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Antioxidant systems, germination and nodulation in chickpea (*Cicer arietinum* L.) genotypes under drought

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Abstract

Drought stress is one of the important factors limiting legumes growth and nodulation in arid and semi-arid regions such as southern Morocco. Hence, the identification of more drought tolerant chickpea varieties is one of crucial approaches to improve its yield in these regions. In this work, we assessed the effects of water deficit (induced by different polyethyleneglycol (PEG6000) osmotic potentials: 0 (control), - 0.2 (HS1), - 0.5 (HS2) and - 0.7 MPa (HS3) on the germination parameters of five chickpea varieties (Zahour, Rizki, Douyet, Farihane and Mazouzia) as well as their growth, nodulation and antioxidant responses to 40 % of field capacity (FC) water deficit in pots for 30 days. The result showed that drought significantly reduced germination parameters in all of the studied varieties. Zahour presented the highest germination percentages (GP) of 37 % and 8 % under HS1 and HS2 respectively, as well as the highest shoot and root lengths. After two months of germination, the plants from HS1 and HS2 seeds have presented more drought tolerance with significant increases in their roots and shoots lengths, nodules number and antioxidant enzymes activities, especially for Zahour and Rizki varieties from HS1 seeds, they maintained high peroxidase (POD) and polyphenoloxidase (PPO) activities and low malonyldialdehyde (MDA) contents. Based on these results, Zahour and Rizki varieties from HS1 seeds were selected as more droughts tolerant, while Farihane were qualified as less drought tolerant.

Keywords: *Antioxidant activity; chickpea; drought; germination; nodulation.*

1. Introduction

The world produced 13.1 million tons (Mt) of chickpea in 2013, essentially India with (6 Mt), Pakistan (0.75 Mt), Turkey (0.6 Mt), Australia (0.3 Mt), and Ethiopia (0.2 Mt) [1]. However, drought is one of the severe factors that limit its productivity and many other crops in the world [2]. The availability of water during the early stages of germination plays a decisive role in the plants life cycle [3]. However, chickpea plays an important role in the preservation of soil fertility, particularly in the dry and irregular rainfalls areas due to its ability to fix atmospheric nitrogen in association with rhizobia [4]. In Morocco, the producers of chickpea are small traditional farmers having a hand of plentiful family work. In spring, chickpea is grown in rotation with cereals, particularly in northern zones of Morocco where the annual rainfall exceeds 350 mm. Generally, assessing drought impacts on seeds germination is done with artificial solutions with low osmotic potentials such as PEG6000 to simulate water deficit [5]. In contrast, the NaCl penetrates into plant cells and can be accumulated in the vacuole for the tolerant plants or in the cytoplasm for sensitive cultivars [6]. Drought negatively affects seeds germinations and growth; high concentration of PEG prevents the entrance of water molecules to the seeds, this type of stress is called physiological drought, reduces epicotyls elongation more than the root growth and consequently decreases the shoot/root ratio [7]. The seedlings root and shoot lengths are very important for plants vigour because roots are directly exposed to low soil moisture and mineral inputs, while the shoot brings the energy for the plant [8]. Drought also induces many physiological and biochemical responses at the whole plant parts such as the decrease in the productivity, growth, synthesis of many stress proteins and metabolites. These responses may vary from legumes species to another as well as between varieties allowing these plants to develop sophisticate strategies to survive drought [9]. This abiotic stress negatively affects the legumes N_2-fixing by reducing the number and effectiveness of rhizobia in the soil and thus delaying nodules formation and activity [10,11]. The inoculation with tolerant rhizobia increases the availability of nutrients in the rhizosphere, positively influences roots growth and morphology and promotes other beneficial plant–microbe interactions related to stress tolerance [9,12]. The oxidative stress induces an increase of the liberation of Reactive oxygen species (ROS), resulting in the decrease of cells membrane stability, proteins integrity and the increase of peroxidases activity [13]. Plants have developed different defense mechanisms in response to abiotic stresses, which consist of an overproduction of antioxidant enzymes such as peroxidase and catalase to control the excess of oxidative stress and to protect the plant cells by eliminating the ROS [14]. Drought stress causes irreversible cellular damage, such as lipids peroxidation [15]. The malondialdehyde (MDA) is one of the final products of the membrane lipids peroxidation [16]. This peroxidation is the most attributed symptom to the oxidative damage; it is often used as indicator of the oxidative stress [17].

The aim of this study was to assess the effect of drought on germination and growth as well as nodulation, antioxidant responses and lipid peroxidation in five spring and winter chickpea varieties.

2. Materials and methods

2.1. Effect of water deficit on germination parameters

The experiment was carried out at the Faculty of Sciences and Techniques, Cadi Ayyad University, Morocco and was divided in two parts, the first one took place in the laboratory and consists of seeds germination test. The seeds of five chickpea varieties: Farihane (F), Douyet (D), Zahour (Z), Rizki (R) and Mazouzia (L) were surface sterilized with 5 % sodium hypochlorite followed by several rinses with sterilized distilled water and germinated for 8 days at 25 ± 2 °C [18] in 9 cm Petri dishes with two filter paper layers. The Petri dishes were filled with 7-8 mL of (0 (control), -0.2 MPa (HS1), -0.5MPa (HS2) and -0.7 MPa (HS3)) solutions of polyethylene glycol (PEG 6000). The PEG solutions were prepared according to the method described by [19]. The seeds considered germinated when the radical length is

approximately 2 mm. Seeds germinated on distilled water served as controls. The Germination Parameters were recorded at the 8 days of experiment:

Germination percentage (GP) = (n /N) × 100.

Where, *n* is the number of germinated seeds and *N* is the number of total seeds. The seedlings shoot and roots lengths were measured after the final count in the standard germination test. The shoots length (SL) was measured from point of the attachment of the cotyledon to the tip of the seedling. Similarly, the roots length (RL) was measured from the point of attachment to the tip of the root [20]. Seedling vigour index was determined using the following formula:

(SVI) = (n) × (SL + RL)

Where, *n* is the number of germinated seeds on the 8th day of germination [21]. Five samples were taken as common factor to compare the vigour of the varieties. The growth rates defined as follows:

GR (%) = (SVIb - SVIa /SVIb) × 100

Where, *SVIb* is seedling vigour index after 60 Days and *SVIa* is seedling vigour index after 8th days for 5 randomly samples. In the second part, the experiment was carried out in greenhouse conditions with an approximate temperature of 33/20 ºC (day/night) under 16 h white fluorescent light (1500 lux) and 8 h dark. The germinated seeds were transferred to pots filled with autoclaved sand and peat with an approximate temperature of 33/20 ºC (day/night) under 16 h white fluorescent light (1500 lux) and 8 h dark the proportions (9:1) respectively in a density of 3 plants per pot. The plants were then inoculated by rhizobia suspension of 10^9 CFU/mL (colony forming unit). Then days after, half of the plants were subjected to drought stress (40 % of FC) for 30 days. Five samples per replication were randomly collected for each variety and treatment to measure the shoot (SL) and the root lengths (RL) as well as the nodules number (NN). Other samples were stored in the freezer for future assessment of physiological and metabolic parameters; Peroxidase (POD), polyphenols oxidase (PPO), catalase (CAT) and malondialdehyde (MDA) content).

2.2. Effect on POD, PPO and CAT activities

Shoot fresh material (0.5g) was homogenized in a mortar with 1.5 mL of (0.1 M pH 7) phosphate buffer containing 5 % of polyvinylpolypyrrolidone (PVPP) [22]. The mixture was centrifuged at 12 000 × g for 30 min at 4 °C. The collected supernatants served as enzymatic extract. For the POD activity, the reactive mixture contained 100 µL of the enzymatic extract, 300 µL of 20 mM guaïacol and 2 mL of (0.1 M, pH 6) phosphate buffer. The reaction was started by adding 200 µL of hydrogen peroxide (H_2O_2) then the oxidation of guaïacol was measured by the increase in absorbance at 470 nm [23]. One unit of peroxidase activity was defined as an absorbance change of 0.01 units per min. For the PPO activity, the reactive mixture contains 100 µL of the enzymatic extract, 500 µL of 1.6 % pyrocatechol in the (0.1 M, pH 6) phosphate buffer (w/v), 250 µL of distilled water and 200 µL of phosphate buffer. After incubation for 3 min in the ambient temperature, the absorbance was measured at 410 nm [23]. Activity was expressed as units of activity (UA) in which one unit of PPO was defined as the change in one unit of absorbance per second. The CAT activity was determined by following of the disappearance of H_2O_2 in the reactive mixture according to [24]. The enzyme extract (100 µL) was added to 2 mL of reactive mixture (50 mM Tris-HCl pH 6.8 buffer, containing 5 mM H_2O_2). The reaction was stopped was by addition of 0.25 mL of 20 % titanium tetrachloride. The absorbance of the reaction mixture was measured at 415 nm. The CAT activity was expressed by mg g^{-1} FM.

2.3. Effect on lipid peroxidation

The lipid peroxidation was estimated by determining the content of malondialdehyde (MDA) according to [25]. Shoot material (0.5g) was homogenized in 0.5 mL of 10 % TCA. The homogenate was then added by 0.5 mL of acetone then centrifuged at 8 000 × g for 15 min. The reaction mixture contains 500 µL of the MDA extract, 1 mL of phosphoric

acid (H_3PO_4) and 1 mL of 0.6 % TBA. The mixture was incubated at 95 °C during 30 min. The reaction was stopped by cooling down in a bath of ice and then 1.5 mL of the 1-butanol was added and followed by a centrifugation in 8 000 × g during 15 min. The absorbance of the supernatants was measured at 532 nm. The MDA content was calculated using extinction coefficient of 155 mM^{-1} $cm^{-1}$and expressed in mg g^{-1} FM.

2.4. Statistical analysis

The design was set up as a factorial experiment, using a completely randomized block design (CRD) with three replicates. Differences among treatments and genotypes, as well as their interaction, were tested using SPSS statistical software, version 21.0. Statistical analysis was performed using ANOVA and compared with least significant difference (LSD) at the 5 % level.

3. Results and Discussions

3.1. Effect of water deficit on germination and growth parameters

3.1.1. Germination percentage

The germination percentage (GP) significantly (p<0.001) decreased under water deficit (HS1, HS2 and HS3) in all of the studied chickpea varieties with significant variation between them (Table 1). This is in agreement with the results reported by [26] who reported that under drought, germination percentage significantly reduces due to the decrease of osmotic potential. The reductions were more pronounced at -0.5 MPa (HS2) and -0.7 MPa PEG6000 (HS3). Zahour variety presented the highest GP of 37 % and 8 % under HS2 and HS3 respectively.

Table 1. Effect of the water deficit on germination percentage (GP) and on the nodules number of Chickpea genotypes (Zahour, Rizki, Douyet, Farihane and Mazouzia).

Varieties	GP %				Nodules number		
	Control	HS1	HS2	HS3	Control	HS1	HS2
Z	87	52	37	8	3.37±0.6i	**21.5±0.5a**	9.97±0.57e
R	88	57	32	7	2.55±0.05j	14.7±0.4d	**22.4±0.3a**
D	70	40	32	4	5±1hi	**22±1a**	13.67±0.58d
F	78	43	32	3	8±0.06f	5.67±0.58g	5.6±0.53g
L	78	43	32	4	2.93±0.1	16.67±0.58c	19.67±0.58b
	dF	F			F		
Varieties	4	31.396 ***			264.6***		
PEG	2	811.542 ***			1982.7***		
Varieties*PEG	8	10.708 ***			347.85***		

: Significance at 0.05 probability level; **: Significance at 0.01; *: Significance at 0.001; NS: Not significant at 0.05.*

3.1.2. Root (RL) and shoot lengths (SL) under water deficit

In the 8th day of germination, we observed a significant decrease (p<0.001) of the root and the shoot lengths under water deficit (HS1 and HS2) in all of the tested varieties with significant variation in their behavior (Table 2). Under HS1, the shoots length has significantly decreased by 50 % to 61 % for Mazouzia and Zahour, respectively more than the roots length which presented only 20 % to 46 %, respectively. The shoots length decreased more than the roots

length under stress in the 8th day of germination in all of the studied varieties, while after two months of the experiment, in the greenhouse where the conditions of drought were extreme (33 °C to 40 % FC) the tendency was reversed, we observed an increase in the roots length than in the shoots (Table 2). Based on these results, plants from germinated seeds under drought conditions (HS1 and HS2) resisted better than those from the controls, indeed the plants from seeds of both treatments showed significant (p<0.001) increase in their roots and shoots length (from 30 to 55 %) in comparison to their respective controls (Table 2). This variation could be explained by the osmotic adjustment and turgor maintenance which contributed to the increase in root/shot ratio generally observed in water-limited conditions [27]. The growth rates between 8th and two months of germination in the control seeds varied from 47 to 69 % (Table 3). This percentage increased under HS1 (83 to 86 %) and HS2 (86 to 91 %) in all of the studied varieties with significant (p<0.05) variation. The highest growth rate and SVI value were presented by Rizki and Douyet (91 % and 90 %), respectively under HS2, while Zahour presented the lowest value of 86 % under this condition. under HS1, Zahour, Rizki and Douyet presented the highest GR percentage of 86 %. In response to stress, the vigour values after two months of germination significantly increased, while those after only 8 days showed significant decrease in comparison to their respective controls (Table 3). The increase in seedlings vigour may be due to the efficiency of the oxygen absorbance [28].

Table 2. Effect of the water deficit on roots length (RL) and on shoots length (SL) after 8th days and 60 days (Zahour, Rizki, Douyet, Farihane and Mazouzia) of chickpea.

	RL 8days (cm)			SL 8days (cm)			RL 60days (cm)			SL 60days (cm)		
Varieties	Control	HS1	HS2	Control	HS1	HS2	Control	HS1	HS2	Control	HS1	HS2
Z	6.5±0.15a	3.54±0.08e	2.8±0.26fg	7.7±0.18a	3±0.17d	1.8±0.06fg	11.27±0.2fg	19.8±0.2a	14±0.1d	15.77±0.3g	25.6±0.3a	17.97±0.4f
R	5±0.05b	3.2±0.18ef	2.2±0.2hi	4.4±0.05b	2.2±0.26e	1.4±0.1gh	11.83±0.06ef	17.44±0.5b	16.4±0.36c	12.7±0.4h	22.4±0.5de	23.2±0.25cd
D	4.6±0.1c	3.4±0.1e	2±0.1i	4.36±0.2bc	2±0.18ef	1.6±0.1fg	6.65±0.3i	14.6±0.47d	12.2±0.25e	17.2±0.7fg	24.97±0.9ab	24.7±0.26abc
F	4.2±0.1cd	3.2±0.2ef	2±0.15i	4.2±0.1bc	2±0.1ef	1±0.06h	10.67±0.6g	8±0.15h	7±0.06i	12.13±0.6h	21.6±0.35e	15.77±0.2g
L	4±0.07d	3.2±0.17ef	2.5±0.07gh	4±0.1c	2±0.1ef	1.1±0.1h	8.37±0.3h	12±0.1ef	16.6±0.36bc	18±0.8f	24±0.3bc	17.67±0.15f
	dF	F			F			F			F	
Varieties	4	88.2***			240.67***			710.07***			160.5***	
PEG	2	1182.1***			2588.16***			890.05***			1158.1***	
Varieties * PEG	8	35.27***			81.78***			276.45***			76.36***	

: Significance at 0.05 probability level; **: Significance at 0.01; *: Significance at 0.001; NS: Not significant at 0.05.*

The nodules number (NN) significantly increased (p<0.001) under water deficit (40 % FC) in all of the studied varieties with significant variation between them (Table 1). Douyet and Zahour from HS1 seeds presented the highest nodulation values of 22 ± 1 and 21.5 ± 0.5 respectively, while Rizki variety from HS2 seeds presented the highest value of 22.4 ± 0.3. Indeed, during drought stress, the biological N_2 fixation decrease is associated with the decrease of the nodules number, their specific activities and their sizes [29]. Treatment of seeds with PEG can lead to enhancement of seed germination, nodulation and consequently to improved stand establishment and crop yield under normal or stressed conditions [30]. A strong correlation was found between drought and germination percentage (r=0.903**), vigour (r=0.470**) and nodules number (r=0.558**).

Table 3. Effect of the water deficit on the vigour and on the growth percentage of Chickpea genotypes (Zahour, Rizki, Douyet, Farihane and Mazouzia).

Varieties	*SVIa* 8days (cm)			*SVIb* 60days (cm)			GR (%)		
	Control	HS1	HS2	Control	HS1	HS2	Control	HS1	HS2
Z	71±1.4a	32±1d	22.8±1.3f	135.2±1.4g	227.2±2a	159.8±1.3e	47	86	86
R	47±0,3b	27±0.5e	18±0,2g	122.6±2.4h	199.4±3.8b	198.2±3b	62	86	91
D	45±1.3b	27.2±1.5e	18.2±0,2g	119.3±2hi	198±2.6b	184.7±1.5c	62	86	90
F	41.7±1.2c	26.2±0,7e	15.43±0,7g	114±1i	148.3±2f	114±1i	63	83	87
L	40±0.2c	26±0,97e	18.2±1g	132±2.6g	180±1c	171.3±2.5d	69	85	89
		dF	F		F		F		
Varieties		4	329.29^{***}		769.2^{***}		126.6^{***}		
PEG		2	3919.8^{***}		3497.16^{***}		4708.8^{***}		
Varieties*PEG		8	110.7^{***}		232.98^{***}		86.96^{***}		

: Significance at 0.05 probability level; **: Significance at 0.01; *: Significance at 0.001; NS: Not significant at 0.05.*

3.2. Effect on antioxidant enzymes activities

The POD activity significantly ($p<0.001$, Table 4) increased in all of the tested varieties compared with their respective controls (Fig. 1). In fact, Plants from germinated seeds under HS1 and HS2 showed more POD activities under stress (40 % FC) than those grown in normal conditions (80 % FC). Zahour and Rizki from seeds HS1 presented the highest POD activities of 22.48 and 18.96 Unit g^{-1} FM under 40 % FC, while Farihane presented the lowest value of 13.48 Units g^{-1} FM under the same conditions. According to statistical analysis, no significant ($p>0.05$) variation has been noted between the two PEG treatments. Rizki and Farihane from HS2 seeds presented the highest and lowest activities of 17.69 and 6.35 Units g^{-1} FM respectively in comparison to their respective controls.

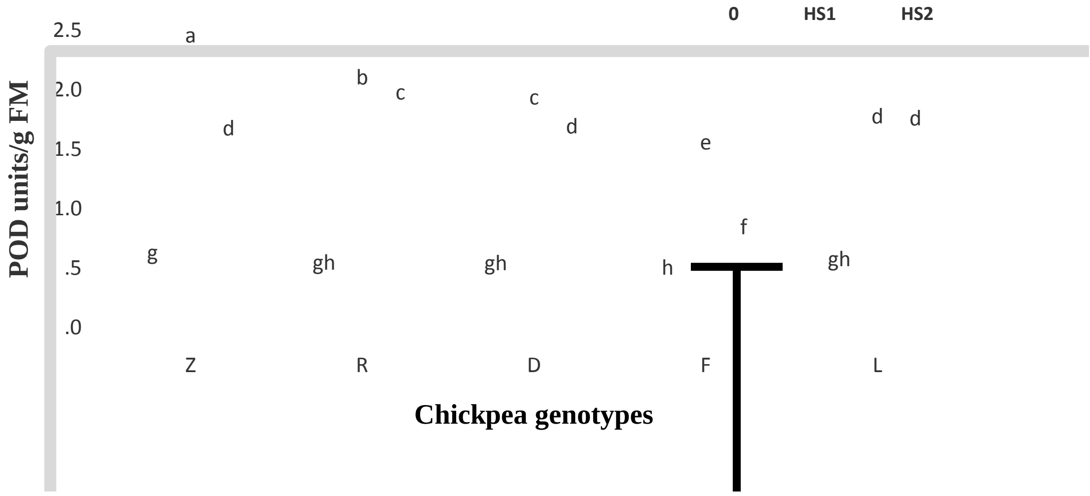

Figure 1. Levels of peroxidase activities (POD) in five genotypes of chickpea (Rizki. Zahour. Douyet. Farihane and Mazouzia). Values are means of three replicates and bars represent standard errors.

We observed a strong correlation between the POD contents and nodules number (r=0.864**). So, under water deficit, we noticed that the increases of the antioxidant enzymes activities such as POD and PPO contribute to the decrease of the reactive oxygen forms such as the H_2O_2. These changes include macromolecular synthesis, enzymes activities, increase in germination power and vigour [31]. Results showed a significant (p<0.001, Table 4) increase in PPO activity in all of the studied varieties with significant variation in their behavior (Fig. 2). Plants from HS1 seed showed more significant increases of the PPO activities under stress (40 % FC) than those from HS2 seed. Zahour from HS1 seeds presented the highest activity with 191 Units g^{-1} FM followed by Rizki with 187.5 Units g^{-1} FM, while Farihane from HS1 showed the lowest value 154.5 Units g^{-1} FM. Hence, the majority of plants from HS2 seeds presented PPO activities higher than 175.35 Units g^{-1} FM. We observed a strong correlation between the PPO contents and nodules number (r=0.795**). The synthesis of the phenolic compounds was activated in plants in response to biotic and abiotic stresses [32].

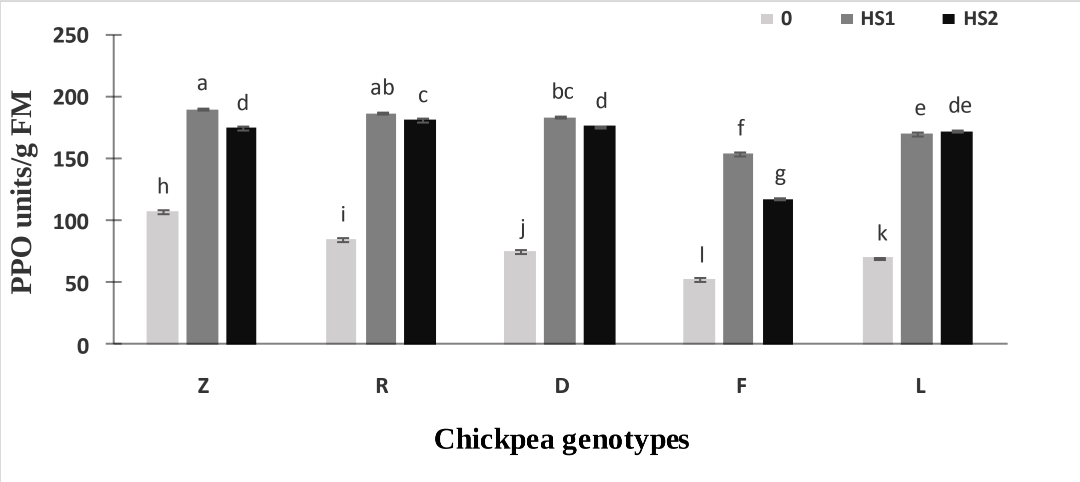

Figure 2. Levels of polyphenols oxidase activities (PPO) in five genotypes of chickpea (Rizki. Zahour. Douyet. Farihane and Mazouzia). Values are means of three replicates and bars represent standard errors.

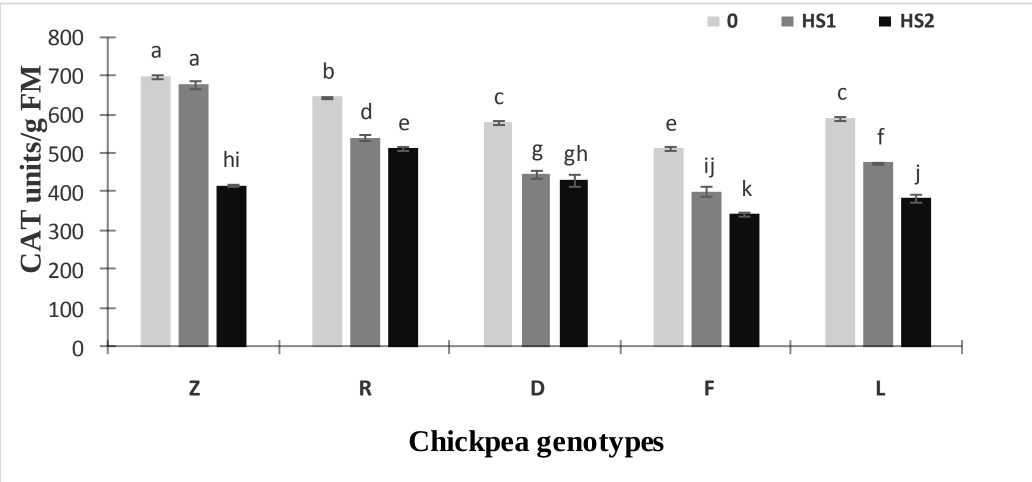

Figure 3. Levels of catalase activities (CAT) in five genotypes of chickpea (Rizki, Zahour, Douyet, Farihane, and Mazouzia). Values are means of three replicates and bars represent standard errors.

The catalase activities significantly (p<0.001, Table 4) decreased in all of the studied varieties under water deficit compared with their respective controls with significant variation between them (Fig. 3). the reductions were more

pronounce in plants from seeds HS2 then in those from seeds HS1. Zahour from HS1 seeds presented the highest CAT activity of 681 Units g^{-1} FM followed by Rizki with 544 Units g^{-1} FM under stress 40 % FC. While Rizki from HS2 seeds showed the highest activity of 516 Units g^{-1} FM. The water deficit perturbs the metabolic processes of the mitochondria and chloroplasts and leads to an overproduction of reactive oxygen species [33]. As a consequence, plants improved their anti-oxidative defense system including POD and CAT enzymes. In addition, CAT has one of the important enzymes in the removal of H_2O_2 generated in peroxisomes by oxidases involved in b-oxidation of fatty acids, photorespiration and purine catabolism and most turnover rates for all enzymes: one molecule of CAT can convert 6 million molecules of H_2O_2 to H_2O and O2 per minute [34, 35, and 36]. The result showed a strong correlation between drought and POD (r=0.637**), PPO (r=0.737**) and CAT (r=0.714**).

3.3. Effect on Lipid peroxidation (MDA content)

The results showed significant (p<0.001, Table 4) increase of the MDA content under stress (40 % FC) in plants from HS1 in comparison to the controls, while no significant variation has been noted for those from HS2 (Fig. 4). Rizki and Douyet from HS2 seeds showed the lowest MDA value of 0.150 mg g^{-1} FM under water deficit. While Farihane presented the highest values of 0.93 mg g^{-1} FM. For the plants from HS1 seeds, the MDA contents were much higher than those from HS2 seeds. Zahour showed the lowest value of 0.187 mg g^{-1} FM, while Farihane and Mazouzia sowed the highest MDA contents of 0.26 and 0.23 mg g^{-1} FM respectively. The effect of the water deficit on the oxidative metabolism in the chickpea showed that the stress treatment affected the cell membrane stability reflected by the increase of the MDA contents in these tissues, so the most resistant varieties accumulated less MDA content such as the varieties Zahour and Rizki with only (0.15 mg g^{-1} FW, Fig. 4).

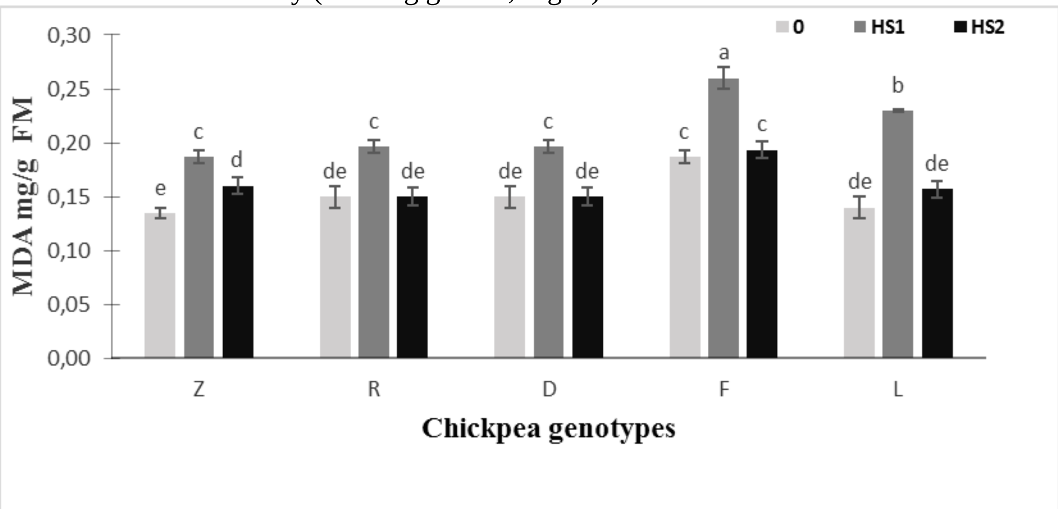

Figure 4. Levels of Malonyldialdehyde (MDA) in five chickpea genotypes (Rizki. Zahour. Douyet. Farihane and Mazouzia). Values are means of three replicates and bars represent standard errors.

Table 4. Results of variance (ANOVA II) of PEG effects and their interactions for the considered parameters.

	dF	POD	PPO	Catalase	MDA
Varieties	4	379.175***	1964.84***	660.89***	62.02***
PEG	2	5624.65***	25820.67***	1874.9***	252.65***
Varieties*PEG	8	135.84***	190.28***	110.27***	6.84***

**: Significance at 0.05 probability level;* ***: Significance at 0.01l;* ****: Significance at 0.001; NS at 0.05.*

The increase of MDA contents was higher in Farihane variety, which presented more disintegrate membranes under stress conditions followed by Mazouzia. We observe a positive correlation between the MDA products and the growth rates (r=0.380*). A low concentration in MDA showed high anti-oxidative capacity, reflecting high tolerance to the abiotic stress [37].

4. Conclusion

Based on our results, water deficit negatively affected the germination percentage in all of the studied varieties. The vigour of germinated seeds under stress was more important. After two months, plants from HS1 seeds showed an improvement in the vigour, growth, nodulation and antioxidant activities when grown under 40 % FC water deficit. In contrast, plants from HS2 seeds showed low MDA contents. Zahour and Rizki from HS1 seeds showed the highest vigour, growth, nodulation, POD, CAT and PPO activities and qualified as more drought tolerant, while Farihane variety showed the most significant reductions in growth germination and vigour. It presented the lowest antioxidant activities and thus was qualified as les drought tolerant.

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