LOW PHOSPHORUS AVAILABILITY DISTURBS GROWTH AND PHOTOSYNTHETIC-RELATED PARAMETERS AND INDUCED OXIDATIVE STRESS IN CHICKPEA (*CICER ARIETINUM* L.)

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Abstract. This study aims at evaluating the effect of low phosphorus (P) availability on growth, photosynthesis, P nutrition, cell membrane stability and antioxidant enzyme activities in two Moroccan chickpea (*Cicer arietinum* L.) varieties, *Bouchra* and *Moubarak*. The experiment was conducted in a growth chamber at 25 ± 1 °C with 16 h photoperiod. P-deficiency was applied for six weeks by watering plant weakly with 1 L of Hoagland nutrient solution containing 0.01 mM P in the form of KH_2PO_4 against 1 mM P as control. The obtained results showed that P-deficiency significantly ($p < 0.05$) reduced the studied agronomic parameters in terms of plant biomass, shoot to root ratio, plant height, number of stems and leaves and leaf area. Furthermore, P-deficiency also decreased ($p < 0.05$) the focused photosynthesis-related parameters, such as total chlorophyll and carotenoid contents, the maximum quantum yield of photosystem II (F_v/F_m) and OJIP fluorescence transients. Moreover, P-starvation significantly ($p < 0.05$) induced an oxidative stress reflected by high contents of malonyldialdehyde and hydrogen peroxide and high electrolyte leakage. The increased oxidative stress was correlated with a significant ($p < 0.05$) increase in the antioxidant activity of superoxide dismutase and peroxidase. Regarding P content, P-deficiency stress markedly reduced P content in both chickpea varieties. Taking all together, P-deficiency induced oxidative stress and reduced photosynthesis efficiency resulted in plant growth reduction in a variety-dependent manner. Indeed, *Bouchra* was found to be the more tolerant to low-P availability compared to *Moubarak*.

Keywords: *maximum quantum yield of photosystem II, phosphorus deficiency, phosphorus use efficiency, membrane stability, antioxidant activity*

Introduction

Legumes are the second most important plant family after the grass family. They represent the most valuable source of proteins for human food and animal feed. Furthermore, in developing countries, grain legumes, such as chickpea (*Cicer arietinum* L.) are vital components of local diet (Kumar et al., 2018). In fact, chickpea represents the third most produced legume regarding its amount after faba bean and pea, with approximately 14.7 million tonnes (Summo et al., 2019). In Morocco, its culture occupies more than 81 982 hectares with an annual production of 75 413 tons

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(FAOSTAT, 2019). Chickpea seeds are highly consumed because of their high-protein content (up to 28.9%). They provide vitamins (A, B1, B2, B3, B5, B6, B12, C, D, E, K and biotin) and several minerals, including copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), phosphorus (P) and calcium (Ca) (Jukanti et al., 2012; Kumar et al., 2018; Wang et al., 2010). Besides its nutritional value, chickpea can fix large amount of atmospheric nitrogen (N_2), up to 140 kg ha^{-1} $year^{-1}$, in symbiosis with many rhizobia species belonging to *Mesorhizobium* genus and in turn, it improves soil N content (Benjelloun et al., 2019). Despite these agro-environmental advantages, the yield of this crop is still decreasing because of many biotic and abiotic stressors, such as salinity, water deficit and soil nutrient deficiency, particularly P (Jha et al., 2020; Kumar et al., 2018; Toker and Mutlu, 2011).

P is the second most important nutrient that plant needs at an adequate quantity for its normal growth and development (Kleinert et al., 2017). In fact, this essential nutrient involved in several key processes, such as energy transfer, enzymatic activities, photosynthesis, respiration and carbohydrate energy metabolism (Graham and Vance, 2003; López-Arredondo et al., 2014). P has also been reported to play a crucial role in cell division and elongation, growth of new tissues and nucleic acid structure (Kavanová et al., 2006; Malhotra et al., 2018; Meng et al., 2021).

In arable soil, P concentration is typically high, up to 3000 mg P kg^{-1} soil (Bargaz et al., 2021). However, although the high amount of this nutrient element, it is mainly found in the soil in unavailable forms for plants. Consequently, P limitation in soil solution constitutes a pressing constraint for many crops. Mandri et al. (2012) reported that legumes, including chickpea, required higher P demand increases, particularly under N_2 fixing symbiosis (up to 20% of total P has posed and allocated to nodules). Their growth was widely reported to be severely affected by P-deficiency (Bargaz et al., 2015). The high reactivity of P with Fe, aluminum (Al) and Ca, to form insoluble compounds, reduces its mobility in the soil solution (López-Arredondo et al., 2014). These reactions provoke a very low-P availability and low-P fertilizer use efficiency in legumes. As a consequence, the limitation of symbiotic nitrogen fixation process, root growth, photosynthesis, translocation of sugars and other functions (Bargaz et al., 2013b; Lazali et al., 2020; Makoudi et al., 2018). P-deficiency was also reported to induce an overproduction of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) (Meng et al., 2021). If not metabolized, ROS could cause damage to cell membranes, nucleic acids and proteins (Ibrahim, 2016). Effectively, in common bean, Bargaz et al. (2013a) showed that P-deficiency increased H_2O_2 content which induced membrane damage as reflected by higher malonyldialdehyde (MDA) content and higher electrolyte leakage. Similar results have been found in rice (Fu et al., 2013). To cope with this treat, plants have evoked various mechanisms. The most common response to oxidative stress is the induction of the antioxidant systems (Bargaz et al., 2013a). In fact, in P-deficient faba bean, the increase in MDA content was correlated with an increase in the activities of polyphenol oxidase (PPO) and peroxidase (PO) (Oukaltouma et al., 2022). Similarly, in common bean, P-deficiency increased the activity of PPO and PO together with an increase in the content of total polyphenol (Bargaz et al., 2013a).

The limitation of grain legumes productivity by P-deficiency is widespread problem which will be increased in the future because of the climate change (Bargaz et al., 2021). To improve the productivity of chickpea in an economical way, the orientation of farmers' choice towards chickpea varieties that are efficient in the use of P is one way,

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among others, of improving the productivity of this crop under the conditions of low-P availability. In this regard, the selections of new varieties and the exploitation of the genetic variation in Moroccan chickpea varieties is a promising way. In this setting, the present study aims at evaluating the effect of P-deficiency on growth, photosynthesis, membrane stability and antioxidant enzymes of two Moroccan chickpea varieties, *Bouchra* (*BO*) and *Moubarak* (*MO*).

Materials and methods

Plant material and culture conditions

Two Moroccan chickpea varieties, *BO* and *MO* were used in the present study. These varieties were selected by National Institute for Agronomic Research INRA- Morocco. They are widely cultivated in different Moroccan agro-systems and are well adapted to different agro-ecological areas with high yield potential, resistance to some diseases and pests. However, climate change has a negative impact on crop production and the chickpea production is facing several constraints which cause serious pre- and post-harvest losses in most of the farming systems. These constraints are mainly abiotic such as drought and soil nutrient deficiency like P. Hence, we investigate here the responses of these two chickpea varieties to low-P availability. This is the first time ever their behavior will be evaluated under low-P availability. Seeds, supplied by the National Institute of Agronomic Research (INRA) Morocco, were surface-sterilized with 5% sodium hypochlorite for 5 min and then rinsed several times with sterile distilled water. The experiment was conducted at the growth chamber of the Polydisciplinary Faculty of Beni Mellal (B2DRN Lab.) with an average temperature of 25 ± 1 °C, a range of relative humidity 60% - 80%, and a photoperiod of 16 h. Six seeds of each variety were allocated to germinate in 18 cm diameter and 15 cm height pots containing sterilized perlite, with the following characteristics: granule size, 0-6 mm; pH 6.5-8.0; EC 0.0-0.1 µS cm^{-1}. One week after sowing, three homogenous seedlings were selected per pot. The seedlings were irrigated with Hoagland nutrient solution once a week according to the field capacity (FC) of the substrate. The composition of the nutrient solution was as follows: KNO_3 (600 µM L^{-1}), K_2SO_4 (0.75 mM L^{-1}), $MgSO_4$ (1 mM L^{-1}), $CaCl_2$ (1.65 mM L^{-1}), Fe-EDTA (16 µmol L^{-1}), H_3BO_3 (4 µM L^{-1}), $MnSO_4$ (6 µM L^{-1}), $ZnSO_4$ (1 µM L^{-1}), $CuSO_4$ (1 µM L^{-1}), Na_2MoO_4 (0.1 µM L^{-1}). P was applied in the form of KH_2PO_4 at concentration of 1 mM P L^{-1} for control against 0.01 mM P L^{-1} for stressed plants according to Alloush (2003). P-deficiency was applied after two weeks of sowing and kept for six weeks. At the flowering stage, eight weeks after sowing, some parameters related to plants growth, P uptake, photosynthesis, oxidative stress and antioxidant response system were assessed. For each variety, three replicates per treatment were executed.

Growth parameters

At the end of the experiment and before the harvest, a set of parameters associated to plant growth were focused. Plant height (PH) was measured using a graduated precision ruler. Leaves number (LN) and stems number (SN) were determined in three random plants of each treatment just before the harvest. Leaf area (LA) was determined on three randomized young and healthy leaves using image J software (http://rsb.info.nih.gov/ij/index.html) as described in Farssi et al. (2021).

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After the harvest, three random plants from each treatment were selected and used for biomass determination. For this purpose, shoots were cut off from the roots and their fresh weight (FW) was determined. Then, the root parts were separated from the substrate, washed thoroughly with deionized water and after wiping, their FW was determined. Both shoot and root parts were subsequently dried at 80 °C for 48 h and their dry weight (DW) were determined. The shoot to root ratio (S to R) was calculated by taking the shoot fresh weight (SFW) and shoot dry weight (SDW) of the plants and dividing them by the root fresh weight (RFW) and root dry weight (RDW) respectively.

Total chlorophyll (Chl) and carotenoid (Car) contents and Chl stability index (CSI)

Total Chl (mg g^{-1} FW) was measured following the method described by Arnon (1949). Fresh leaf material (100 mg) was homogenized in 5 mL acetone (80% v/v) using a mortar and pestle. After 10 min of centrifugation at 5000 ×g, supernatant was used to read the absorbance at 480 nm, 663 nm and 645 nm against 80% acetone as a blank. Three replicates per treatment per varieties were considered. Total Chl and Car contents were determined using *Equations 1* and *2*, respectively:

$$\text{Total Chl (mg g}^{-1}\text{ FW)} = [(8.02 \times \text{OD}_{663} + 20.2 \times \text{OD}_{645}) \times \text{V} \div (1000 \times \text{W})] \quad \text{(Eq.1)}$$

where V represents volume of the aliquot and W weight of tissue.

$$\text{Car (mg.g}^{-1}\text{FW)} = \text{Acar} \div \text{Em} \times 100 \quad \text{(Eq.2)}$$

where $\text{Acar} = \text{OD}_{480} + 0.14 \times \text{OD}_{663} - 0.638 \times \text{OD}_{645}$ and Em = 2500.

CSI was determined as described by Vivek et al. (2020) using *Equation 3:*

$$\text{CSI}(\%) = \frac{\text{Total Chl content (P deficient)}}{\text{Total Chl content (P sufficient)}} \times 100 \quad \text{(Eq.3)}$$

Quantum yield of photosystem II (PS II) and OJIP fluorescence transients

The photochemical efficiency of the PS II was evaluated based on the ratio of variable fluorescence to maximum fluorescence (F_v/F_m), which represents the quantum efficiency of the photochemistry of PS II. F_v/F_m was determined using a portable fluorescence meter (Handy PEA, Hansatech, England), after 20 min of adaptation to darkness (Mouradi et al., 2016).

The OJIP kinetics were determined in five leaves after their dark adaptation for 20 min. Measurement of the rapid kinetics of Chl fluorescence allowed to estimate several photosynthetic parameters associated with the photochemical and non-photochemical activity of PS II. For these parameters, the maximum fluorescence level, F_m, is determined at the "*P*" transition, and the variable fluorescence levels at the "*O"*, *"J"* and *"I"* transitions were estimated at 50 µs, 2 ms and 30 ms respectively.

P content

P content (mg g^{-1} DW) was determined colorimetrically using the molybdate blue method (Murphy and Riley, 1962). Dried samples (0.5 g) from shoots and roots were incinerated for 6 h at 600 °C. The ash obtained was recovered by adding 3 mL of HCl

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(10 N), and then the solution was filtered and the filtrate was adjusted to 100 mL with distilled water. Subsequently, 1 mL of the filtrate was added to 4 mL of distilled water and 5 mL of a mixture of 2.5% (w/v) sodium molybdate and 0.15% (w/v) hydrazine sulfate, and the whole solution was heated in a water bath at 95 °C for 10 min. Afterwards, the OD was determined at 825 nm using a SP-UV1000 Spectrophotometer. A standard curve was established with KH_2PO_4 solutions. Three measurements per treatment were considered.

Different components of P use efficiency (PUE)

Relative P use efficiency (RPUE)

The RPUE (%) of the two used varieties was determined as the ratio between the DW of plants grown under deficient P condition and those grown under sufficient P conditions using *Equation 4* described in (Ozturk et al., 2005):

$$\text{RPUE } (\%) = \left(\frac{\text{DW}_{\text{deficient Pi}}}{\text{DW}_{\text{sufficient Pi}}}\right) \times 100 \quad \text{(Eq.4)}$$

Agronomic P use efficiency (APUE)

The APUE (g DW g^{-1} P_i) was determined using *Equation 5* established by Neto et al. (2016):

$$\text{APUE}(\text{g DW g}^{-1}\ \text{Pi}) = \frac{\text{DW}_{\text{sufficient Pi}} - \text{DW}_{\text{deficient Pi}}}{\text{Difference in the total available Pi between sufficient and deficient Pi conditions}} \quad \text{(Eq.5)}$$

P uptake

P uptake (µg $plant^{-1}$) was determined according to Irfan et al. (2018) with some modifications following *Equation 6:*

$$\text{P uptake } (\mu\text{g plant}^{-1}) = [\text{P}]\ (\mu\text{g g}^{-1}) \times \text{DW } (\text{g plant}^{-1}) \quad \text{(Eq.6)}$$

P uptake efficiency (PUpE)

PUpE (mg P g^{-1} P_i) for plant grown under sufficient or deficient P conditions was calculated as described by Neto et al. (2016) using *Equation 7* or *Equation 8:*

$$\text{PUpE}_{\text{sufficient Pi}} = \frac{\text{P}_{\text{sufficient Pi condition}} \times \text{DW}_{\text{sufficient Pi condition}}}{\text{Pi applied for each plant under sufficient Pi condition}} \quad \text{(Eq.7)}$$

or

$$\text{PUpE}_{\text{deficient Pi}} = \frac{\text{P}_{\text{deficient Pi condition}} \times \text{DW}_{\text{deficient Pi condition}}}{\text{Pi applied for each plant under deficient Pi condition}} \quad \text{(Eq.8)}$$

P utilization efficiency (PUtE)

PUtE (g DW g^{-1} P) was calculated as the plant biomass production per unit P accumulated in the plant according to Neto et al. (2016) using *Equation 9* or *Equation 10*.

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$$PUtE_{\text{sufficient Pi condition}} = \frac{DW_{\text{sufficient Pi condition}}}{[P]_{\text{sufficient Pi condition}} \times DW_{\text{sufficient Pi condition}}} \quad \text{(Eq.9)}$$

or

$$PUtE_{\text{deficient Pi condition}} = \frac{DW_{\text{deficient Pi condition}}}{[P]_{\text{deficient Pi condition}} \times DW_{\text{deficient Pi condition}}} \quad \text{(Eq.10)}$$

Physiological P use efficiency (PPUE)

The PPUE (mg^2 DW mg^{-1} P) corresponds to DW produced for a given plant P concentration was determined following *Equation 11* or *Equation 12* which were developed by Hammond et al. (2009):

$$PPUE_{\text{sufficient Pi condition}} = \frac{DW_{\text{sufficient Pi condition}}}{[P]_{\text{sufficient Pi condition}}} \quad \text{(Eq.11)}$$

or

$$PPUE_{\text{deficient Pi condition}} = \frac{DW_{\text{deficient Pi condition}}}{[P]_{\text{deficient Pi condition}}} \quad \text{(Eq.12)}$$

Oxidative stress markers and cell membrane integrity

Electrolyte leakage (EL) was determined as described by Ghoulam, Foursy and Fares (2002). 0.25 g of fresh leaf samples were washed three times with distilled water to eliminate superficial minerals, placed in tubes containing 10 mL of demineralized water and incubated under agitation at 25 °C. After 24 h of incubation, using a conductivity meter, the initial electrical conductivity (EC_i) was determined at 25 °C. Subsequently, the same samples were autoclaved for 20 min at 121 °C. After cooling down, the samples were stirred for 30 min at 25 °C, then the final electrical conductivity (EC_f) was measured. The EL was determined using *Equation 13:*

$$EL(\%) = \frac{EC_i}{EC_f} \text{x } 100 \quad \text{(Eq.13)}$$

MDA was estimated according to the method described by (Savicka et al., 2018). 50 mg of fresh leaves was ground in 3 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 14000×*g* for 15 min at 4 °C. 1 mL of supernatant was added to 2.5 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. The mixture was incubated in a water bath at 95 °C for 30 min, and then cooled in an ice bath. The OD was then determined at 532 and 600 nm. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as mmol MDA g^{-1} FW.

Hydrogen peroxide (H_2O_2) content was determined according to the method of Velikova, Yordanov and Edreva (2000). Around 100 mg of fresh leaf material was ground in 2 mL TCA (20%) and centrifuged at 15000 ×*g* for 15 min at 4 °C. The supernatants were then recovered to determine the H_2O_2 content. To 0.5 mL of the extract, we added 0.5 mL of potassium phosphate buffer (10 mM, pH 7) and 1 mL of 1 M potassium iodine. After 1 h of incubation in the dark, the OD was determined at 390 nm. H_2O_2 content was expressed as µmol H_2O_2 g^{-1} FW.

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Antioxidant enzyme activities

Superoxyde dismutase (SOD) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beyer and Fridovich (1987) with some modifications. The reaction mixture contained 2550 µL of 0.1 M phosphate buffer (pH 7.8), 75 µL of 55 mM methionine, 300 µL of 0.75 mM NBT, 60 µL of 0.1 mM riboflavin, and 50 µL of the enzyme extract. The reaction was initiated by exposing the samples to fluorescent lamps at 25 °C for 5 min, then the OD was read at 560 nm. One enzyme unit was defined as the amount of enzyme required to cause a 50% inhibition of the rate of reduction of NBT.

Peroxidase (PO) activity was assessed following the method described in Hwang et al. (1999). The reaction mixture contained 2290 µL of 60 mM phosphate buffer (pH 6.8), 100 µL of 20 mM H_2O_2, 100 µL of 18 mM guaiacol and 40 µL of the enzyme extract. Guaiacol oxidation was measured by following the increase in the absorbance at 470 nm (ε = 26.6 mM cm^{-1}) for 5 min.

Statistical analysis

Data were statistically analyzed using SPSS software Version 25 (IBM Corporation). It concerned a two-way analysis of variance (ANOVA II). Comparison between means was also performed using Tukey's test at the level of $p < 0.05$. Data were expressed as the mean of three replicates ± standard error. Principal component analysis (PCA) was done using XLSTAT software version 2018 (Addinsoft, Paris, France), considering variables centered on their means and normalized with a standard deviation of 1.

Results

Growth parameters

Effect of P-deficiency on plant biomass and S to R ratio

The effect of P level on growth of chickpea plants is indicated in *Table 1*. The obtained results indicated that P-deficiency significantly ($p < 0.05$) impaired plant biomass in both varieties. Moreover, this effect was more pronounced in *MO* than *BO*. Indeed, the reduction in *MO* reached 43%, 51%, 38% and 38% respectively for SFW, RFW, SDW and RDW. For S to R ratio, results showed non-significant ($p > 0.05$) effect of P level.

Effect of P-deficiency on LN, SN, PH and LA

Results illustrated in *Table 2* revealed that P-shortage significantly ($p < 0.05$) reduced LN, PH and LA in the two studied varieties and SN in *MO*. The reduction rates of LN and SN were significantly higher in *MO* than in *BO*. Indeed, as compared to unstressed control, LN and SN were decreased in *MO* by 19% and 40% respectively. However, for PH and LA the highest reduction rates of 29% and 43% respectively were noted in *BO*. ANOVA II showed that the effect of P-deficiency was significant for all parameters (*Table A1*).

Total Chl, Car and CSI

Under control conditions, a higher total Chl content was recorded in *BO* variety ($p < 0.05$; *Fig. 1A*) while Car contents were similar in the two varieties ($p > 0.05$;

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Fig. 1B). However, P-limitation caused a significant ($p < 0.05$) decrease in total Chl and Car, especially in *MO* varieties where the highest reduction rates of 44% and 36%, respectively were observed as compared to unstressed chickpea plants.

Results related to CSI (*Fig. 1C*) indicated that this parameter showed similar values under P-sufficient conditions in both varieties ($p > 0.05$). However, P-starvation significantly ($p < 0.05$) reduced the CSI in the two studied varieties with more obvious effect on *MO* as compared to *BO*.

Chl fluorescence OJIP kinetics

The polyphasic OJIP curve of fluorescence in the two chickpea varieties was measured and plotted on a logarithmic time scale as indicated in the *Figure 2*. Regardless P treatment, fluorescence intensity increased with the time in all of the two studied varieties. However, the values recorded under 1 mM and 0.01 mM P treatments were significantly different ($p < 0.05$) in the two studied chickpea varieties. Indeed, we found that the pattern of Chl fluorescence curves was affected by P-deficiency in the two chickpea varieties. The OJIP curve reflects the general properties of PSII. F_m has decreased under 0.01 mM P compared to 1 mM P, and the reduction rate was higher in *MO* (28%) than in *BO* (10%).

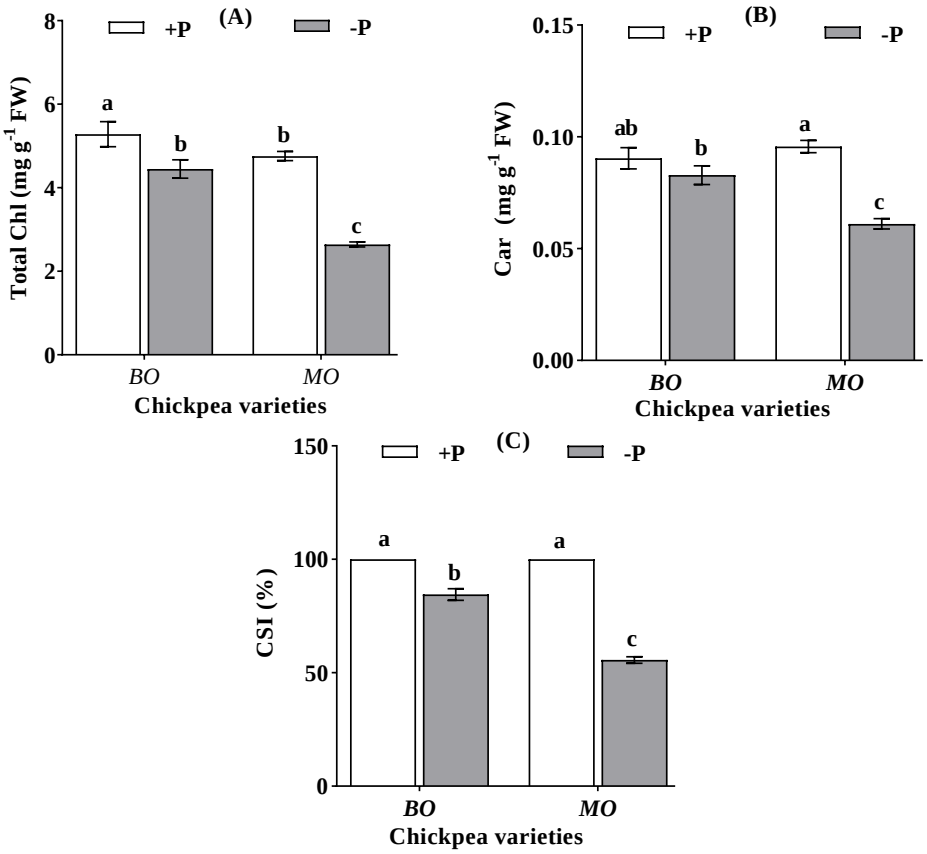

Figure 1. *Total chlorophyll (Chl) content (A), carotenoids (Car) content (B) and chlorophyll stability index (CSI) (C) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply. Values are means of three replicates ± standard errors; Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test*

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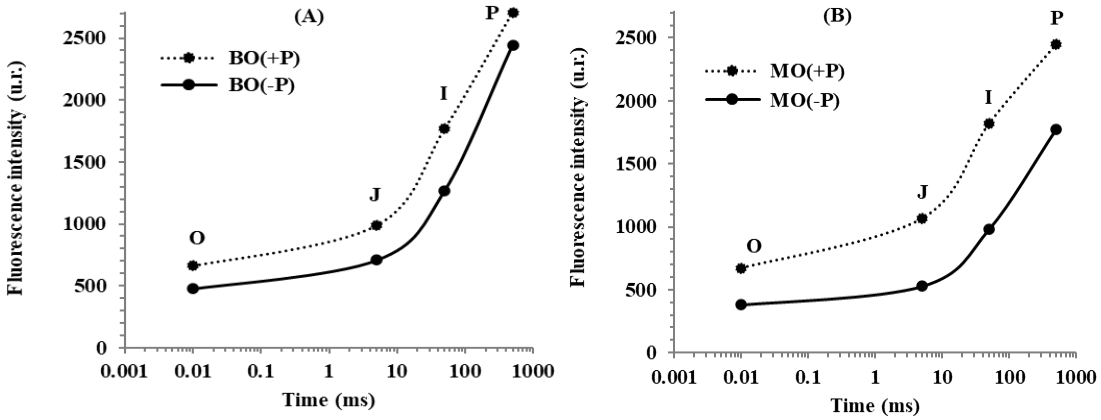

Figure 2. *The OJIP curves of chlorophyll fluorescence as a function of time (ms) measured after adaptation to darkness of two Moroccan chickpea varieties of Bouchra (BO) (A) and Moubarak (MO) (B) grown under sufficient (1 mM) (dashed line) or deficient (0.01 mM) P supply (solid line). The maximum fluorescence level, F_m, is determined at the "P" transition, and the variable fluorescence levels at the "O", "J" and "I" transitions will be estimated at 50 μs, 2 ms and 30 ms respectively. Values are means of three replicates*

Table 1. *Shoot (SFW) and root (RFW) fresh weights, shoot (SDW) and root (RDW) dry weights and shoot to root ratio (S to R) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply*

	SFW (g plant^{-1})		RFW (g plant^{-1})		SDW (g plant^{-1})		RDW (g plant^{-1})		S to R (plant^{-1} DW)	
	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
BO	3.00±0.12^{a}	1.90±0.14^{c}	2.46±0.07^{a}	1.76±0.30^{b}	0.41±0.02^{a}	0.27±0.01^{c}	0.23±0.02^{a}	0.15±0.02^{b}	1.83±0.15^{a}	2.00±0.40^{a}
MO	2.52±0.12^{b}	1.43±0.06^{d}	2.16±0.37^{ab}	1.05±0.08^{c}	0.34±0.03^{b}	0.21±0.03^{d}	0.17±0.02^{b}	0.10±0.01^{c}	2.05±0.25^{a}	1.96±0.13^{a}

Values are means of three replicates ± standard errors. Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test

Table 2. *Leaves number (LN), stems number (SN), plant height (PH) and leaf area (LA) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply*

	LN (Plant^{-1})		SN (Plant^{-1})		PH (Cm)		LA (Cm^2)	
	+P	-P	+P	-P	+P	-P	+P	-P
BO	17.00±0.58^{a}	15.00±0.88^{b}	3.67±1.16^{ab}	2.67±0.24^{ab}	24.33±0.44^{a}	17.20±0.44^{c}	4.66±0.24^{a}	2.65±0.24^{c}
MO	16.00±0.88^{ab}	13.00±0.88^{c}	3.33±0.58^{a}	2.00±0.58^{b}	25.67±1.00^{a}	21.00±1.00^{b}	3.86±0.08^{b}	2.62±0.14^{c}

Values are averages of three replicates ± standard errors and the letters indicate statistically significant values. Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test

Minimum Chl fluorescence F_0 and maximum quantum yield of PS II (F_v/F_m)

The photochemical efficiency of PS II was determined by analyzing the fluorescence parameter (F_0 and F_v/F_m) which represents the maximum quantum yield efficiency of PSII as the *Figure 3B* indicated. The obtained results showed a significant effect of treatment for both parameters ($p < 0.05$; *Fig. 3*). For F_v/F_m, the behavior of the two varieties studied was not significantly ($p > 0.05$) different. F_0 increased as the P level decreased ($p < 0.05$) in the two studied varieties.

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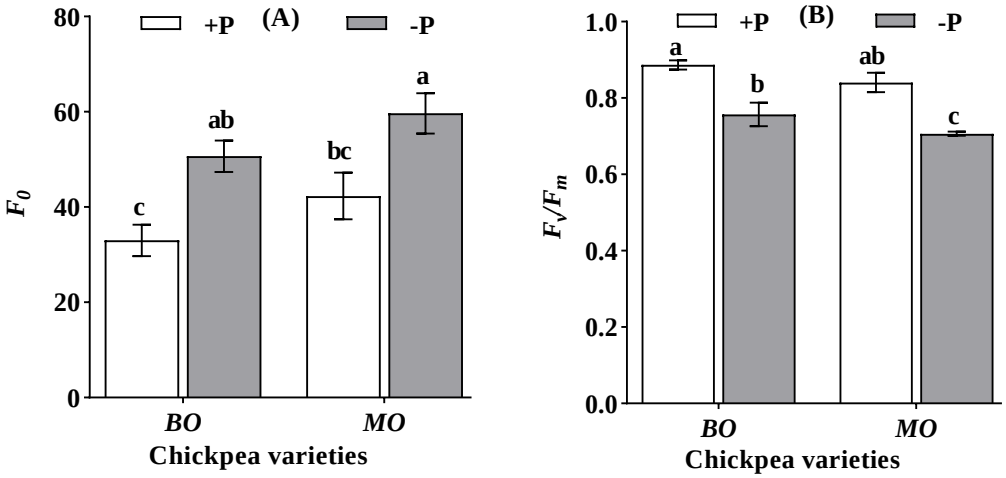

Figure 3. *The minimum chlorophyll fluorescence F_0 (A) and quantum yield of photosystem PSII (F_v/F_m) (B) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply. Values are means of three replicates ± standard errors; Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test*

P content in shoots and roots of chickpea plants

The effect of P-deficiency on shoot and root P contents is represented in *Figure 4*. The findings indicated that P-starvation reduced significantly ($p < 0.05$) P contents in both parts of the two studied chickpea varieties with a significant difference between them ($p < 0.05$) in shoot part. Indeed, compared to the corresponding controls, *BO* variety was more affected by P-deficiency with reduction rates of 46% and 43%, respectively for shoots and roots (*Fig. 4*). However, the reduction rates in *MO* were 40% for the shoot and only 32% for the root.

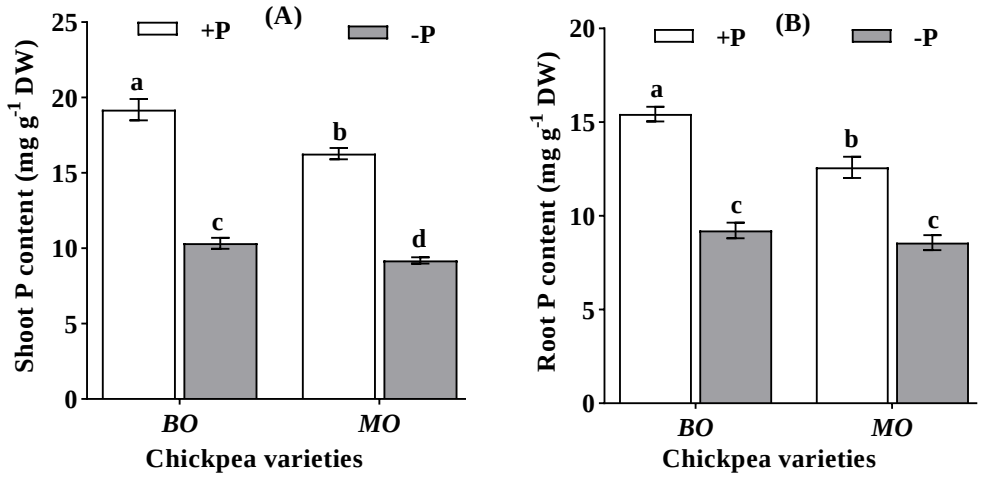

Figure 4. *Shoot P content (A) and root P content (B) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply. Values are means of three replicates ± standard errors. Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test*

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Different components of PUE

RPUE and APUE

Results related to RPUE (*Table 3*) indicated that P treatment has a significant effect ($p < 0.05$) with non-significant differences between the studied varieties ($p > 0.05$). The same behavior was noted for the APUE. Even that there was non-significant difference between the two varieties, *BO* variety has shown the highest values for both RPUE and APUE as compared to *MO*.

Table 3. *Relative P use efficiency (RPUE) and agronomic P use efficiency (APUE) in two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply*

	RPUE (%)	**APUE (g DW g^{-1} Pi)**
BO	67 ± 4.43^{a}	0.050 ± 0.009^{a}
MO	62 ± 4.54^{a}	0.046 ± 0.007^{a}

Values are means of three replicates ± standard errors; lowercase letters indicate non-significant differences ($p > 0.05$) between means according to Tukey's test

P uptake

P-deficiency significantly ($p < 0.05$) reduced P uptake in the two chickpea varieties. *BO* was found to be significantly ($p < 0.05$) the less affected one (*Table 4*). Indeed, this variety showed the highest P uptake value of 8.20 µg $plant^{-1}$ against 5.60 µg $plant^{-1}$ in *MO* variety.

Table 4. *P uptake, P uptake efficiency (PUpE), P utilization efficiency (PUtE) and physiological P use efficiency (PPUE) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply*

	P uptake (µg $plant^{-1}$)		**PUpE (mg P g^{-1} Pi)**		**PUtE (mg DW mg^{-1} Pi)**		**PPUE (mg^2 DW mg^{-1} Pi)**	
	+P	**-P**	**+P**	**-P**	**+P**	**-P**	**+P**	**-P**
BO	21.90 ± 0.44^{a}	8.20 ± 0.15^{c}	5.05 ± 0.10^{c}	188.85 ± 3.47^{a}	28.94 ± 0.96^{d}	51.19 ± 1.29^{b}	18.43 ± 1.62^{b}	21.50 ± 1.09^{a}
MO	14.78 ± 1.29^{b}	5.60 ± 0.48^{d}	3.41 ± 0.30^{d}	128.95 ± 10.96^{b}	34.64 ± 0.23^{c}	56.33 ± 1.64^{a}	17.74 ± 1.60^{b}	19.18 ± 2.33^{ab}

Values are means of three replicates ± standard errors. Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test

PUpE

Results presented in *Table 4* showed that PUpE was significantly ($p < 0.05$) increased in the two studied chickpea varieties in response to P-deficiency with a significant difference between them ($p < 0.05$). In fact, the PUpE in *BO* reached 188.85 mg P g^{-1} Pi. However, the values did not exceed 128.95 mg P g^{-1} Pi in *MO*. According to ANOVA II (*Table A1*), P level, varieties and their interaction were significant for PUpE.

PUtE

Results illustrated in *Table 4* indicated that P-starvation significantly ($p < 0.05$) increased PUtE in the two studied chickpea varieties with a significant difference between them ($p < 0.05$). Indeed, under either deficient or sufficient P conditions, PUtE

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was higher in *MO* more than *BO* with the lower value noted in *BO*. ANOVA II (*Table A1*) indicated that variety and P were significant ($p < 0.05$).

PPUE

Results illustrated in *Table 4* indicated that P-starvation significantly ($p < 0.05$) increased PPUE in *BO* according to Tukey's test. However, non-significant effect was noted for *MO* variety under both P treatments ($p > 0.05$).

Oxidative stress markers and cell membrane integrity

Results in *Table 5* indicated that P-deficiency induced a significant increase ($p < 0.05$) in the contents of MDA and H_2O_2 and in EL percentage in the two studied varieties. The recorded contents were significantly ($p < 0.05$) different between both varieties for H_2O_2 contents only. Indeed, under low-P availability, the H_2O_2 content of 4.83 µmol g^{-1} FW was noted in *MO* variety, while in *BO* this amount did not exceed 3.42 µmol g^{-1} FW.

Table 5. *Electrolytes leakage (EL) malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) and peroxidase (PO) of two Moroccan chickpea varieties Bouchra (BO) and Moubarak (MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply*

	EL (%)		MDA (mmol g^{-1} FW)		H_2O_2 (µmol g^{-1} FW)		SOD (EU mg^{-1} protein)		PO (EU mg^{-1} protein)	
	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
BO	14.76±0.32^{b}	16.81±0.66^{a}	0.02±0.01^{b}	0.03±0.01^{a}	1.46±0.08^{d}	3.42±0.04^{b}	33.81±0.47^{b}	49.76±1.97^{a}	34.03±0.74^{b}	56.96±3.81^{a}
MO	14.42±0.92^{b}	18.20±0.92^{a}	0.01±0.00^{b}	0.03±0.00^{a}	1.88±0.13^{c}	4.83±0.61^{a}	28.91±0.51^{c}	54.99±4.24^{a}	31.41±1.02^{c}	65.71±5.15^{a}

Values are means of three replicates ± standard errors. Different and same small letters indicate significant ($p < 0.05$) and non-significant differences ($p > 0.05$), respectively, between the means according to Tukey's test

Antioxidant enzyme activities

SOD activity revealed the significant ($p < 0.05$) effect of P treatments on both varieties (*Table 5*). In fact, SOD activity was higher under P-deficient than P-sufficient conditions. The induction of SOD activity was more pronounced in *MO* as compared to *BO*.

Similar to SOD, P-deficiency induced the activity of PO in the two investigated chickpea varieties (*Table 5*). Indeed, under P starvation, PO activity was significantly ($p < 0.05$) increased by 67% and 109%, respectively in *BO* and *MO* relative to their corresponding controls. Non-significant differences were noted between the both verities ($p > 0.05$) for both enzymes under P-deficient condition.

PCA analysis and Pearson's correlation matrix

For more information on the possible correlations between the different parameters studied and to show the behavior of chickpea plants with respect to the P-deficiency that was applied, a principal component analysis (PCA) was carried out (*Fig. 5*). Our analysis yielded a correlation matrix (*Fig. 6*) specifying the correlation coefficients between the different pairs of variables studied, and a graph corresponding to a projection of these variables on a two-dimensional basis (*Fig. 5*). The percentage of variability represented on the first (F1) and the second (F2) axis is very high (85.94%). The first axis alone accounts for 76.10% of the existing variability, compared with 8.84% for the second axis. Our results demonstrated that the deficient P (0.01 mM) shows a highly negative correlation

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with most variables like growth and photosynthesis parameters (Chl, Car, and F_v/F_m). Moreover, low-P availability shows higher positive correlation with H_2O_2, MDA and EL and antioxidant enzymes activities (SOD and PO). However, a significant positive correlation between growth parameters and photosynthetic pigment levels (Total Chl, Car), F_v/F_m, shoot and root P contents were observed.

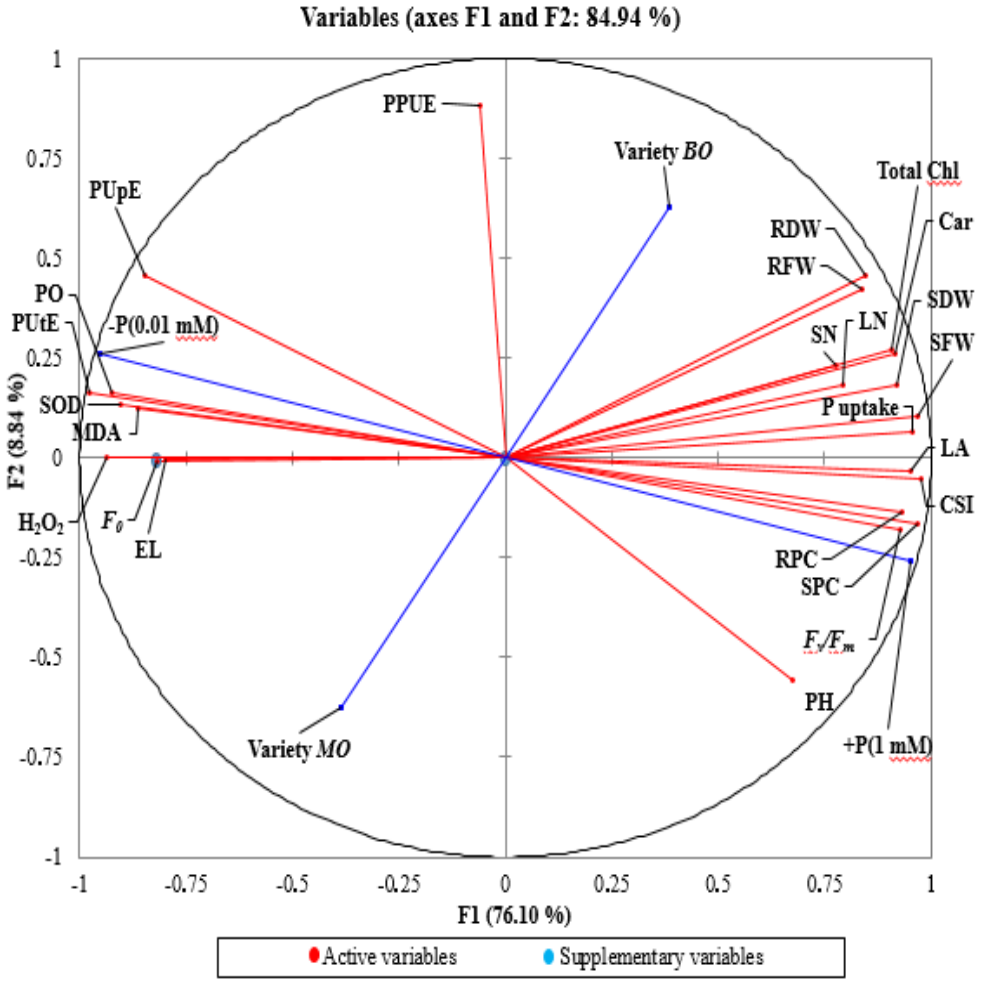

Figure 5. *Principal components analysis (PCA) between assessed parameters of two Moroccan chickpea varieties (BO and MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply. Shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW), leaves number (LN), stems number (SN), plant height (PH), leaf area (LA), total chlorophyll (Chl), carotenoids (Car), Chl stability index (CSI), The minimum chlorophyll fluorescence (F_0), Quantum yield of photosystem PSII (F_v/F_m), shoot P content (SPC), root P content (RPC), P uptake, P uptake efficiency (PUpE), P utilization efficiency (PUtE), physiological P use efficiency (PPUE, electrolyte leakage (EL), malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) and peroxidase (PO)*

Discussion

P nutrition in plant and particularly in legumes is a determinant factor because of the high demand of P during biological nitrogen fixation process. However, this effect varies greatly between species as well as among varieties within the same species (Bilal et al., 2018). Thus, selection of genotypes with high tolerance to P-deficiency may

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represent an efficient approach to get high legume's productivity under P starvation. For this reason, in the present study, we analyzed the genetic variation of two Moroccan chickpea varieties (*BO* and *MO*) using a large set of parameters related to growth, P nutrition, photosynthesis, oxidative stress and antioxidant system in order to evaluate their tolerance to P-deficiency. Plant growth evaluated by fresh and dry biomass, plant height and leaf area were remarkably restricted under P-deficiency in the two studied varieties. We also speculate that the *MO* variety was the most affected by P-deficiency with reductions rates of 43%, 51%, 38% and 38%, respectively for SFW, RFW, SDW and RDW. Additionally, P-starvation reduced the LN and SN, PH and LA in the two studied chickpea varieties with a significant difference between them. Indeed, the comparison between the varieties revealed that *MO* presented the highest reduction rates for almost all the above-mentioned traits. The significant biomass reduction under P-deficiency agrees with previous study carried out by Ahmed et al. (2020), in which the results showed that the biomass of chickpea plants increases significantly ($p < 0.001$) with soil P content. In this study, SDW of plants varied from 4.73 g plant^{-1} in P content of 6.73 ppm, to 38.3 g plant^{-1} in available P level of 23.52 ppm. Similar results have been found on common bean (Mandri et al., 2012), where the authors indicated that the restriction of P from 0.25 mM to 0.075 mM reduced shoots and roots biomass by 16% and 43%, respectively. Likewise, same results have been showed in other legumes like mashbean, mungbean, soybean (Chaudhary et al., 2008), and faba bean (Liu et al., 2016) and in other plant species, such as tomato (Fujita et al., 2003) and maize (Liu et al., 2016). According to Mollier and Pellerin (1999), P-deficiency inhibit the emergence of new axile roots as well as the elongation of first-order laterals root, which was partially explain our results about the reduction in root biomass during response to P-limitation stress.

In our study, there was a significant positive correlation between plant growth reduction and P content in the shoots and roots of two studied chickpea verities. Indeed, the P content was interestingly lower in shoot than in root for all the two studied varieties, indicating that P-deficiency affects not only P assimilation by the root but also its translocation to the shoots. It has been reported that P plays a crucial role in energy transfer, growth of new tissue and cells division (Bayuelo-Jiménez et al., 2011; Sulieman and Tran, 2015). Therefore, P is an essential macronutrient required for plant growth, and its restriction in this work was clearly explained the growth reduction in the two studied varieties, since the close significant positive correlation observed between shoot P content and SFW ($r = 0.93$) and RFW ($r = 0.75$), LA ($r = 0.95$), LN ($r = 0.75$) and SN ($r = 0.70$) (*Fig. 6*). Our findings are similar to those published by Chen, Lee and Yeh (2018) on lisianthus, by Chaudhary et al. (2008) on mashbean, mungbean and soybean, by Tang et al. (2001) on common bean and by Rodríguez et al. (1998) on sunflower. Another interesting finding by Sulieman et al. (2009) on common bean indicated that when plants were stressed by low-P availability, the results were dramatic reduction in plant biomass, LA index and seed yield. In addition to P content, there is a wide variation in the different components of PUE during response of chickpea varieties to low-P availability stress. Indeed, RPUE index was 1.13-fold higher in *BO* variety as compared to *MO* variety. Furthermore, the APUE was varied significantly between *MO* and *BO* varieties, with a specific high value was recorded in *BO* chickpea variety. Our results were concord with those of Irfan et al. (2018) and Neto et al. (2016), who found that the PUE, represented by RPUE and APUE, was species or even variety dependent manner. Furthermore, at low Pi supply, PUpE was significantly ($p < 0.05$) increased,

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particularly in *BO*, where the value reached 188.85 mg P g^{-1} Pi as compared to *MO* (128.95 mg P g^{-1} Pi).

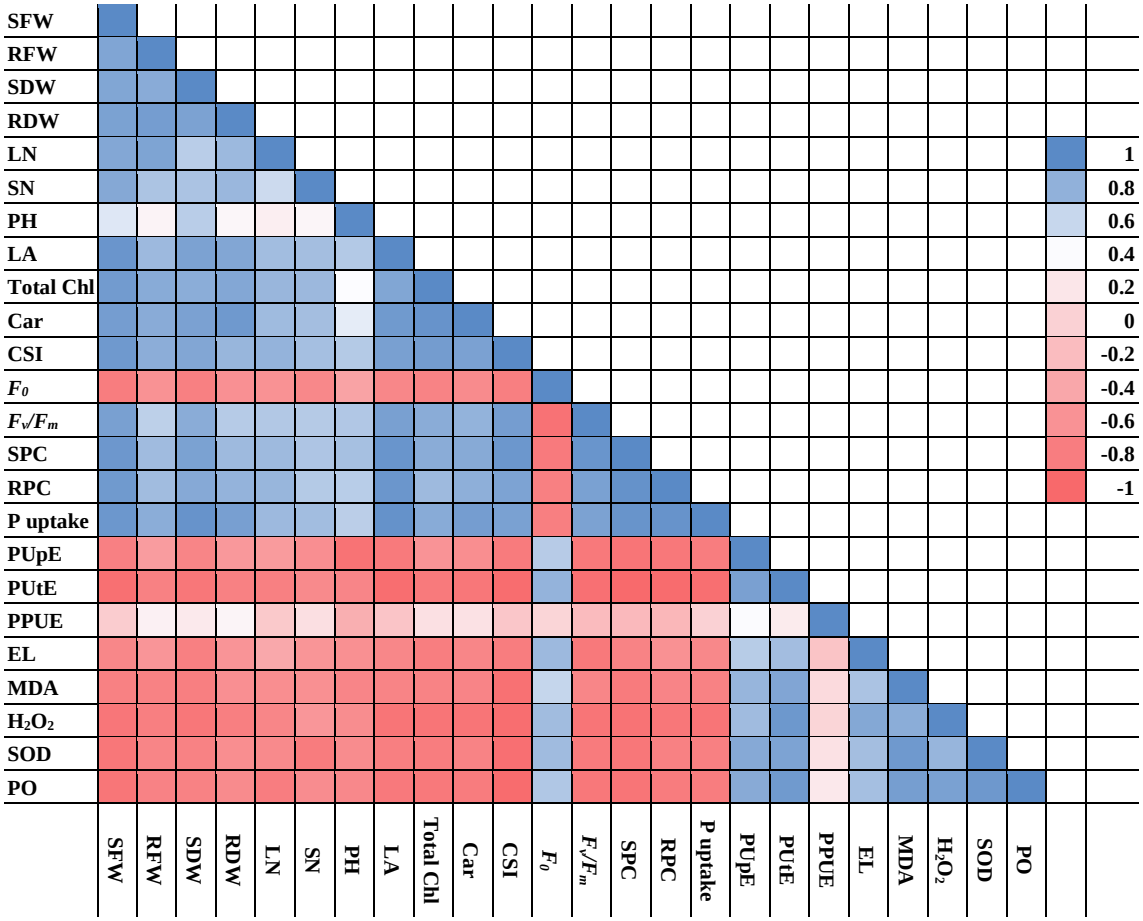

Figure 6. *Pearson's correlation matrix between assessed parameters of two Moroccan chickpea varieties (BO and MO) grown under sufficient (1 mM) or deficient (0.01 mM) P supply. Correlations are displayed in blue (positive) and in read (negative); color intensity is proportional to correlation coefficient. Shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW), leaves number (LN), stems number (SN), plant height (PH), leaf area (LA), total chlorophyll (Chl), carotenoids (Car), Chl stability index (CSI), The minimum chlorophyll fluorescence (F_0), Quantum yield of photosystem PSII (Fv/Fm), shoot P content (SPC), root P content (RPC), P uptake, P uptake efficiency (PUpE), P utilization efficiency (PUtE), physiological P use efficiency (PPUE), electrolyte leakage (EL), malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) and peroxidase (PO)*

Moreover, as P uptake efficiency reflect the ability of plants to assimilate Pi from the soil (da Silva et al., 2016; Neto et al., 2016; Nguyen and Stangoulis, 2019), it will also reflect high dry mass production. In our study, P uptake was higher in control, while P-deficiency significantly ($p < 0.05$) restricted it in the two studied varieties, with a significant ($p < 0.05$) difference between them. Indeed, under P-deficiency, *BO* showed the highest P uptake value (8.20 µg $plant^{-1}$) as compared to *MO* (6.60 µg $plant^{-1}$), where the P uptake was reduced by 62% relative to control. Generally, such a positive

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correlation (*Fig. 6*) between P uptake and plant biomass (r = 0.82) either under stressed or unstressed conditions has been found in several plants' species including coffee and rice (Neto et al., 2016; Vandamme et al., 2016).

On the other hand, Wissuwa, Gamat and Ismail (2005) suggested that the low biomass accumulation under P-deficiency could be the result of photosynthesis reduction. The same results were found in the present study, where P-deficiency not only disrupted chickpea plant growth, but also negatively affected photosynthetic activity. Indeed, total Chl and Car contents were markedly reduced under P limitation, which in turn impaired plant growth. This reduction in photosynthetic pigments content was more prominent in the *MO* variety as compared to *BO*. The reduction in photosynthetic pigments in the present study could be explained by the reduction in P content, which was evident from the close significant positive correlation found between P content and total Chl (r = 0.82) and Car (r = 0.84). In addition, P limitation, in our study, also decreased F_v/F_m and F_0 in the two chickpea varieties. Our findings corroborate with those of Wissuwa et al. (2005).

More interestingly, Meng et al. (2021) demonstrated that photosynthesis reduction under P-deficiency is the key causes of plant biomass reduction. According to Farissi et al. (2018), the reduction in maximum quantum of PSII (F_v/F_m) could be the result of Chl degradation. This was evident in our study as indicated by the high significant positive correlation between total Chl and *Fv/Fm* (r = 0.83). Furthermore, P-deficiency reduced fluorescence intensity but increased the time to reach the maximum fluorescence intensity as compared to control plants.

As P represents the main constituent of cell membrane, its absence may disturb the membrane stability and functionality (Bargaz et al., 2021). In the same line, the present study found that the P-deficiency caused a significant increase in the content of MDA and H_2O_2 and the percentage of EL, as the key membrane destruction markers, thus in return it induced an oxidative stress and destructed the membrane integrity in the two studied chickpea varieties. Also, the significant increase in the oxidative stress markers observed under P-deficiency was accompanied with an increase in the activity of both SOD and PO in both *BO* and *MO*, with a significant difference between them. Indeed, the activity of both SOD and PO was higher in the tolerant variety *BO* than in the sensible one *MO*. The caused oxidative stress, reflected by the higher MDA and H_2O_2 contents and EL percentage, has been reported in several plant species, such as common bean (Bargaz et al., 2013a), faba bean (Oukaltouma et al., 2022) and rice (Fu et al., 2013). To overcome P-induced oxidative stress, plants evoked several mechanisms. One of the widespread mechanisms is the induction of the antioxidant system like improving the activities of antioxidant enzymes (Bargaz et al., 2015). In the same line, Oukaltouma et al. (2022) correlated the increase in the oxidative stress markers, in terms of MDA content, in P-deficient faba bean with a significant increase in the activities of polyphenol oxidase and peroxidase. Similarly, in common bean, Bargaz et al. (2013a) found that P-deficiency raised H_2O_2 content, which was induced membrane damages reflected by higher MDA content and EL. Kaya et al. (2020) observed that P-deficiency significantly increased EL and leaf H_2O_2 accumulation, but reduced SOD, PO and catalase activities in maize plants. Taken together, our findings suggested that the low-P availability remarkably induced an oxidative stress, which could be reduced photosynthetic activity, P uptake, PUE and as results, it decreased all studied growth traits.

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Conclusion

In conclusion, the results of this study indicate that the Moroccan chickpea varieties studied exhibited different levels of adaptability to P-deficiency. Indeed, P-deficiency significantly reduced plant biomass, plant height, leaf and stem number, and leaf area and the depressive effects were more obvious in *BO* than in the *MO*. P-deficiency reduced significantly P content and increased MDA and H_2O_2 content, EL, and the activity of SOD and PO. This nutrient constraint disturbed photosynthesis by reducing photosynthesis parameters in terms of total Chl and Car contents, Chl fluorescence (F_v/F_m) and OJIB kinetics. The comparison between the two studied varieties indicated that, *MO* variety was the more sensitive one to P-deficiency stress compared to *BO*.

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Conflict of interests. The authors declare no conflict of interests.

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APPENDIX

Table A1. *Results of two-way analysis of variance (ANOVA II) of P treatment and variety effects and their interactions for the considered parameters: Shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), root dry weight (RDW), shoot to root ratio (S to R), leaves number (LN), stems number (SN), plant height (PH), leaf area (LA), total chlorophyll (Chl), carotenoids (Car), Chl stability index (CSI), The minimum chlorophyll fluorescence (F_0), Quantum yield of photosystem PSII (Fv/Fm), shoot P content (SPC), root P content (RPC), P uptake, P uptake efficiency (PUpE), P utilization efficiency (PUtE), physiological P use efficiency (PPUE), electrolyte leakage (EL), malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) and peroxidase (PO)*

Dependent variables	Independents variables					
	Varieties		Phosphorus		Interactions	
	dF	F	dF	F	dF	F
SFW	1	16.39**	1	88.00***	1	0.00^{NS}
RFW	1	4.29^{NS}	1	13.89**	1	0.01^{NS}
SDW	1	8.19*	1	32.66***	1	0.00^{NS}
RDW	1	6.48*	1	15.86**	1	0.20^{NS}
S to R (DW)	1	0.11^{NS}	1	0.02^{NS}	1	0.27^{NS}
LN	1	4.17^{NS}	1	10.67*	1	0.17^{NS}
SN	1	1.50^{NS}	1	8.17*	1	0.17^{NS}
PH	1	9.42*	1	49.42***	1	2.21^{NS}
LA	1	4.94^{NS}	1	75.77***	1	4.25^{NS}
Total Chl	1	29.77**	1	40.89***	1	10.88*
CSI	1	7.17*	1	204.81***	1	7.16*
Car	1	14.00**	1	36.94***	1	0.30^{NS}
F_0	1	4.46^{NS}	1	16.26***	1	0.001^{NS}
F_v/F_m	1	5.27^{NS}	1	39.07***	1	0.02^{NS}
SPC	1	19.81**	1	308.27***	1	3.86^{NS}
RPC	1	15.10**	1	129.04***	1	6.01*
REUP	1	0.53^{NS}	1	126.91***	1	0.53^{NS}
P uptake	1	44.76***	1	248.10***	1	9.68*
PUpE	1	28.66**	1	724.00***	1	25.68**
PUtE	1	22.10**	1	363.21***	1	0.06^{NS}
PPUE	1	1.56^{NS}	1	0.88^{NS}	1	0.72^{NS}
EL	1	0.50^{NS}	1	15.28**	1	1.35^{NS}
MDA	1	0.37^{NS}	1	25.29**	1	0.75^{NS}
H_2O_2	1	8.46*	1	60.57***	1	2.45^{NS}
SOD	1	0.01^{NS}	1	79.15***	1	0.00^{NS}
PO	1	0.38^{NS}	1	76.79***	1	4.27^{NS}

*Significance at 0.05 probability level; **significance at 0.01 probability level; ***significance at 0.001 probability level; NS: non-significant at 0.05. Lower-case letters indicate significant differences at 0.05 (Tukey's test)

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