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Role of acid phosphatase and enzymatic and non-enzymatic antioxidant systems in tolerance of alfalfa (*Medicago sativa* L.) populations to low phosphorus availability

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Abstract. This study aims at evaluating the tolerance of four alfalfa (*Medicgao sativa* L.) populations to low phosphorus (P) in rooting medium. The experiment was carried out under controlled conditions. The seedlings of 15 old days were subjected to P deficiency using Ca_3HPO_4, insoluble form and P sufficiency using KH_2PO_4, as soluble form, at a final concentration of 250 µmol P plant^{-1}. week^{-1}. After 60 days P deficit, several agro–physiological and biochemical traits were measured and determined in both conditions. The obtained results indicated that the P–starvation significantly ($P < 0.001$) reduced the agroeconomic traits evaluated such as plant dry weight and leaf area. The root and shoot P contents were found ($P < 0.001$) decreased by low–P availability in the rooting medium. This constraint induced significant ($P < 0.001$) increase in phosphatase acid activity and caused lipid peroxidation and oxidative damage to cells, evaluated through malondialdehyde and hydrogen peroxide contents. Our results showed also, that low P availability significantly ($P < 0.001$) increased the enzymatic antioxidant responses reflected by the activities of superoxide dismutase (SOD), guaiacol peroxidase and catalase. The non-enzymatic antioxidant molecule such as proline and total polyphenols were found significantly increased in alfalfa stressed plants. The behavior of alfalfa populations tested was significantly different ($P > 0.05$). The *OL* population was found to be the least affected and the *DEM* was most sensitive one, whereas the populations *TATA* and *RICH* showed a moderate tolerance. Our study advises that the tolerance of Moroccan alfalfa populations to low P–availability was associated with increased acid phosphate activity and ability to induce enzymatic and non-enzymatic antioxidant responses leading to cell detoxification from reactive oxygen species (ROS).

Key words: alfalfa, acid phosphatase, antioxidant activity, oxidative stress, phosphorus, ROS.

INTRODUCTION

Legumes are an important source of proteins for both humans and animal livestock. They contribute to the incorporation of nitrogen in agro–pastoral ecosystems with valuable economic influence, to reduce or limit expansive and eco–unfriendly chemical fertilizers (Farssi et al., 2021; Lazali et al., 2021). Legumes are also used in intercropping or rotation with many plant species because of their multiple beneficial functions (Mouradi et al., 2018). Indeed, when cultivated in intercropping, legumes showed multiple advantages such as biological control, nutrient cycling, and increase in total yield (Martins da Costa et al., 2018; Mouradi et al., 2018; Oukaltouma et al., 2021). Therefore, exploiting the N_2–fixing ability of legumes could be a good way to improve the growth and productivity in legume–cereal intercropping system.

However, Abiotic constraints accentuated in recent decades by climate change are the primary cause of crop losses in the Mediterranean region. In fact, the richness of the genetic heritage of many leguminous species including alfalfa (*Medicago sativa* L.) is threatened by several abiotic factors, such as prolonged periods of drought, soil and irrigation water salinity, and soil P deficiencies (Farissi et al., 2011; Bargaz et al., 2013b; Farissi et al., 2013) In fact, previous investigation on legumes showed that phosphorus limitations leading to an obvious reduction in different plant aspects including growth, photosynthesis, and nutrient use efficiency (Farssi et al., 2021; Lazali et al., 2021). In the same way, low P availability negatively affected P nutrition and atmospheric nitrogen fixation in forage and grain legumes, and importantly the effect was more aggravated under the combined effect of P and osmotic stresses (Oukaltouma et al., 2021). The culture of legumes is therefore concentrated in the northern regions with a favorable climate. Because of the climate change conditions, it is perceived that plant production globally, including legumes, is seriously threatened and the sensitivity of legumes to several abiotic stresses is a major obstacle to obtaining large quantity and quality products.

Low P availability, particularly in N_2–fixing symbiosis, has a significant effect on legumes' yield (Lazali & Drevon, 2018). Indeed, this symbiosis poses supplementary P demand (20% of total to nodules) and any P starvation may affect the activity of rhizobia and consequently the symbiosis efficacy (Drevon, 2017). However, soluble P enhances the growth of *Vigna unguiculata* L. plants, their total P content and nodulation (Benlahrech et al., 2018). In the same sense, Bekel et al. (2019) reported that the P fertilizers significantly influenced both total and effective number of nodules in *Arachis hypogaea* L. plants. Based on the positive correlations between acid phosphatase activity and P use efficiency, it would be worthwhile to take into account the role of acid phosphatases in building an effective legume-rhizobia symbiosis as a possible mechanism in P-deficiency tolerance (Lazali et al., 2021). In fact, Tran et al. (2010) reported that under low-P availability, acid phosphatases are thought to be crucial for the metabolism of organic P in both intracellular and extracellular plant tissues. It has been suggested that secreted or cell wall-associated acid phosphatases recycle Pi from endogenous phosphomonoesters that have leaked from the cytoplasm across the plasma membrane or scavenge Pi from organic-P compounds found in the rhizosphere (Shane et al., 2014).

Furthermore, the reactivity of P with some soil cations, such as iron, aluminum, and calcium, which results in the formation of insoluble compounds, limits its mobility in soil solutions. These interactions result in reduced P availability and low phosphate fertilizer efficiency in plants. As a result, the SNF process, root growth, photosynthesis, rhizobia proliferation, and nodule development are all limited (Neila et al., 2014; Boudanga et al., 2015).

In recent years, the most important technique for reducing the effects of environmental restrictions on legume production has been to select plant genotypes tolerating to abiotic stress (Latrach et al., 2014). In fact, the exploitation of the genetic diversity existing in local germplasm constitutes a promising approach to enhance plant productivity under unfavorable conditions. In this context, our study aims at evaluating the tolerance to low P availability in four Moroccan alfalfa (*Medicago sativa* L.) populations. The emphasis was on agro–physiological and biochemical properties related to the tolerance to this environmental constraint. The role of phosphatase and enzymatic and non-enzymatic antioxidant system were focused.

MATERIALS AND METHODS

Plant material and growth conditions

The biological material used in this study consists of four Moroccan alfalfa (*Medicago sativa* L.) populations; *OUED LMALEH*, *DEMNATE*, *TATA* and *RICH*. Seeds were supplied by The National Institute for Agronomic Research, Morocco. Local populations of alfalfa are commonly used in the Moroccan traditional agroecosystems, oasis and mountain, and powerfully involved in the socio-economic development chain of local families as the nutrition source for their livestock. They have been cultivated for many centuries and are still extensively used by farmers in these traditional agroecosystems. Continuous natural and human selection has led, by this time, to their adaptation to the local habitats with distinction in the agro–morphological traits of the landraces, which have reached Hardy–Weinberg equilibrium (Farissi et al., 2013). The seeds were germinated in 20 cm diameter and 15 cm height plastic pots containing sterilized perlite as a substrate. The experiment was conducted in a growth chamber at 28 ± 2 °C day/night, 60% – 80% relative humidity, and a photoperiod of 16 h. After 15 days of sowing, the young seedlings were irrigated by capillarity with a nutrient solution with two P forms, insoluble P using Ca_3HPO_4s (limited available P) *versus* soluble (KH_2PO_4). Both soluble and insoluble P forms were adjusted to reach 250 µmol P plant^{-1} week^{-1}. The composition of the nutrient solution (Hoagland & Arnon, 1950) used was as follows: [KNO_3 (600 µmol), $MgSO_4$ (1,000 µmol), K_2SO_4 (750 µmol), $CaCl_2$ (1,650 µmol), Fe-ethylenediaminetetraacetic acid (EDTA) (16 µmol), MnSO4 (6 µmol), H_3BO_3 (4 µmol), $ZnSO_4$ (1 µmol), $NaMoO_4$ (0.1 µmol), and $CuSO_4$ (1 µmol)]. By using 0.1 M HCl or 0.1 M NaOH, the pH of the nutrient solution was, respectively, reduced or raised to reach 7 before use. After 60 days of P stress, the plants were collected, measured, and several agro-physiological and biochemical traits were analyzed prevailing plant growth and development. Each pot was planted with five plants and each treatment was represented by three replicates, resulting in a total of 24 pots and 120 plants plants.

Plant biomass and leaf area

The shoot and root fresh weights (FW) were determined immediately after harvest. The dry weight (DW) was then measured using precision balance after their drying at 80 °C for 48 h. The leaf area was estimated using MESURIM software (version 3.4.4.0) using a digital scanner.

Phosphorus contents

The P contents were determined using 0.5 g of the dry matter of each plant parts after incineration at 600 °C for 6 h. The ash obtained was treated in 3 mL of HCl (10 N) and filtered. Then, the P concentration were measured using the molybdate blue colorimetric assay (Murphy & Riley, 1962). After color development at 100 °C for 10 minutes, the optical density was measured at 820 nm. A standard curve was established with KH_2PO_4 solutions.

Acid phosphatase activitiy

Samples of fresh matter (50 mg) were ground in mortar using 500 µL of sodium acetate extraction buffer (0.1 M, pH 5.5), 2.5% polyvinylpyrrolidone (PVP) and 5 µL of β mercaptoethanol. The homogenates were centrifuged at 12,000 × g for 30 min at 4 °C. The acid phosphatase activity was measured using 100 µL of enzymatic extract mixed with 200 µL of p–NPP (*p*–nitrophenyl phosphate) and incubated for 30 min at 37 °C. Then, 1 mL of 1N NaOH was added to stop the reaction. The acid phosphatase activity (µmol *p*–NPP min^{-1} mg^{-1} protein) was measured by a spectrophotometer at 410 nm wavelength (Araújo et al., 2008).

Protein content in all enzyme preparations was determined using bovine serum albumin (BSA) as standard (Bradford, 1976).

Oxidative stress markers and membrane cell intergity assessments

The lipid peroxidation was estimated by malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) accumulation. MDA was measured using 50 mg of fresh leaves added to 2 mL of 0.1% trichloroacetic acid (TCA). After centrifugation at 14.000 rpm for 15 min, 1 mL of supernatant was added to 2.5 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was placed at 95 °C for 30 min and cooled down by ice. The absorbance was measured at 532 and 600 nm. The MDA content was calculated by the extinction coefficient ε = 155 mM^{-1} cm^{-1} (Savicka & Škute, 2010).

The H_2O_2 content was determined as described by Velikova et al. (2000) 100 mg of fresh leaves were mixed with 5 mL of 0.1% TCA. After centrifuged at 12,000 × g for 15 min, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The absorbance was measured at 390 nm. The H_2O_2 content was expressed as µmol $H_2O_2.g^{-1}$ FW.

For the electrolyte leakage (EL), 0.1 g of young leaflets were washed three times with distillated water to eliminate surface–adhered electrolytes then placed in closed flasks filled with 10 mL of distillated water. The flasks were after that incubated for 24 h at 25 °C on a rotary shaker. The initial electrical conductivity (Li) was measured by using a conductivity meter. Then the samples were autoclaved at 120 °C for 20 min. The final electrical conductivity (Lf) was measured after 25 °C equilibration, The percentage of EL was calculated as follow (Lutts et al., 1996):

$$EL\ (\%) = (Li\ /Lf) \times 100$$

Enzymatic antioxidant actvities

100 mg of fresh leaves were crushed in 1 mL of phosphate buffer (20 mM, pH 7). After centrifugation at 15,000 × g for 20 min at 4 °C, the supernatant was used for the determination of the POD (EC 1.11.1.7) enzymatic activity according to (Beyer & Fridovich, 1987). The reaction mixture consisted of 200 µL of H_2O_2 at 0.3%, 300 µL of guaiacol at 20 mM, 2 mL of phosphate buffer (0.1 M, pH 6), 1 mL of distilled water and 10 µL of enzymatic extract. The POD activity was measured after 3 min, at 470 nm. The activity was calculated using the guaiacol extinction coefficient 26.6 mM^{-1} cm^{-1}, and expressed as µmol H_2O_2 min^{-1} mg^{-1} proteins.

SOD (EC 1.15.1.1) activity was determined as described (Chagas et al., 2008). 50 µL of crude enzymatic extract in phosphate buffer (20 mM, pH 7) was added to a solution containing 13 mM L–methionine, 75 µM *p*–nitro blue tetrazolium chloride (NBT), 100 µM EDTA and 2 µM riboflavin in a 50 mM potassium phosphate buffer (pH 7.8). The reaction was performed in assay tubes upon illumination using a 30 W fluorescent lamp at 25 °C for 15 min. The blue formazan produced by NBT photoreduction was spectrophotometrically measured at 620 nm. An enzyme unit was equal to the amount to inhibit 50% of NBT. SOD activity was expressed as enzymatic U min^{-1} mg^{-1} proteins.

CAT (EC 1.11.1.6) activity was determined using 250 µL of the extract was added to 2 mL of the assay mixture (50 mM Tris–HCl buffer pH 6.8, containing 5 mM H_2O_2) (Gong et al., 2001). Then after 10 min at 20°C, 250 µL of 20% titanic tetrachloride (v/v, in concentrated HCl) were added to stop the reaction. CAT activity was read at 415 nm and calculated by comparing the absorbance against a standard curve of 0.25 to 2.5 mM) H_2O_2. CAT activity was expressed as µmol H_2O_2 min^{-1} mg^{-1} proteins.

Protein content in all enzyme preparations was determined using BSA as standard (Bradford, 1976).

Non-enzymatic antioxidant molecules

Non-enzymatic antioxidant molecules were evaluated through proline and total polypenol contents. The proline content was determined using extract from 100 mg FW with 2 mL of 40% methanol: water. Afer incubation at 85 °C for 30 min, 1 mL extract was mixed with 1 mL of a mixture of glacial acetic acid andorthophosphoric acid at 6M (3: 2; v/v) and 25 mg ninhydrin. After 1 h incubation at 100°C, the tubes were cooled and 5 mL toluene was added. The optical density of the upper phase was measured at 528 nm. The proline content was determined using a standard curve obtained using reference proline solutions (Bates et al., 1973).

For total polyphenol content, 100 mg of fresh samples were grinded in 1 mL of methanol (80%). After centrifugation at 12,000 × g for 20 min at 4 °C, the supernatants were recuperated. The content of total polyphenols was determined through the Folin–Ciocalteu method and their concentration was described as mg gallic acid equivalents (GA) g^{-1} FW (Singleton & Rossi, 1965).

Statistical analysis

Statistical analysis was executed using SPSS version 22. A two-way analysis of variance (ANOVA) was adopted. Means comparison was performed using Tukey's test. XLSTAT software version 2014 (Addinsoft, Paris, France) was used to determine the correlations.

RESULTS

Effect on plant biomass and leaf area

The starvation of P availability in rooting medium caused significant ($P < 0.001$) reductions in plant shoot and root dry weights. The lowest reductions were noted in *OL* populations in comparison with other populations which showed the same behavior according to Tukey's test ($P > 0.05$). In fact, under low P availability, the shoot dry weight recorded in *OL* was 32.80 mg plant^{-1} with a reduction of 28.90% comparatively to *OL* plants grown under P-sufficient conditions. However, the remaining populations, *DEM*, *TATA*, *RICH* showed the reductions of 52.80, 41.09 and 39.96% respectively for the same growth trait (Fig. 1, A). Regarding, the root dry weights (Fig. 1, B), the effect was more pronounced ($P > 0.05$) in *DEM* population with reductions of 41.44% relative to control. *OL* was found to be the least affected one ($P < 0.05$) with the reduction percentages did note exceed 19.39%. However, *TATA* and *RICH* showed an intermediate behavior.

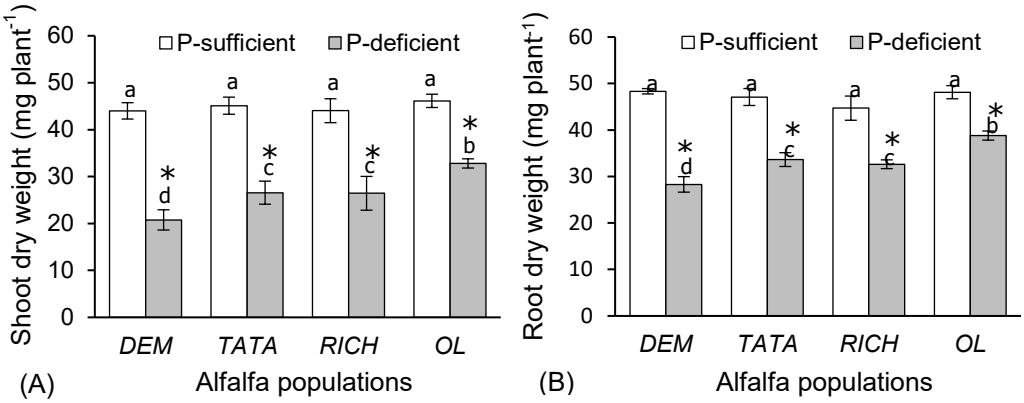

Figure 1. Shoot (A) and root (B) dry weights in alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

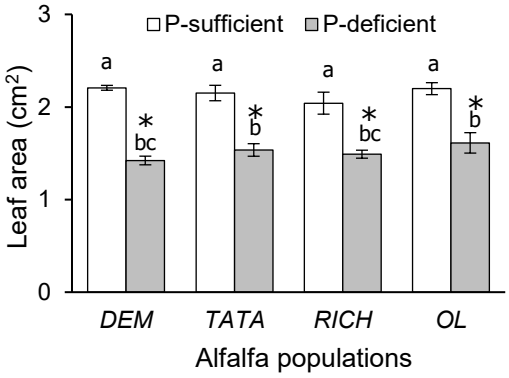

Figure 2. Leaf area of alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

For leaf area (Fig. 2), the significant ($P < 0.001$) reductions were caused by the low P availability in rooting medium. OL population maintained the highest leaf area value

(1.61 cm^2), but with no significant differences ($P > 0.05$) in comparison with the remaining populations according to Tukey's statistical test.

Phosphorus contents

The Fig. 3 illustrates the shoot and root P concentration under the stressed and non-stressed conditions. The obtained results mentioned that the highest P contents of 238 and 333 mg g DW^{-1} were noted in shoots and roots of *OL* population respectively under P stress, followed by *TATA* and *RICH*, whereas the lowest P contents of 105 and 179 mg g DW^{-1} were noted in *DEM* population under the same conditions.

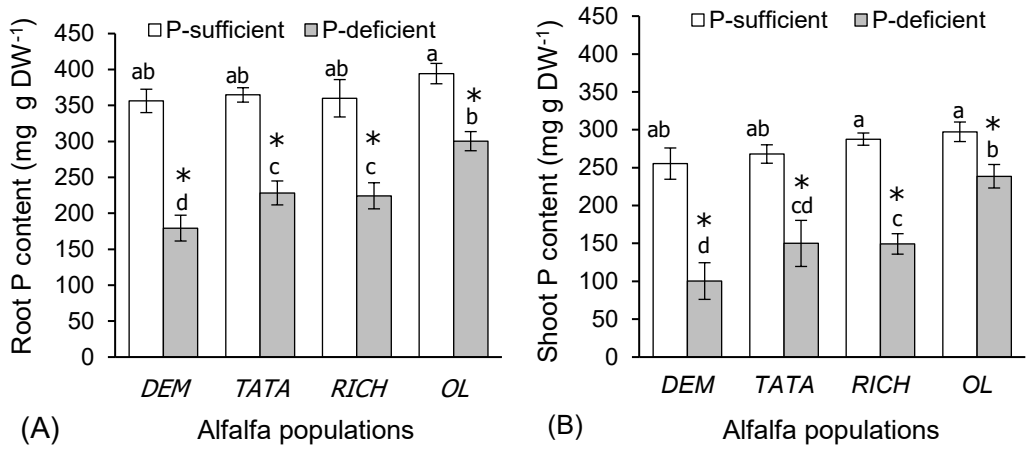

Figure 3. P contents in roots (A) and shoots (B) of alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

Phosphatase acid activity

Low P availability induced significant ($P < 0.001$) increase in phosphatase acid activity in alfalfa roots. The highest activity ($P < 0.05$) was noted in *OL* population (19.85 µmol *p*–NPP min^{-1} mg^{-1} proteins). Nevertheless, the activities not exceeding 15.72 µmol *p*–NPP min^{-1} mg^{-1} proteins were recorded in *TATA* and *RICH* populations with non-significant differences ($P > 0.05$) between theme, according to the statistical grouping test considered. However, the lowest ($P < 0.05$) acid phosphatase specific activity was noted in *DEM* population 12.68 µmol *p*–NPP min^{-1} mg^{-1} proteins.

Effect on membrane cell integrity and oxidative stress markers

The EL contents reflecting the cell membrane integrity were found increased ($P < 0.001$) under low P availability (Table 1). The cell membrane damages were most prominent in the *DEM*, *TATA* and *RICH* populations with percentages of 22.98, 21.27 and 23.32% However, the lowest EL contents ($P < 0.05$) were noted in *OL* population 18.07%.

Table 1. EL, MDA and H_2O_2 contents in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test

Alfalfa population	EL (%)		MDA (µmol g FW^{-1})		H_2O_2 (µmol g FW^{-1})	
	P-sufficient	*P-deficient*	*P-sufficient*	*P-deficient*	*P-sufficient*	*P-deficient*
DEM	10.22 ± 0.94*a*	22.98 ± 0.97*a*	13.32 ± 0.56*a*	48.72 ± 2.74*a*	0.45 ± 0.035*a*	1.19 ± 0.072*a*
TATA	09.19 ± 1.13*a*	21.27 ± 1.67*a*	15.04 ± 1.53*a*	47.45 ± 1.75*a*	0.29 ± 0.042bc	0.99 ± 0.051*b*
RICH	08.47 ± 1.21*a*	23.32 ± 1.73*a*	16.21 ± 0.82*a*	41.28 ± 3.11*b*	0.43 ± 0.028*a*	1.03 ± 0.061*b*
OL	11.33 ± 1.73*a*	18.07 ± 0.69*b*	17.93 ± 1.23*a*	39.11 ± 0.73*bc*	0.33 ± 0.063 bc	0.88 ± 0.073*c*

Table 2. Superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT) specific enzymatic activities in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test

Alfalfa population	SOD U min^{-1} mg^{-1} proteins		POD µmol H_2O_2 min^{-1} mg^{-1} proteins		CAT µmol H_2O_2 min^{-1} mg^{-1} proteins	
	P-sufficient	*P-deficient*	*P-sufficient*	*P-deficient*	*P-sufficient*	*P-deficient*
DEM	21.08 ± 1.17*a*	32.11 ± 1.17*c*	9.32 ± 1.16*a*	20.63 ± 0.74*d*	14.25 ± 1.03*a*	32.19 ± 2.72*c*
TATA	18.13 ± 1.55*ab*	42.23 ± 1.55*b*	10.04 ± 1.33*a*	22.55 ± 1.75bc	15.08 ± 1.42a	36.99 ± 2.05*b*
RICH	23.32 ± 1.98*a*	39.09 ± 1.98*b*	12.21 ± 1.92*a*	23.21 ± 2.11*b*	14.43 ± 1.28*a*	37.03 ± 3.61*b*
OL	19.07 ± 1.62*ab*	48.16 ± 1.62*a*	8.93 ± 1.43*a*	26.19 ± 0.73*a*	13.33 ± 1.63*ab*	44.88 ± 1.73*a*

The oxidative markers, MDA and H_2O_2, were found significantly ($P < 0.001$) accumulated under low P availability in alfalfa stressed plants (Table 1). The significant accumulations were observed in *DEM* pollution followed by *TATA* and *RICH* populations. The contents ranged from 41.28 to 48.72 g FW^{-1} for MDA and from 0.99 to 1.19 g FW^{-1} for H_2O_2. Nevertheless, the *OL* population was significantly ($P > 0.05$) revealed to be the least affected one according to Tukey's test with the values of 39.11 µmol g FW^{-1} and 0.88 µmol g FW^{-1} for MDA and H_2O_2 respectively.

Low P availability induced significant ($P < 0.001$) increase of the enzymatic antioxidant activities in P–stressed alfalfa plants (Table 2). The highest activities were noted in OL population. The values recorded were 48.16 U min^{-1} mg^{-1} proteins, 26.19 µmol H_2O_2 min^{-1} mg^{-1} proteins and 44.88 µmol H_2O_2 min^{-1} mg^{-1} proteins respectively for SOD, POD and CAT. However, the lowest values were observed in DEM population (32.11 U min^{-1} $^{mg-1}$ proteins, 20.63 µmol H_2O_2 min^{-1} mg^{-1} proteins and 32.19 µmol H_2O_2 min^{-1} mg^{-1} proteins for the mentioned enzymes respectively). Whereas, intermediate values were noted for the remaining alfalfa populations.

Effect on non-enzyamtic antioxidant molécules:

Starvation of P availability in rooting medium significantly ($P < 0.01$) increased the non-enzymatic antioxidant molecules in alfalfa plants (Table 3). The lowest proline and total polyphenol contents were noted in *DEM* population, 314 µg g^{-1} FW and 57.63 mg GA g^{-1} FW respectively. However, OL population accumulated the highest contents 534 µg g^{-1} FW and 77.19 mg GA g^{-1} FW in comparison with the other alfalfa populations ($P > 0.05$).

Table 3. Proline and total polyphenol contents in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

Alfalfa population	Proline, µg g^{-1} FW		Total polyphenols, mg GA g^{-1} FW	
	P-sufficient	*P-deficient*	*P-sufficient*	*P-deficient*
DEM	232 ± 23*b*	314 ± 23*c*	32.24 ± 1.16*b*	57.63 ± 2.54*d*
TATA	245 ± 21*b*	413 ± 15*b*	38.17 ± 1.33*ab*	61.55 ± 2.25*c*
RICH	276 ± 34*ab*	402 ± 21*b*	36.09 ± 1.92*ab*	65.21 ± 3.12*b*
OL	272 ± 64*ab*	534 ± 19*a*	32.11 ± 1.43*b*	77.19 ± 2.18*a*

DISCUSSION

Phosphorus (P) is a very important nutrient required for optimum crop production (Ibrahim et al., 2021). It involved in many physiological and biochemical process governing plant growth and development. The high reactivity of P with some cations such as iron, aluminum, and calcium, to form insoluble compounds, reduces its mobility in the soil solution. These reactions caused a very low–P availability and low efficiency of phosphate fertilizers used by plants (Farssi et al., 2021). As a consequence, the limitation of the growth plant and development. In fact, we report in the present study that the low-P availability in rooting medium significantly reduced the plant biomass and leaf area (Fig. 1 and Fig. 2). The comportment of the alfalfa populations in this study was significantly different. The OL population was found the least affected and the DEM

population was the most sensitive one, whereas the two remaining alfalfa populations were found to be moderately affected. The effect of low–P availability was documented in many species. Indeed, the reduction in plant tillering and biomass barley (*Hordeum vulgare* 'Quench') was observed under P deficiency conditions (Carstensen et al., 2018). Same results were noted in leguminous species such as *Vicia faba* L. (Bargaz et al., 2012). The reduction in plant growth under low P availability was associated with the plant P contents. In fact, the lowest P contents were noted in the most sensitive population (Fig. 2). In fact, we observed significant and positive correlation between shoot biomass and their P contents ($r = 0.94$) and between root biomass their P contents ($r = 0.96$). Also, positive correlations were noted for leaf area and the contents of shoots and roots in P, $r = 0.87$ and $r = 0.93$, respectively.

In response to P deficiency, plants have several morphological, physiological, biochemical, and molecular adjustments to improve their P uptake (Plaxton & Tran, 2011; Farssi et al., 2021). In our study, the low P availability induced acid phosphatase activity in alfalfa stressed plants (Fig. 4). The activity was more pronounced in the least affected alfalfa population. Significant and negative correlations were noted for the alfalfa plant biomass and phosphatase activity ($r = -0.66$). The induction of phosphatase activity under low–P availability was documented in many species including *Medicoga sativa* L. In fact, under low soil P supply alfalfa roots released more phosphatases and carboxylates, principally tartrate, into the rhizosphere (He et al., 2020). Similarly, acid phosphatase activity of cell wall in leaves and roots of low-P tolerant stylo (Stylosanthes) mutant, TPRC2001-84, were 46.6% and53.6% higher than in non-mutant control (RY2) under P deficiency (Liu et al., 2018). The increase of activity in the mutant may contribute to increasing P use efficacy under P stress by cell wall P scavenging and recycling (Liu et al., 2018). An increase in this activity was reported in *Vicia faba* L. under low–P availability (Makoudi et al., 2018).

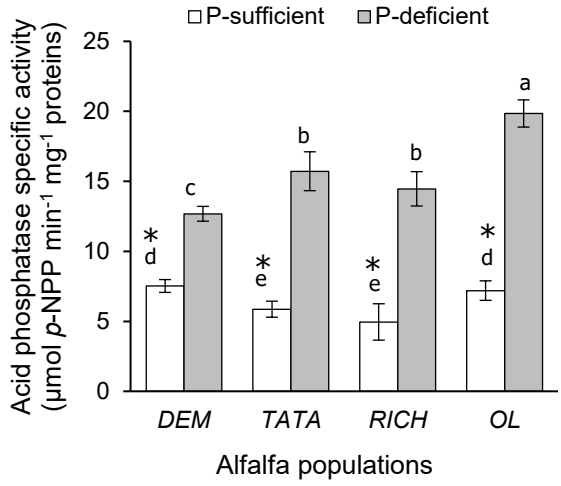

Figure 4. Acide phosphatase specific activity in alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $P < 0.001$. Different and same small letters above histograms indicate significant ($P < 0.05$) and no significant differences ($P > 0.05$), respectively, between the means according to Tukey's test.

P is an essential in ATP, NADPH, nucleic acids, sugar phosphates, and phospholipids. These compounds have possessive role in cell membrane composition and integrity. Our findings indicate that low–P availability affects the membrane cell integrity and induced oxidative stress evaluated by the accumulation of EL, MDA and H_2O_2 (Table 1). The lowest contents ($P < 0.05$) were noted in the most tolerant

population (OL), whereas the highest contents were noted in the most sensitive population (DEM). The generation of reactive oxygen species (ROS) is considered because of plant exposure to many stresses including P deficit (Mouradi et al., 2018a; 2018b). In the lack of effective protective mechanism, ROS can seriously damage plants by lipid peroxidation, protein degradation, breakage of DNA and programmed cell death (Bargaz et al., 2013a). To overcome with the increased ROS level, plants developed enzymatic and non-enzymatic antioxidant process leading to cell detoxification from ROS. The antioxidant systems involve SOD, POD, and CAT. In our study, significant increases in the activities of these enzymes were noted (Table 2). The tolerance of alfalfa populations tested to low–P availability is positively correlated with the induction of antioxidant enzymatic activity. Similar results were reported in *Phaseolus vulgaris* L. (Bargaz et al., 2013a) and in *Brassica napus* L. (Chen et al., 2015). SOD changes superoxide (O_2^-) into H_2O_2, POD and converts H_2O_2 to H_2O and O_2 using electron donors (Kapoor et al., 2019). In the same sense, our findings demonstrated that the P deficiency increase the non-enzymatic antioxidant molecules. The proline and total polyphenol contents were more accumulated in the least sensitive population (OL). Proline actions in plants may resume in chelation of metals, antioxidant ability and/or a signaling roles (Hayat et al., 2012). The proline accumulation can activate stress–responsive genes coding for other antioxidant compounds (El Moukhtari et al., 2021). Silva et al. (2018) reported that proline induces also the production of phenolic compounds in transgenic tobacco subjected to water deficit. This finding agrees with our results. In fact, the accumulation of the proline in alfalfa stressed plants was positively correlated with the total polyphenol contents. Previous findings on polyphenol accumulation were noted in other legume species such as common bean (Bargaz et al., 2013a). This increase suggests a possible role that phenols could play in during P–deficiency. Indeed, the accumulation of phenolic compounds has important antioxidant properties in protecting membranes by neutralizing lipid radicals (Takahama & Oniki, 2000). The statement we noted agrees with previous findings on proline and polyphenols that are triggered in alfalfa in response to abiotic stress such as salinity (El Moukhtari et al., 2020; 2021)

CONCLUSIONS

The present study suggests significant variability in Moroccan alfalfa populations against low–P availability. The OL population was found to be the least affected population and the DEM was the most sensitive one. However, TATA and RICH displayed a moderate tolerance to P–deficiency conditions. The alfalfa P stress tolerance was linked to the induction of acid phosphatase activity, the enhancement of P solubilization and uptake, the maintain of cell membrane integrity and the induction of non-enzymatic and enzymatic antioxidant responses against the accumulation of ROS.

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