FC)) and five levels of nitrogen (control, based on soil test results; 25% less than the recommended level; 50% less than the recommended level; 25% more than the recommended level; and 50% more than the recommended level). The moisture content of the pots was maintained at 30%, 50%, and 70% of FC during the experiment. Results of the soil analysis test are presented in Table 1. Nutrients needed for each pot were calculated separately considering the weight of soil per hectare. The recommended level of nitrogen, based on the soil test, was 180 kg ha^{-1} (0.09 g of nitrogen per kilogram of soil). The recommended levels of potassium sulfate and triple superphosphate, based on the soil test, were 0.1 and 0.05 g per kilogram of soil, respectively. Nitrogen treatments included N1 = 0.04, N2 = 0.06, N3 = 0.09, N4 = 0.11, and N5 = 0.13 g of nitrogen per kilogram of soil. N3 was the control treatment, based on soil test results. Nitrogen was supplied from urea and added to the pots after the first irrigation. Pots were hand-weeded during the season. The studied cultivar was Hyola401. The pots were kept in a greenhouse with a 14/10 h light/dark photoperiod at 25 ± 2 °C. Plastic pots with a capacity of 5 kg were selected, and 5 kg of soil was added to each of them. Drought stress treatments were applied after seedlings emerged at 2-leaf stage. Samples were collected at three stages (4–6, 6–8, and 8–10 leaf).

2.1. Total protein assay

In order to extract protein, 0.2 g of fresh plant tissue was pulverized in a mortar using liquid nitrogen, and then 1 mL of buffer Tris-HCl (0.05 M, pH 7.5) was added. The obtained mixture was centrifuged for 20 min at 13,000 rpm at 4 °C, and the supernatant was used for enzyme activity measurements (Sudhakar et al., 2001).

2.2. Catalase (CAT) assay

Catalase activity was assayed according to Kar and Mishra (1976). Then 60 µL of protein extract was added to Tris buffer (50 mM, pH 7) H_2O_2 5 mM in the ice bath, and then the absorbance curve was considered at a wavelength of 240 nm. Enzyme activity was obtained for OD/mg protein in fresh tissue.

Table 1. The characteristics of the soil used in the experiment.

Bulk density (g/cm^3)	Texture	Sand (%)	Silt (%)	Clay (%)	K (mg kg^{-1})	P (mg kg^{-1})	OC (%)	pH	EC (ds m^{-1})
1.25	Loamy sand	84	14	2	170	8.5	0.62	7.88	0.625

2.3. Peroxidase (PO) assay

Peroxidase activity was measured as described by Kar and Mishra (1976): 50 µL of protein extract was added to 2.5 mL of extraction buffer containing 100 µM Tris buffer and 100 mM and 5 mM hydrogen peroxide and 10 mM pyrogallol in an ice bath. Absorbance changes were read at a wavelength of 425 nm.

2.4. Polyphenol oxidase (PPO) assay

Polyphenol oxidase enzyme activity was measured by the method of Kar and Mishra (1976); 100 µL of protein extract was solved in 1.5 mL of 0.2 M Tris and 0.3 mL of 0.02 M pyrogallol, and the resulting complex was placed in a water bath (bain-marie) at 25 °C for 5 min, and then the absorbance rate at 420 nm was recorded.

2.5. Proline assay

Proline was measured in the youngest leaves using the method proposed by Bates et al. (1973). Thus, 0.1 g of leaf tissue was pulverized in 2 mL of 3.3% sulfosalicylic acid and was then centrifuged at 4000 rpm for 10 min at 4 °C. Then, 2 mL of ninhydrin reagent and 2 mL of pure glacial acetic acid were added to 2 mL of the resulting extract in separate tubes. The tubes were placed in a bain-marie for 1 h. Each tube was then vortexed for 15–20 s after adding 4 mL of toluene. After the formation of two separate phases, the colored upper phase was separated, and the absorbance was measured using a spectrophotometer at a wavelength of 520 nm.

Statistical analysis was performed using SAS software. Mean comparison was also performed using Duncan's multiple range test at $P \leq 0.05$.

3. Results

3.1. Catalase (CAT)

ANOVA results showed that drought stress, nitrogen, and their interaction significantly affected CAT activity at all three stages of sampling (4–6, 6–8, and 8–10 leaf stages) (Tables 2–4). As shown in Table 5, the maximum CAT activity (794.04 OD mg protein min^{-1}) at 4–6 leaf stage was obtained at 50% of FC and the application of 90 kg ha^{-1} of nitrogen. Minimum CAT activity (230.952 OD mg protein min^{-1}) at the same stage was also obtained under favorable moisture conditions (70% of FC) and the application of 0.09 kg of nitrogen per kilogram of soil. The results also indicate that the lowest level of CAT activity under moisture conditions 30% of FC was observed in N3 (control) treatment, and increasing levels of nitrogen (higher than control treatment) did not reduce it (Table 5). Evaluating the level of CAT activity at 6–8 leaf stage also showed that the CAT activity increased at all levels of nitrogen application, and the highest CAT activity (710.76 OD mg protein min^{-1}) was observed under 30% of FC moisture conditions. CAT activities at all levels of nitrogen application, except the N5 treatment, at the same stage under 70% of FC moisture conditions belonged to the same statistical group (Table 6).

Table 2. ANOVA of characteristics studied under drought stress and nitrogen application at the 4–6 leaf stage.

SOV	Df	CAT	PPO	PO	Proline	Dry matter
Stress	2	543,408**	557,096**	2,673,421**	10.711**	0.000576**
Nitrogen	4	70,200.32**	357,589.2**	145,779**	0.52807**	0.00022**
Stress × nitrogen	8	96,710.94**	513,657**	357,772.93**	0.08017**	0.000004**
Error	30	60.128	4396	10,040.607	0.04344	0.00000144
CV (%)		1.54	3.13	2.38	10.23	4.85

*and**: significant at 5% and 1% levels of probability, respectively; ns: not significant.

Table 3. ANOVA of characteristics studied under drought stress and nitrogen application at the 6–8 leaf stage.

SOV	Df	CAT	PPO	PO	Proline	Dry matter
Stress	2	188,112**	14,278.36**	498,060.91**	4.3744**	0.0007383**
Nitrogen	4	60,995.01**	17,776.16**	820,377.60**	0.7668**	0.0004039**
Stress × nitrogen	8	47,225.63**	32,647.36**	39,549.87**	0.0630**	0.0000050**
Error	30	454.467	170.2606	3169.85	0.01056	0.0000022
CV (%)		5.93	4.88	7.27	7.28	4.58

*and**: significant at 5% and 1% levels of probability, respectively; ns: not significant.

Table 4. ANOVA of characteristics studied under drought stress and nitrogen application at the 8–10 leaf stage.

SOV	Df	CAT	PPO	PO	Proline	Dry matter
Stress	2	35,476.30**	98,509.49**	86,704.57**	4.80849**	0.0014856**
Nitrogen	4	34,222.08**	31,0551.70**	312,481.2**	0.94587**	0.0001403**
Stress × nitrogen	8	32,514.88**	19,287.293**	19,497.95**	0.02419ns	0.0000078**
Error	30	57.7178	338.951	264.157	0.19917	0.00000218
CV (%)		2.81	5.706	5.14	8.96	3.54

*and**: significant at 5% and 1% levels of probability, respectively; ns: not significant.

Table 5. Means of drought stress × nitrogen on studied parameters at the 4–6 leaf stage.

	CAT (ΔOD min^{-1} mg^{-1} protein)	PPO (ΔOD min^{-1} mg^{-1} protein)	PO (ΔOD min^{-1} mg^{-1} protein)	Soluble sugar (mg g^{-1} FW)	Proline (µg g^{-1} FW)	Shoot dry weight (g)
S1N1	618.64^{d}	183.87^{j}	519.31^{g}	0.667^{g}	0.662^{e}	0.022^{gh}
S1N2	287.55^{h}	155.97^{k}	607^{f}	0.79^{g}	1.10^{d}	0.029^{cd}
S1N3	197.69^{k}	197.69^{ij}	796.11^{e}	1.353^{b}	1.11^{d}	0.031^{c}
S1N4	512.12^{f}	206.34^{i}	629.92^{f}	0.962^{d}	1.30^{cd}	0.035^{b}
S1N5	464^{g}	264.63^{g}	348.88^{jk}	0.833^{ef}	1.54^{c}	0.038^{a}
S2N1	794.04^{a}	710.52^{c}	1953.29^{a}	1.12^{c}	1.56^{c}	0.018^{i}
S2N2	780^{b}	380.57^{f}	1469^{b}	1.17^{c}	2.06^{b}	0.023^{fg}
S2N3	718.92^{c}	531.31^{e}	1036^{c}	1.52^{a}	2.34^{b}	0.025^{e}
S2N4	734.98^{b}	734.98^{b}	1031.11^{c}	1.17^{c}	2.32^{b}	0.027^{de}
S2N5	573^{b}	573^{d}	766.32^{e}	0.938^{d}	2.36^{b}	0.029^{c}
S3N1	284.45^{l}	284.54^{g}	396.02^{i}	1.30^{b}	2.72^{a}	0.013^{l}
S3N2	284.26^{l}	284.26^{g}	433.1^{h}	1.52^{a}	2.80^{a}	0.015^{k}
S3N3	230.95^{j}	230.95^{h}	328.36^{k}	1.14^{c}	2.80^{a}	0.019^{ij}
S3N4	278^{i}	278^{g}	336.14^{ij}	0.891^{de}	2.82^{a}	0.020^{hi}
S3N5	771.70^{b}	768.11^{a}	835.3^{d}	0.51^{h}	2.89^{a}	0.025^{ef}

This may indicate that an increase in nitrogen levels under favorable moisture conditions is a stress on the plants and they attempt to neutralize excess nitrogen by increasing CAT activity. The trend in CAT activity changed at the 8–10 leaf stage, and the maximum CAT activity under 30% of FC moisture conditions was observed in N2 treatment. The CAT activity at the 8–10 leaf stage (Table 7) decreased under severe drought stress conditions (30% of FC) with an increasing application of nitrogen, in contrast to the 6–8 leaf stage (Table 6). The regression results of nitrogen and drought stress on catalase were different during different stages of growth. The regression relationship between drought stress (70% FC and 30% FC) and nitrogen on CAT activity at the 4–6 leaf stage was not significant; however, there was a negative relationship between drought stress (50% FC) and nitrogen on CAT activity (Figure 1a). The regression relationship was significant between CAT activity and nitrogen under severe drought stress conditions (30% of FC) at the 6–8 leaf stage (Figure 1b). There was a linear relationship between drought stress and nitrogen on CAT activity at the 8–10 leaf stage (Figure 1c). For each degree of increase in nitrogen, CAT activity decreased 1454.73, 1971.2, and 1443.7 units under 30%, 50%, and 70% of FC moisture conditions (Figure 1c).

Table 6. Means of drought stress × nitrogen on studied parameters at the 6–8 leaf stage.

	CAT (ΔOD min^{-1} mg^{-1} protein)	PPO (ΔOD min^{-1} mg^{-1} protein)	PO (ΔOD min^{-1} mg^{-1} protein)	Soluble sugar (mg g^{-1} FW)	Proline (µg g^{-1} FW)	Shoot dry weight(g)
S1N1	218.18^{h}	187.58^{g}	1205^{b}	0.658^{b}	0.642^{h}	0.034^{c}
S1N2	216.49^{h}	155.42^{h}	788.67^{d}	0.635^{bc}	0.851^{g}	0.036^{bc}
S1N3	190.3^{h}	163.64^{h}	778.17^{de}	0.719^{a}	0.896^{fg}	0.037^{b}
S1N4	212.75^{h}	224.52^{f}	689.78^{ef}	0.437^{h}	1.06^{ef}	0.043^{a}
S1N5	332^{f}	511.14^{a}	722.09^{g}	0.480^{g}	1.25^{d}	0.045^{a}
S2N1	441.67^{d}	249.48^{de}	1519.42^{a}	0.637^{bc}	0.998^{fg}	0.028^{de}
S2N2	340^{ef}	255.02^{d}	1056^{c}	0.650^{b}	1.19^{de}	0.030^{d}
S2N3	488.16^{c}	335.56^{bc}	864.07^{d}	0.608^{cd}	1.29^{d}	0.031^{c}
S2N4	604.73^{b}	352.4^{b}	653.84^{f}	0.595^{de}	1.33^{d}	0.035^{bc}
S2N5	371.44^{e}	320.58^{c}	676.17^{f}	0.338^{i}	1.618^{c}	0.037^{b}
S3N1	204.37^{h}	327.61^{c}	1136.28^{bc}	0.415^{h}	1.59^{c}	0.019^{g}
S3N2	280.30^{g}	229.90^{ef}	477.35^{g}	0.519^{f}	1.61^{c}	0.023^{f}
S3N3	360.11^{ef}	266.05^{d}	404.76^{g}	0.584^{de}	1.87^{b}	0.027^{e}
S3N4	420.44^{d}	254.54^{d}	460.32^{g}	0.577^{e}	2.37^{a}	0.028^{de}
S3N5	710.67^{a}	173.50^{gh}	468.83^{g}	0.591^{de}	2.54^{a}	0.028^{de}

Table 7. Means of drought stress × nitrogen on studied parameters at the 8–10 leaf stage.

	CAT (ΔOD min^{-1} mg^{-1} protein)	PPO (ΔOD min^{-1} mg^{-1} protein)	PO (ΔOD min^{-1} mg^{-1} protein)	Soluble sugar (mg g^{-1} FW)	Shoot dry weight (g)
S1N1	301^{d}	479.64^{c}	479.6^{c}	0.338^{ef}	0.045^{d}
S1N2	329.37^{c}	212.34^{g}	212^{fg}	0.346^{ef}	0.049^{c}
S1N3	236.46^{e}	178.56^{h}	188.4^{g}	0.39^{de}	0.052^{b}
S1N4	197.15^{g}	97.17^{i}	97.14^{i}	0.524^{a}	0.056^{a}
S1N5	204.42^{fg}	210.41^{g}	210.47^{fg}	0.507^{ab}	0.058^{a}
S2N1	335^{c}	765^{a}	765^{a}	0.507^{ab}	0.033^{fg}
S2N2	245.52^{e}	300.55^{e}	265^{e}	0.466^{abc}	0.037^{e}
S2N3	243.37^{e}	265^{f}	226.26^{f}	0.367^{cd}	0.039^{e}
S2N4	217.15^{f}	226.29^{g}	226.67^{f}	0.418^{cd}	0.043^{d}
S2N5	116.39^{h}	124.52^{i}	124.51^{fg}	0.410^{cd}	0.044^{d}
S3N1	352.33^{b}	663.45^{b}	663.41^{b}	0.296^{f}	0.029^{h}
S3N2	380.79^{a}	491.05^{c}	457.71^{c}	0.344^{ef}	0.031^{h}
S3N3	356.45^{b}	360^{d}	360^{d}	0.452^{bc}	0.033^{fg}
S3N4	323.39^{c}	228.33^{g}	228.33^{f}	0.507^{ab}	0.034^{f}
S3N5	210^{fg}	237.01^{fg}	237.01^{f}	0.516^{a}	0.034^{f}

Means with at least one letter in common are not significantly different at the 5% probability level (using least significant range, DMART-LSR).

3.2. Polyphenol oxidase (PPO)

ANOVA results also showed that drought stress, nitrogen, and their interaction significantly affected PPO activity at all three stages of sampling (Tables 2–4). Maximum PPO activity (768.116 OD.mg.protein min^{-1}) at 4–6 leaf stage was observed under severe drought stress conditions (30% of FC) and the application of N5. Increasing severity of drought stress also increased PPO activity at all levels of applied nitrogen (Table 5). Unlike 2–4 leaf stage, the maximum PPO activity at 4–6 leaf stage was observed under favorable moisture conditions (70% of FC) and the application of N5. At 6–8 leaf stage under favorable moisture conditions (70% of FC) PPO activity increased with the increased use of nitrogen (N4 and N5), compared to control treatment (N3), while under severe stress conditions (30% of FC) PPO activity decreased with the increased use of nitrogen, compared to the control treatment. These changes are shown in Table 6. Mean comparison results showed that the maximum PPO activity (765 OD mg protein min^{-1}) at the 8–10 leaf stage was obtained under mild drought stress conditions (50% of FC) and the application of N1. Increased use of nitrogen under both 50% and 70% FC moisture conditions was followed by a decrease in PPO activity (Table 7). The linear equation was significant between PPO activity and nitrogen only under favorable moisture conditions (70% of FC), and increases in nitrogen led to increased PPO activity at the 4–6 leaf stage (Figure 2a). The regression equations were changed at the 6–8 leaf stage, and increased nitrogen caused a rise in PPO activity under both 70% and 50% FC moisture conditions; however, the equation was negative under severe stress conditions (Figure 2b). There was a linear relationship between nitrogen and POL activity, and increasing nitrogen led to decreasing POL activity under different moisture conditions (Figure 2c).

3.3. Peroxidase (PO)

Maximum PO activity was observed at the 4–6 leaf stage under mild drought stress conditions (50% of FC) and

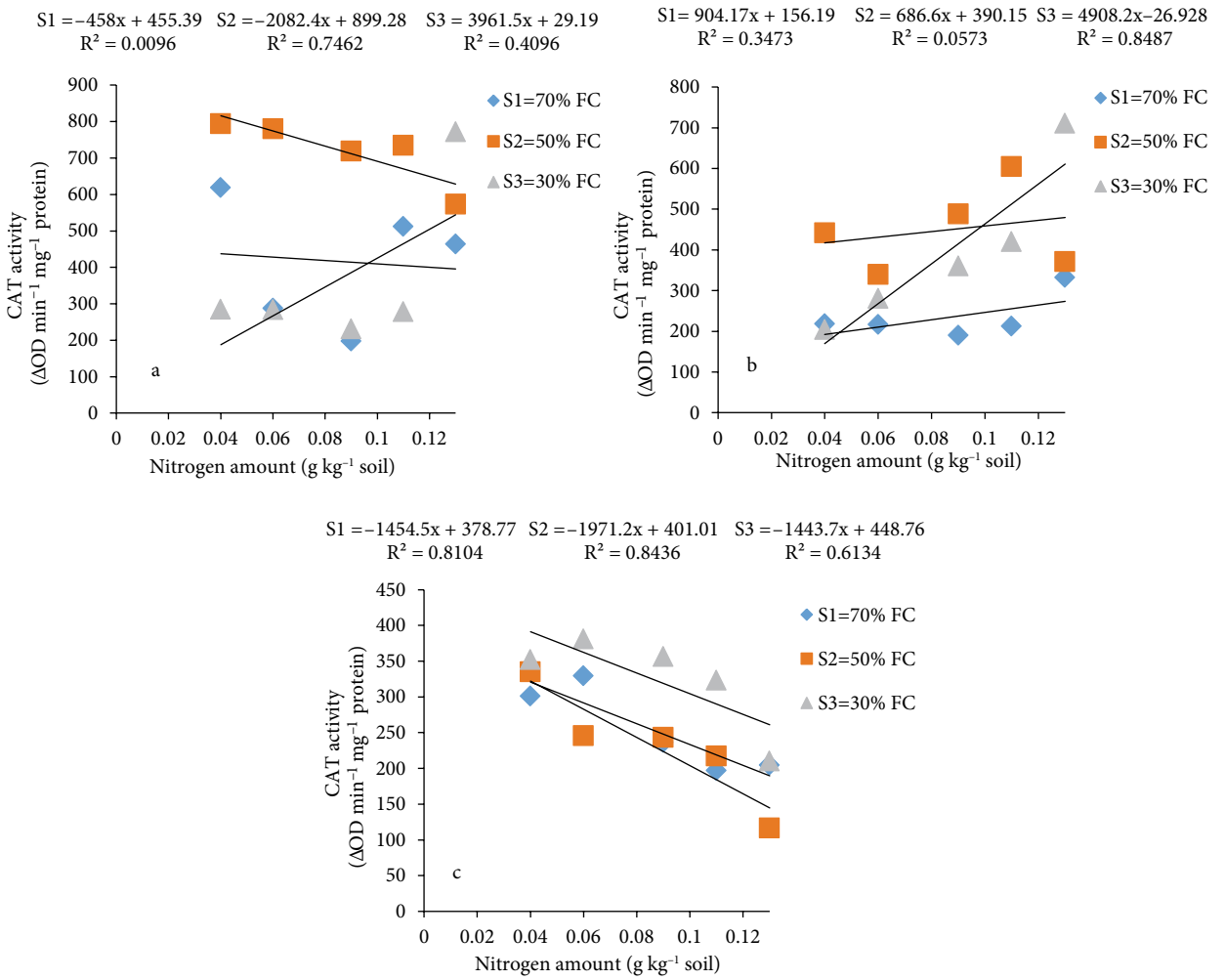

Figure 1. Relationships between nitrogen and CAT activity under drought stress at the 4–6 (a), 6–8 (b), and 8–10 (c) leaf stages.

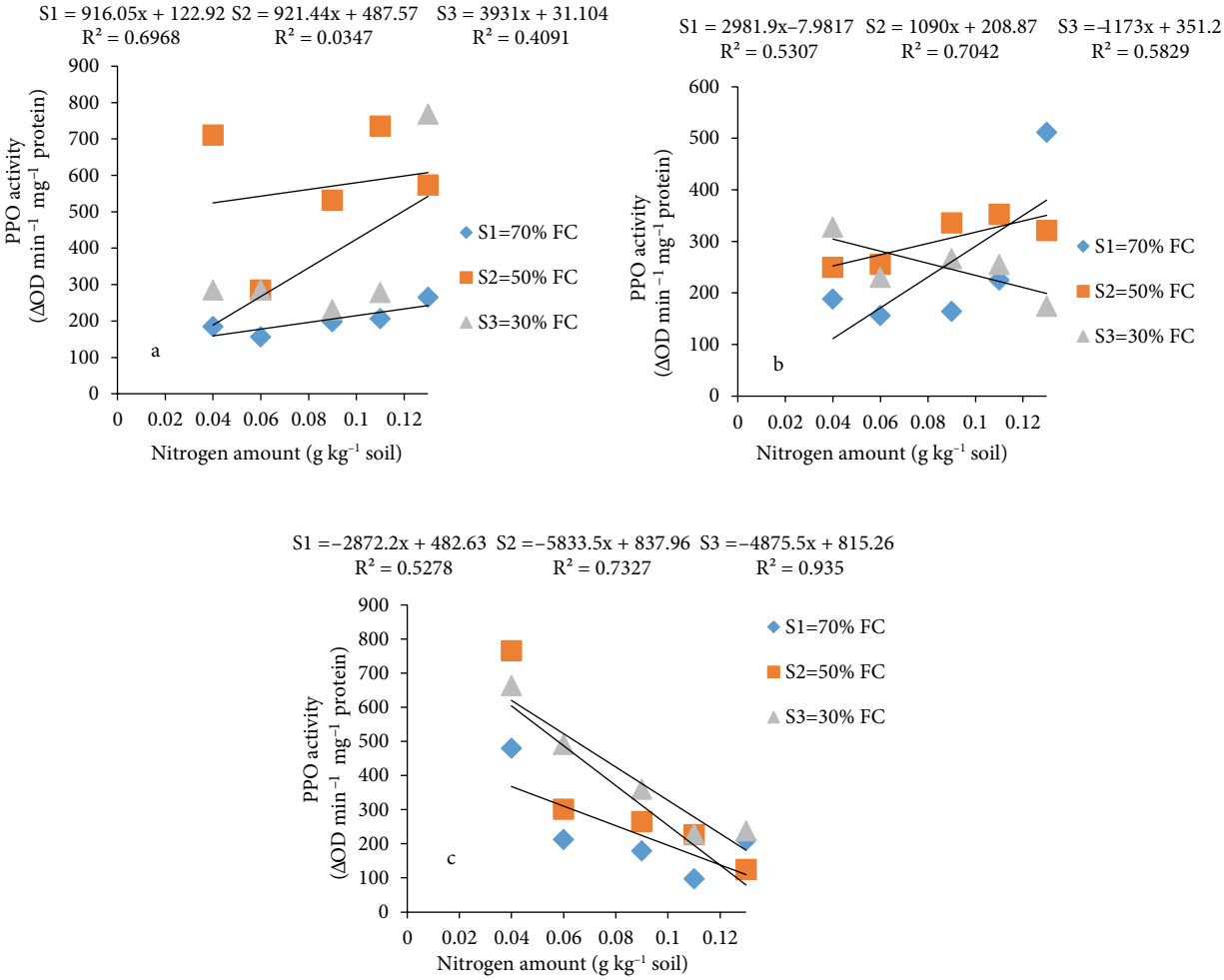

Figure 2. Relationships between nitrogen and POL activity under drought stress at the 4–6 (a), 6–8 (b), and 8–10 (c) leaf stages.

the application of N1. Minimum PO activity was also observed under severe drought stress conditions (30% of FC) and the application of N3. Changes in PO activity under mild drought stress conditions (50% of FC) were such that activity decreased with an increase in applied nitrogen; however, the rate of change in PO activity under severe drought stress conditions (30% of FC) was lower compared to that under mild drought stress conditions (50% of FC). In advanced stages of plant growth the trend in PO activity also changed, and maximum PO activity (1519.42 OD mg protein min^{-1}) was observed at the 6–8 leaf stage under mild drought stress conditions (50% of FC) and a nitrogen application rate of 50% less than control (N1). The changes in PO activity under mild drought stress conditions (50% of FC) at all stages of sampling were such that it decreased with increases in applied nitrogen (Tables 5–7). As a result of increasing nitrogen under 50% FC, PO activity was significantly reduced, but there was no significant regression in the relationship between nitrogen level and PO activity under favorable moisture and severe drought stress conditions (Figure 3a). When we evaluated the relationship between POD activity and nitrogen at the 6–8 and 8–10 leaf stages, it was obvious that there was a negative relationship between them under different moisture conditions, and the amount of reduction at the 6–8 leaf stage was greater than that at the 8–10 leaf stage (Figures 3b and 3c).

3.4. Proline

ANOVA results (Tables 2 and 3) showed that drought stress, nitrogen, and their interaction significantly affected proline content at both 4–6 and 6–8 leaf stages, while the simple effects of treatments were significant for proline content at 8–10 leaf stage (Table 4). Increasing severity of drought stress increased the proline content as well. Table 5 shows that under favorable moisture conditions (70% of FC), increased nitrogen application

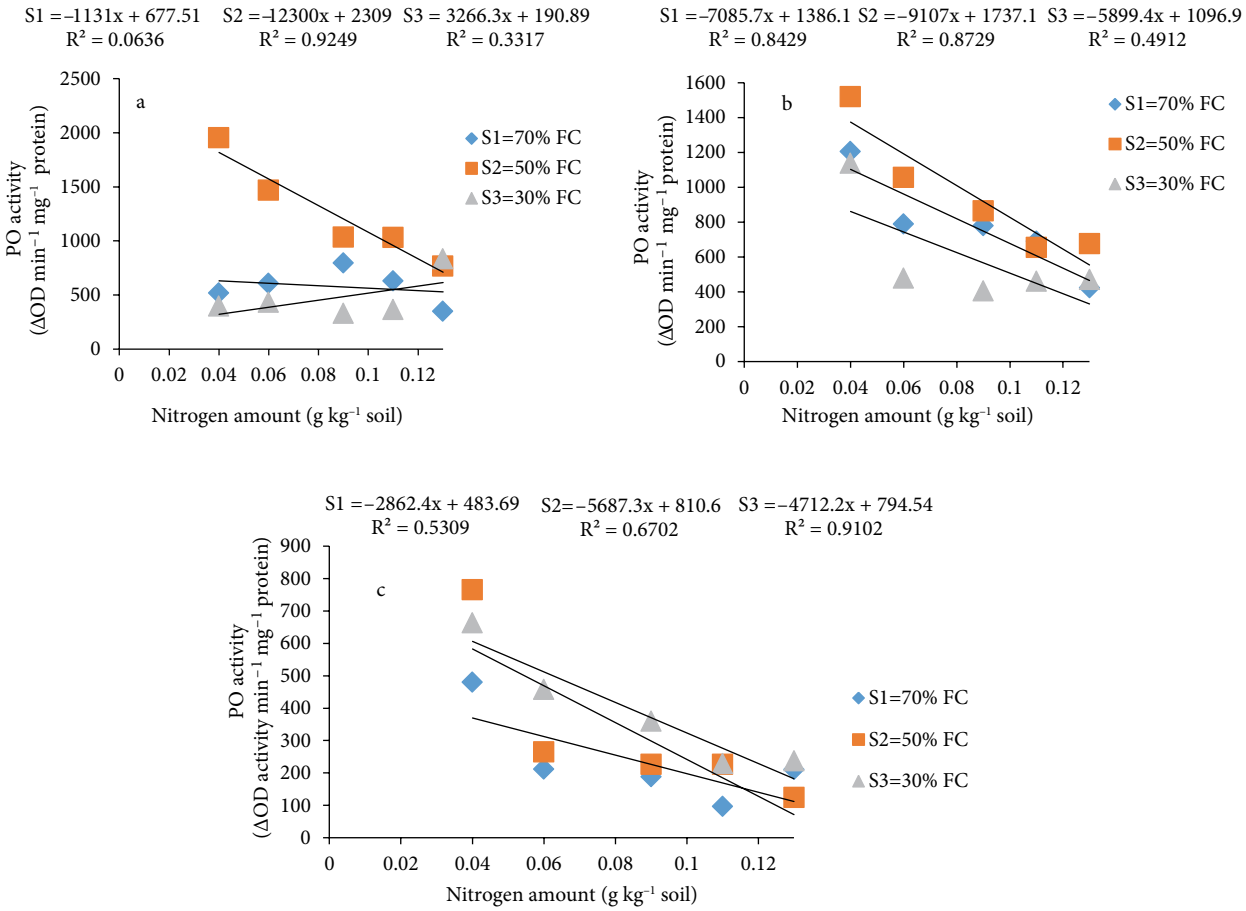

Figure 3. Relationships between nitrogen and PO activity under drought stress at the 4–6 (a), 6–8 (b), and 8–10 (c) leaf stages.

led to an increase in proline production. The same trend was also observed under mild drought stress conditions (50% of FC). Under severe drought stress conditions (30% of FC), increased nitrogen application did not have a significant effect on proline production increase (Table 5). Increasing severity of drought stress also led to an increase in proline production at 6–8 leaf stage. The highest proline content (2.547 μg.g^{-1} FW) was observed under severe drought stress conditions (30% of FC) and the application of N5 (Table 6). Mean comparison results for the simple effects of drought stress on proline content at 8–10 leaf stage also showed that proline production increased with increasing drought stress severity. There was a positive linear relationship between leaf nitrogen amount and proline amount at 4–6 and 6–8 leaf stages, and proline increased as a result of nitrogen additions under different moisture conditions (Figures 4a and b). Proline production under 50% and 30% of FC moisture conditions increased by 51.4% and 51.67%, respectively, compared to favorable moisture conditions (70% of FC) at the 8–10 leaf stage (Figure 5a). The highest proline content at the 8–10 leaf stage was also observed in N5 treatment (Figures 5b).

3.5. Dry matter

Drought stress significantly decreased shoot dry matter at all three stages of sampling (4–6, 6–8, and 8–10 leaf stages). Nitrogen also had a significant effect on dry matter (Tables 1–3). Effects of nitrogen and drought stress on dry matter at the 4–6 leaf stage showed that increased nitrogen application led to an increase in dry matter produced in all drought stress treatments (Table 5). The highest dry matter (0.038 g) was obtained by the application of 0.13 g of nitrogen per kg of soil under 70% of FC moisture conditions. Drought stress, however, decreased dry matter production at 6–8 and 8–10 leaf stages. Changes in dry matter production were also incremental and significant with increased applications of nitrogen at both 6–8 and 8–10 leaf stages (Tables 6 and 7). A significant positive relationship was found between nitrogen and dry matter under different moisture conditions, and increasing nitrogen levels led to increased shoot dry matter at different growth stages (Figures 6a–c).

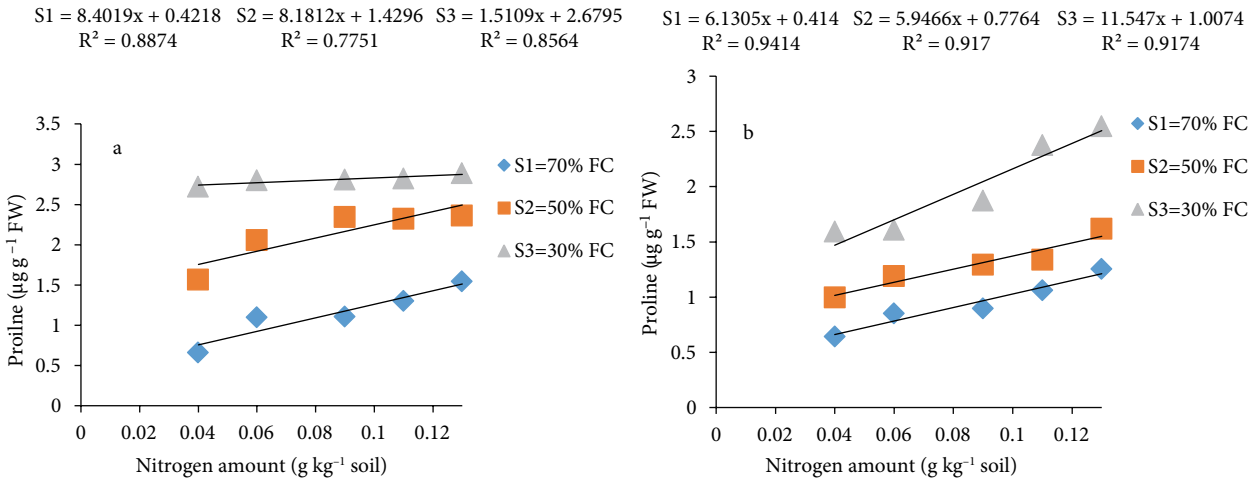

Figure 4. Relationships between nitrogen and proline amount under drought stress at the 4–6 (a), 6–8 (b), and 8–10 (c) leaf stages.

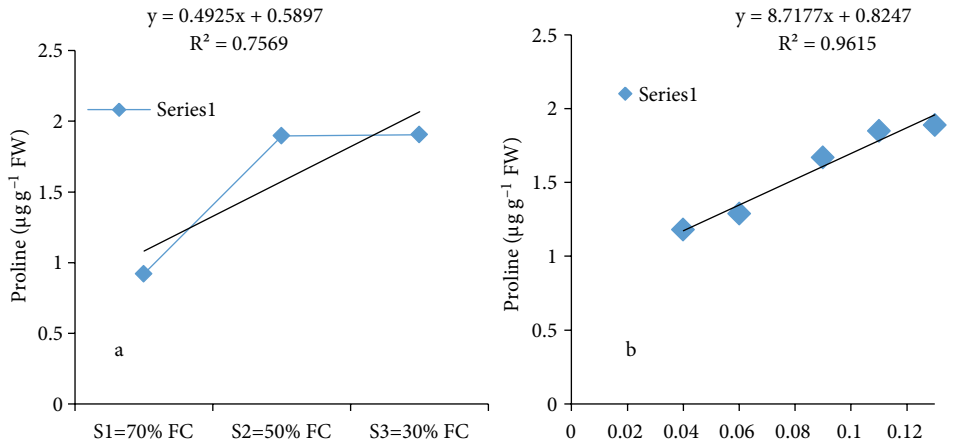

Figure 5. Relationships between drought stress (a) and nitrogen (b) with proline amount at the 8–10 leaf stages.

4. Discussion

When plants are exposed to drought stress they use a series of physiological and biochemical reactions to reduce the effects of stress. In addition, antioxidant enzyme systems including CAT, PPO, and PO have an important role in scavenging of ROS, and their activity increases under stress conditions (Zhao et al., 2005). Table 5 indicates that increasing the severity of drought stress up to 50% of FC at the 4–6 leaf stage led to a significant increase in CAT activity, and that plants tried to neutralize the free radicals produced under stress conditions by increasing CAT production. Drought stress severity increases up to 30% of FC decreased CAT production; however, an increase in nitrogen application led to an increase in CAT production under severe drought stress conditions. The CAT activity decrease under severe drought and salinity stress conditions could be attributed to an increase in the accumulation of H_2O_2 (Tanou et al., 2009). An overview of changes in CAT activity at different stages indicates that levels of CAT decrease in advanced stages of plant growth; this could mean a decrease in CAT ability to scavenge ROS. Filippou et al. (2011) also stated that the implementation of drought stress for 14 days induced CAT activity, compared to control.

PPO activity also increased with increasing drought stress severity, but changes were different at various growth stages. Maximum PPO activity (768.116 OD mg protein min^{-1}) was observed at the 4–6 leaf stage under severe drought stress conditions (30% of FC) and the application of N5 (Table 5). Increased nitrogen application at the 4–6 leaf stage also led to an increase in PO activity, but it was followed by a decrease in PO activity at the 6–8 and 8–10 leaf stages (Tables 5–7). Increased application of nitrogen under various levels of drought stress at both the 6–8 and 8–10 leaf stages in this study decreased PO activity (Tables 6 and 7). Maximum PO activity was also observed

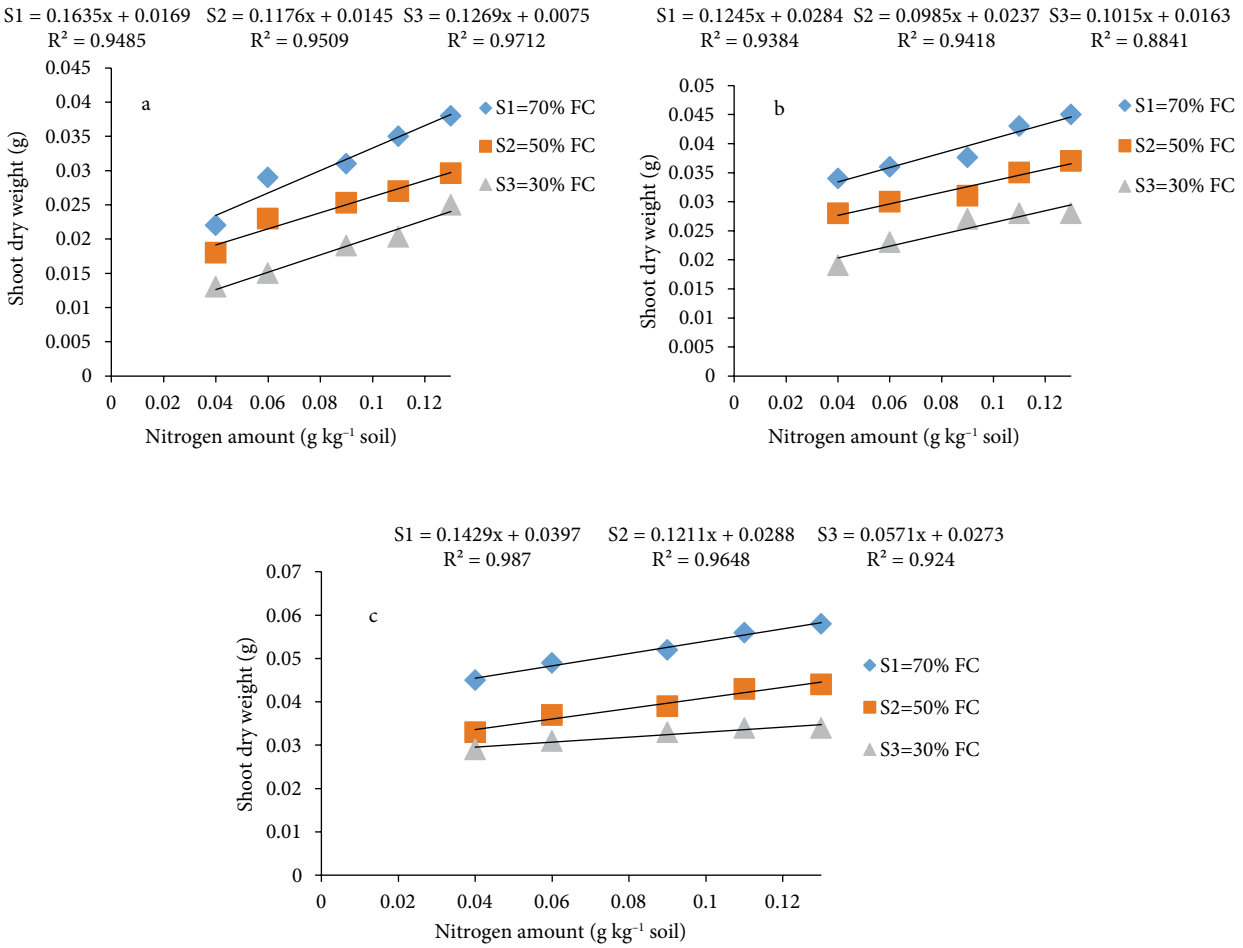

Figure 6. The interaction effect of drought stress and nitrogen on dry matter at the 4–6 (a), 6–8 (b), and 8–10 (c) leaf stages.

at the 4–6 leaf stage under mild drought stress conditions (50% of FC) and the application of N1. It appears that an increase in plant nitrogen nutrition leads PO to perform poorly in its key role as a ROS scavenger.

Studies on the physiological adaptation of crops indicates that changes in superoxide dismutase, PO, and CAT activity are associated with the genotype, amount of water, and stage of development (Sairam and Srivastava, 2001; Dhanda et al., 2004; Ramachandra et al., 2004). Changes in superoxide dismutase, PO, CAT, and MDA activity under drought stress conditions are different depending on tolerance of the variety to drought, drought stress implementation technique, and severity (Arora et al., 2002). Results obtained in other experiments indicate both an increase in and variability of superoxide dismutase, PO, and CAT activity in drought-tolerant varieties compared to susceptible varieties (Mencon et al., 1995). In general, antioxidant enzyme activities will initially increase and then decrease with the advancement of crop growth under stress conditions; however, the timing of such changes may differ depending on the extent of a variety's tolerance to drought (Zhang et al., 2007). Water stress and nitrogen deficit increased H_2O_2 production and MDA concentration in *Arabidopsis* (Shin et al., 2005) and decreased antioxidant enzyme activities in corn leaves (Sun et al., 2001). Increased nitrogen could also reduce lipid peroxidation by increasing the activity of antioxidant enzymes and decreasing MDA concentrations in order to maintain the photosynthetic processes in leaves under drought stress conditions (Jiang et al., 2005).

Proline concentrations increase in response to water deficit (Hanson et al., 1977; Hasegawa et al., 1994; Yeo, 1998), and many reports suggest a positive correlation between proline accumulation and increased tolerance to drought and salinity stresses (Rensburg and Kruger, 1994; Kishor et al., 1995). Other empirical evidence suggests that proline accumulation is more an indication of stress damage than an indication of stress tolerance (Liu and Zhu, 1997). However, it seems that proline concentration could be a useful indicator of drought-stress tolerance in plants (Iannucci et al., 2000; Ain-Lhout et al., 2001). The overall results of different sampling stages showed that

increasing severity of drought tolerance together with increased nitrogen application led to a rise in proline production. An increase in nitrogen application had a positive impact on proline accumulation and enhanced drought tolerance (Zhou et al., 2011). Increases in proline concentration under stress conditions may be the result of protein breakdown and a decrease in its use due to the reduction in plant growth. Proline accumulation, on the other hand, may provide the cells with an opportunity to continue the absorption of water by decreasing both the osmotic and water potential in cells. The accumulation of compatible protective solutions such as proline in response to drought and salinity stress helps to facilitate the absorption of water (Ashraf and Foolad, 2007).

Canola seedlings had the ability to tolerate drought, but drought stress led to a decrease in dry matter production. Production of more dry matter could be a result of higher photosynthesis in leaves under favorable moisture conditions (Majid and Simson, 1997). The pattern of dry matter accumulation is influenced by certain factors; nitrogen and the availability of nitrogen during the growth period play an important role in the proper establishment of plants (Diepenbrock, 2003). Increased application of nitrogen in the current study also led to an increase in dry matter production. Increased application of nitrogen could further increase dry matter production in canola under various moisture regimes (Kamkar et al., 2011). The true nature of nitrogen impact is its influence on photosynthesis and CO_2 assimilation (Kappen et al., 1998).

The results of this study indicate that CAT, PPO, and PO activities decreased with advancement of plant growth stages, and differences in the response of these enzymes to changing levels of nitrogen during various growth stages were large compared to proline. The variability of changes in antioxidant enzyme activities during various growth stages indicates that plants take advantage of a variety of mechanisms to neutralize the negative effects of drought stress. Changes in proline concentrations due to drought stress and nitrogen application were such that increasing drought stress severity and the nitrogen application rate led to an increased production of proline, which indicates that increased levels of nitrogen may also have a role in tolerance to drought stress through an increase in proline production.

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