Contrasting Growth, Photosynthesis, Antioxidant Responses and Water Use Efficiency in Two *Medicago sativa* L. Genotypes under Different Phosphorus and Soil Water Conditions

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**Abstract:** Genotypic variations of alfalfa (*Medicago sativa* L.) to both phosphorus (P) deficiency and water deficiency are evident on the Loess Plateau of China. Here, we compare the adaptive mechanisms between an introduced cultivar (Arkaxiya) and a landrace (Longzhong) subjected to P- and water-limited conditions. The two genotypes were grown in a soil medium with 0, 4.2, 8.4 and 16.8 µg applied P per gram dry soil. Three water treatments were imposed (maintained at 75–90%, 45–55% and 30–35% of pot capacity (PC)) 28 days after sowing (DAS). At high soil P and high soil water content (SWC), high rates of net photosynthesis (*Pn*) contributed to greater plant growth and P-use efficiency (PUE) in the introduced Arkaxiya compared to the landrace Longzhong. However, at low SWC, Longzhong had enhanced antioxidative defense (mainly SOD and CAT) compared to Arkaxiya. In addition, shorter shoot length and greater branching in Longzhong than Arkaxiya may also facilitate adaptation to low SWC. The contrasting adaptive mechanisms of the two genotypes provide a number of early-screening parameters associated with plant growth for the selection and introduction of alfalfa targeted at different rainfall and available P environments.

**Keywords:** alfalfa; antioxidative defense; drought stress; gas exchange; phosphorus deficiency; shoot morphology

1. **Introduction**

Alfalfa is an important forage legume for the semiarid regions of the Loess Plateau of China [1,2]. In the past 25 years, the areas growing alfalfa on the Loess Plateau have expanded dramatically to meet the huge demand for forage for animal industry. However, the productivity of alfalfa is reduced by low soil-available phosphorus (P) and limited rainfall [3], and further restricted by reductions in both soil moisture and available P with increasing stand age [2,4,5]. The negative effects of water and P limitation will increase with the predicted increase in the incidence and severity of droughts with climate change on the Loess Plateau [6], as well as the future depletion of the non-renewable P resource [7]. Therefore, it is necessary to explore the adaptive mechanisms and performance of alfalfa under both P- and water-limited conditions [4,8,9].

Stomatal closure by environmental constraints, such as drought stress [10] and P deficiency [11], is likely to induce oxidative stress in plants. Oxidative stress inevitably reduces CO₂ assimilation and increases electron transfer from photosynthetic electron carriers toward O₂, and increases the reactive...
oxygen species (ROS), namely the superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (·OH) [12–14]. The disturbance of ROS homeostasis in plant cells can alter normal cellular metabolism and is associated with reduced plant growth [15].

The overproduction of ROS in plants is a common feature in response to various abiotic stresses. Fortunately, plants have evolved ROS-scavenging systems to mitigate the oxidative damage caused by ROS, including non-enzymatic and enzymatic constituents [16]. Of the non-enzymatic constituents, ascorbate and glutathione (GSH) are the most important low molecular mass and soluble antioxidants [17]. The superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are the main enzymatic compounds involved in ROS scavenging [16]. SOD catalyze, the dismutation of O$_2^-$ to H$_2$O$_2$, is considered the first defense line against ROS. The H$_2$O$_2$ is further detoxified by CAT and enzymes of the ascorbate-GSH cycle, such as APX and GR. Furthermore, the antioxidant defense of the plant to oxidative stress depends on plant genotype [15,18], development stage [19] and stress duration and intensity [18].

Comparative studies of antioxidant responses among genotypes to abiotic stresses, predominantly drought stress [15,19,20], but also P deficiency [17], are common. Further, P application improves the antioxidative defense in drought-stressed plants [14], implying that there is a complex interaction of antioxidative defense between water shortage and P deficiency. To date, few studies have focused on comparative studies among genotypes on the antioxidant responses to both water shortage and P deficiency. Previous research has shown that enhanced antioxidative defense decreases the peroxidation of lipid membranes and induces increased plant growth and productivity [15,18]. The oxidative damage to the photosynthetic machinery and the partial stomatal closure induced by drought and P deficiency is likely to reduce the rate of photosynthesis [14]. Shoot morphological adjustment, such as a reduction in leaf area, plant height and stem diameter to reduce the demand for water and P consumption, will also occur with prolonged abiotic stress [14]. As the combination of moisture and P limitation frequently occur in agricultural systems worldwide, the selection of more tolerant crop genotypes under limited moisture and P conditions is expected to be an effective way to improve crop productivity [9]. Therefore, comparative studies among plant genotypes on morphological, physiological and biochemical responses to the combination of limited water and P will be of immense importance in crop breeding and selection to minimize the negative effects of soil water and P limitation.

There are approximately three alfalfa genotypes released in China every year, indicating slow alfalfa breeding progress. Few of these genotypes released in China are adapted to the low water and low P environments on the Loess Plateau, the dominant alfalfa production area of China. Breeding and the introduction of new genotypes is an efficient way to increase the productivity of alfalfa stands, and a deeper understanding of the morphological, physiological and biochemical adaptation of alfalfa to the combined limitations of low soil moisture and low soil P is urgently needed. In the present study, two genotypes were used based on their widespread use in the cultivated areas and long-term adaptation to different rainfall regions of the Loess Plateau. The landrace, Longzhong, was selected by local famers and has been grown for more than 70 years in areas with 250–400 mm annual precipitation [21], while Arkaxiya is a genotype from the USA introduced in 1974 and grown in areas with 400–650 mm annual precipitation [22].

To understand the alfalfa response and adaptation to both water and P limitations, we compared the effect of the interaction of soil P and soil moisture on growth, morphology, gas exchange and antioxidant defense in the landrace and the introduced genotype. Additionally, the water-use efficiency and P-use efficiency of the two genotypes were evaluated. The following hypotheses were proposed: (i) the landrace will be more resistant in low soil P and low soil moisture compared to the introduced genotype, while the latter will grow better in soils with higher levels of available P and soil water; (ii) the different shoot morphological traits in the two genotypes are involved in their adaptation to soil P and soil moisture; (iii) genotypic variation in plant growth performance will be associated with different rates of photosynthesis, water-use efficiency and P-use efficiency; and (iv) differences in
antioxidative defense will be induced by low soil moisture and low soil P in the two genotypes that can explain their different growth performances.

2. Materials and Methods

2.1. Plant Materials and Experimental Set-Up

The experiment was conducted from April to June 2013 at the Yuzhong Experiment Station of Lanzhou University, Gansu Province, China (35°51’N, 104°07’E altitude 1620 m). Two alfalfa (Medicago sativa L.) genotypes, Arkaxiya (introduced genotype) and Longzhong (landrace), were used in this experiment. Seeds of both genotypes were obtained from the College of Grassland Science of Gansu Agricultural University, Lanzhou. All pot was arranged in a block design under an automatic rainout shelter (50 m long × 24 m wide × 5.7 m height) that covered the pots when rain occurred.

The air-dried soil was mixed with vermiculite in the ratio of 4:1, and then sieved by passing through a 2-mm mesh. Before filling, the soil mixture was pre-mixed with essential nutrients with final concentrations being (µg g⁻¹ dry soil) 126 N, 63 K, 21 Ca, 13.5 Mg, 37 Cl, 0.14 B, 0.014 Mn, 0.014 Zn, 0.009 Cu and 0.002 Mo. Phosphorus was added as calcium superphosphate (containing (%) 18.1 P₂O₅, 12.8 SiO₂, 2.2 Al₂O₃, 1.5 K₂O, 20.2 CaO, 1.5 MgO and 0.1 MnO) in powder form and pre-mixed in the soil mixture to reach four added-P levels: 0, 4.2, 8.4 and 16.8 µg P g⁻¹ dry soil. Finally, a total of 7.14 kg air-dried soil mixture was filled into each non-draining plastic pot (220 mm in diameter and 250 mm in height). The initial available P and pot capacity (PC) of the soil mixture was 6.9 µg g⁻¹ dry soil and 25.7% (w/w). Soil available P was determined using the Olsen-P method [23]. Seeds were vernalized at 4°C for 24 h, and then germinated in an incubation cabinet. In total, 20 seeds were sown in each pot and thinned to seven seedlings after two cotyledons emerged at 14 days after sowing (DAS). All seedlings were inoculated with Sinorhizobium meliloti ACCC17512, which was purchased from the Agricultural Culture Collection of China (Beijing). After thinning, the soil moisture for all plants was kept at 80–90% PC by watering every two days with deionized water until the commencement of the water treatments. At 28 DAS, the water treatments were imposed by withholding water until three average soil water contents (SWC) were reached: (i) SWC = 75–90% PC, well-watered (WW); (ii) SWC = 45–55% PC, moderate water shortage (MS); and SWC = 30–35% PC, severe water shortage (SS). The water was withheld until the SWC decreased close to predetermined levels of 75%, 45% and 35% PC, respectively, then re-watered to 90%, 55% and 30% PC and repeated. The three water regimes were maintained by watering pots to weight with deionized water every day after the commencement of the water treatments. At 28 DAS, the water treatments were imposed by withholding water until three average soil water contents (SWC) were reached: (i) SWC = 75–90% PC, well-watered (WW); (ii) SWC = 45–55% PC, moderate water shortage (MS); and SWC = 30–35% PC, severe water shortage (SS). The water was withheld until the SWC decreased close to predetermined levels of 75%, 45% and 35% PC, respectively, then re-watered to 90%, 55% and 30% PC and repeated. The three water regimes were maintained by watering pots to weight with deionized water every day after 18.00 h (Beijing Standard Time (BST)). At 28 and 59 DAS, the increasing fresh weight of alfalfa in each treatment was included in the overall pot weight based on an empirical equation between fresh weight and plant height established in our laboratory. The experiment was a randomized block design, with three replicates for each treatment. Each replicate consisted of 24 pots (two genotypes, three water treatments and four applied P treatments).

2.2. Gas Exchange Measurements

One day prior to final harvest (70 DAS), the net photosynthetic rate (Pn), stomatal conductance (gs) and transpiration rate (Tr) were measured with a portable open-flow gas exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, USA), between 09.30 and 11:00 h BST, on fully-expanded leaves that were all at a similar development stage. During this time, relative humidity, temperature, CO₂ concentration and photo flux density in a red-blue LED chamber of LI-6400 were maintained at ~50%, ~25 °C, 380 µmol mol⁻¹ and 1000 µmol m⁻² s⁻¹, respectively. Each replicate of the gas exchange was the mean of five readings per leaf taken shortly one after the another. The parameter for each gas exchange was a mean of five readings per leaf (five-technique replication). After measurement, the part of the leaf used to measure the gas exchange was cut and immediately scanned by a scanner (Epson Perfection V300 Photo, Seiko Epson Corp., Tokyo, Japan), and calculated with Imag j software (32-bit Java for Windows 7, National Institutes of Health, Bethesda, MD, USA). All gas exchange parameters were
standardized for leaf area and the intrinsic water use efficiency (WUE\textsubscript{i}) was calculated as the ratio of P\textsubscript{n} to transpiration rate (Tr).

2.3. Plant Measurements

Plants were harvested at 70 DAS. The plant height, branch number and stem diameter were measured. After removing and washing the roots from the soil mixture, the plant was immediately separated into leaves, stems, flowers and roots. Leaf area was measured immediately after harvest using the Epson scanner mentioned above. All samples were dried in an oven at 70 °C for 72 h and the weight of dry mass recorded.

2.4. Water-Use Efficiency and P-Use Efficiency

The water consumption was obtained by recording the added water from sowing to harvest. The water-use efficiency (WUE) was calculated by the following equation: WUE = total dry mass (DM, g)/water use (kg) from sowing to harvest. Phosphorus-use efficiency (PUE) represented the amount of total DM produced per unit of P applied, that is, PUE = Δ total DM (g)/amount of applied soil P (mg). The Δ total DM was the difference in total DM between the three levels of applied P and nil-applied P.

2.5. ROS and Lipid Peroxidation

Two forms of ROS, O\textsubscript{2}\textsuperscript{-} and H\textsubscript{2}O\textsubscript{2}, were measured. The production rate of O\textsubscript{2}\textsuperscript{-} in leaves was measured by monitoring nitrite formation from hydroxylamine, in the presence of O\textsubscript{2}\textsuperscript{-} [24]. Fresh leaves (0.3 g) were homogenized with 5 mL of 50 mM potassium phosphate (pH 7.8, ice-cold) and centrifuged at 5000 g for 600 s at 4 °C. The incubation mixture contained 1 mL of 1 mM hydroxylamine hydrochloride (dissolved in 50 mM potassium phosphate buffer (pH 7.8) as solvent) and 1 mL of supernatant. After incubation at 25 °C for 1200 s, 17 mM sulphanilamide and 7 mM α-naphthylamine were added. After additional incubation at 25 °C for 1200 s, the absorbance was measured in aqueous solution at 530 nm. H\textsubscript{2}O\textsubscript{2} was measured by monitoring the absorbance of the titanium–peroxide complex at 415 nm [25], calibrated against a standard curve with known H\textsubscript{2}O\textsubscript{2} concentrations.

Lipid peroxidation was estimated by determining malondialdehyde (MDA) as described by Zhao et al. [26]. Approximately 0.3 g of fresh leaves were ground in 5 mL of 10% trichloroacetic acid (TCA) which contained 0.5% thiobarbituric acid (TBA). The mixture was heated in a water bath at 100 °C for 0.25 h and then quickly cooled in an ice bath. After centrifugation at 4000 g for 0.25 h, the absorbance of the supernatant was recorded at 450, 532 and 600 nm. The MDA content was calculated by its absorbance [26].

2.6. Antioxidant Enzymes Assay

Fresh leaves (0.3 g) were ground up in a mortar and pestle in liquid N\textsubscript{2}. The powder was transferred to an Eppendorf tube and 4 mL of ice-cold extraction buffer, which consisted of 50 mM potassium phosphate buffer (pH 7.0), and 1% polyvinylpyrrolidone (PVP) and 1 mM EDTA were added. The homogenate was centrifuged at 12,000 g at 4 °C for 1200 s, and the supernatant was collected as crude enzyme extract for the direct assay of antioxidative enzyme activity.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) as described by Giannopolitis and Ries [27]. The reaction buffer (final volume of 2 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 100 µL crude enzyme extract. The amount of enzyme that caused 50% inhibition of reduction of NBT at 560 nm was defined as one unit of SOD activity (U).

Catalase (CAT, EC 1.11.1.6) activity was assayed by the disappearance of H\textsubscript{2}O\textsubscript{2} (extinction coefficient 39.4 mmol\textsuperscript{-1} cm\textsuperscript{-1}) at 240 nm for 180 s as described by Aebi [28]. The 2 mL reaction buffer contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H\textsubscript{2}O\textsubscript{2} and 200 µL crude enzyme extract. The reaction was initiated by adding the enzyme extract.
Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by monitoring the decrease in absorption at 290 nm (extinction coefficient 2.8 mmol$^{-1}$ cm$^{-1}$) for 60 s, as described by Amako et al. [29]. The 1 mL reaction buffer contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H$_2$O$_2$, 0.5 mM ascorbic acid and 200 µL crude enzyme extract. The reaction was initiated by H$_2$O$_2$ addition.

Glutathione reductase (GR, EC 1.6.4.2) activity was measured by the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) [30]. The reaction buffer (final volume of 1 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 0.15 mM NADPH, 2 mM Na$_2$DTA, 0.5 mM oxidized glutathione (GSSG) and 150 µL enzyme extract. The GR activity was calculated using the molar extinction coefficient of NADPH (6.2 mM$^{-1}$ cm$^{-1}$).

2.7. Statistical Analyses

The experiment was a three-factorial (genotype, applied P and water treatment) randomized complete block design. All measured variables were analyzed by general analysis of variance (ANOVA) in GenStat 17.0 statistical package (VSN International Ltd., Rothamsted, UK). There were no transformations required to meet ANOVA assumptions. The significant differences were determined using ANOVA at $p < 0.05$% and differences between treatments were identified using Duncan’s multiple range test. All figures were drawn by SigmaPlot 12.5 software (Systat Software, San Jose, CA, USA).

3. Results

3.1. Plant Growth and Dry Mass Allocation

Plant growth responses to applied P varied between the two genotypes (Table 1, Figure 1 and Figure S1). The total dry mass of both genotypes increased with soil P supply ($p < 0.05$) and reached maximum values at the highest applied P level (16.8 µg P g$^{-1}$ dry soil). The total dry mass of Arkaxiya was significantly higher than that of Longzhong at applied P levels of 8.4 and 16.8 µg P g$^{-1}$ dry soil in the WW and MS treatments, but only at 16.8 µg P g$^{-1}$ dry soil in the SS treatment. There was no difference in total dry mass between the two genotypes with nil-P applied in soil at all three soil water treatments.

A similar pattern of responses to applied P in dry mass was observed in the shoots (including leaves and stems, see Figure S1) and roots (Table 1, Figure 1). In the WW treatment, a similar amount of dry mass was allocated to roots as to leaves and stems (Table S1 and Figure S1), whereas a greater proportion of dry mass was allocated to roots with limited water supply (MS and SS treatments, Table S1). In the WW and MS treatments, both genotypes were flowering at harvest in the applied P treatments, while the alfalfa was still vegetative at nil-applied P (Figure S1). In the SS treatment, there were no flowers except in Arkaxiya at 8.4 and 16.8 µg P g$^{-1}$ dry soil (Figure S1).
Table 1. Significance of the sources of variability.

| Character                        | Source of Variability |
|----------------------------------|-----------------------|
|                                  | G         | P       | W       | G × P      | G × W      | P × W     | G × P × W |
| Total dry mass (g plant⁻¹)       | *** (0.050) | *** (0.071) | *** (0.061) | *** (0.100) | *** (0.087) | *** (0.123) | n.s.     |
| Shoot dry mass (g plant⁻¹)       | *** (0.037) | *** (0.053) | *** (0.046) | * (0.074) | * (0.064) | *** (0.091) | n.s.     |
| Root dry mass (g plant⁻¹)        | *** (0.024) | *** (0.036) | *** (0.031) | *** (0.051) | *** (0.044) | *** (0.061) | ** (0.087) |
| Plant height (mm)                |              | *** (1.5) | *** (2.1) | *** (1.8) | n.s. | n.s. | *** (3.6) | n.s.     |
| Number of branches per plant     | *** (0.15) | *** (0.21) | *** (0.18) | n.s. | n.s. | n.s. | n.s.     |
| Total leaf area (cm² plant⁻¹)    |              | *** (8.5) | *** (7.3) | n.s. | n.s. | n.s. | n.s.     |
| Stem diameter at base (mm)       | *** (0.17) | *** (0.24) | *** (0.21)| * (0.34) | *** (0.29) | *** (0.41) | n.s.     |
| Net photosynthetic rate (Pn) (µmol CO₂ m⁻² s⁻¹) | *** (0.51) | *** (0.72) | *** (0.62) | n.s. | *** (0.88) | *** (1.25) | n.s.     |
| Stomatal conductance (gs) (mmol H₂O m⁻² s⁻¹) | * (6.3) | * (8.9) | *** (7.7) | n.s. | n.s. | n.s. | n.s.     |
| Transpiration rate (Tr) (mmol H₂O m⁻² s⁻¹) | *** (0.09) | *** (0.12) | *** (0.11) | n.s. | n.s. | ** (0.21) | n.s.     |
| Intrinsic water use efficiency (WUEₙ, p₊Tr) | n.s. | *** (0.22) | n.s. | n.s. | *** (0.27) | *** (0.38) | n.s.     |
| Water-use efficiency (WUE) (g total DM kg⁻¹ H₂O) | * (0.057) | *** (0.081) | *** (0.070) | n.s. | * (0.099) | *** (0.139) | * (0.197) |
| P-use efficiency (PUE) (g total DM mg⁻¹ applied P) | ** (0.010) | *** (0.012) | *** (0.012) | n.s. | * (0.017) | *** (0.021) | n.s.     |
| Production rate of reactive oxygen species (O₂⁻) (nmol g⁻¹ FW min⁻¹) | n.s. | *** (0.28) | *** (0.24) | n.s. | * (0.34) | n.s. | n.s.     |
| Hydrogen peroxide concentration (H₂O₂) (µmol g⁻¹ FW) | * (0.22) | *** (0.31) | *** (0.27) | n.s. | ** (0.38) | n.s. | n.s.     |
| Malondialdehyde (MDA) concentration (nmol g⁻¹ FW) | * (0.26) | *** (0.37) | *** (0.32) | n.s. | ** (0.45) | n.s. | n.s.     |
| Superoxidae dismutase (SOD) activity (U g⁻¹ FW) | n.s. | *** (5.74) | *** (4.94) | * (8.12) | *** (7.03) | n.s. | n.s.     |
| Catalase (CAT) activity (µmol g⁻¹ FW min⁻¹) | n.s. | *** (1.9) | *** (1.6) | n.s. | *** (2.3) | ** (3.3) | n.s.     |
| Ascorbate peroxidase (APX) activity (µmol g⁻¹ FW min⁻¹) | ** (9.1) | *** (12.9) | *** (11.1) | n.s. | n.s. | * (22.3) | n.s.     |
| Glutathione reductase (GR) activity (µmol g⁻¹ FW min⁻¹) | n.s. | *** (0.028) | *** (0.024) | n.s. | n.s. | n.s. | n.s.     |

Significant effects are indicated for genotypes (G), phosphorus application (P), soil water treatment (W) and their interactions (n.s.—not significant, * p < 0.05, ** p < 0.01, *** p < 0.001). The numbers in parentheses are the least significance differences (LSD) at p = 0.05 level.
A similar pattern of responses to applied P in dry mass was observed in the shoots (including leaves and stems, see Figure S1) and roots (Table 1, Figure 1). In the WW treatment, a similar amount of dry mass was allocated to roots as to leaves and stems (Table S1 and Figure S1), whereas a greater proportion of dry mass was allocated to roots with limited water supply (MS and SS treatments, Table S1). In the WW and MS treatments, both genotypes were flowering at harvest in the applied P treatments, while the alfalfa was still vegetative at nil-applied P (Figure S1). In the SS treatment, there were no flowers except in Arkaxiya at 8.4 and 16.8 μg P g⁻¹ dry soil (Figure S1).

3.2. Shoot Morphology

The limited-water treatments reduced the plant height, individual branch number, total leaf area and stem diameter at the base in both genotypes (Table 1, Figure 2). In the SS treatment, plant height in Arkaxiya was generally higher than Longzhong, while individual branch number was reduced. The plant height of both genotypes was increased with applied P in the WW and MS treatments, while it remained at a similar height in the SS treatment (Table 1, Figure 2). For both genotypes, the individual branch numbers increased at 4.2 μg P g⁻¹ dry soil in the WW treatment and 8.4 μg P g⁻¹ dry soil in the MS treatment, but did not increase further when more P was applied (Table 1, Figure 2). In the SS treatment, the branch numbers reached a maximum at the highest applied P (16.8 μg P g⁻¹ dry soil) (Figure 2).

Figure 1. Changes in total dry mass (DM) (a), shoot DM (b) and root DM (c) of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars) grown for 10 weeks with four applied P levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS). Values are means ± one standard error of the mean (n = 3). Different letters between bars represent significant differences between means at p < 0.05.
Figure 2. Changes in plant height (a), number of branches per pot (b), total leaf area (c) and stem diameter at base (d) of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars), grown for 10 weeks with four applied P levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS). Values are means ± one standard error of the mean (n = 3). Different letters between bars represent significant differences between means at p < 0.05.

The total leaf area varied from 24 to 168 cm² plant⁻¹ in the different water and P treatments, increasing with the maintenance of higher SWC and P supply (Figure 2). There was no significant difference in total leaf area between the two genotypes in all three water treatments, except for that at 16.8 µg P g⁻¹ dry soil in the WW treatment (Table 1, Figure 2). The mean diameter of the stem base varied from 2.6 to 5.5 mm in the water and P treatments (Figure 2). For both genotypes, the diameter at base of the stem increased at 4.2 µg P g⁻¹ dry soil in the WW treatment and 8.4 µg P g⁻¹ dry soil in MS treatment, and did not increase further when more P was applied (Figure 2). In the WW treatment, the stem diameter at the base was higher in Longzhong than Arkaxiya at nil-P applied in soil, and this difference was not observed as soil P applied increased (Figure 2d). In MS and SS treatment, the stem diameter at base was higher in Arkaxiya than Longzhong at 8.4 and 16.8 µg P g⁻¹ dry soil, and there was no difference at 0 and 4.2 µg P g⁻¹ dry soil.
3.3. Gas Exchange

The net photosynthetic rate ($Pn$) of both genotypes increased with increased applied P, and the increase in $Pn$ was higher with the increase in applied-P in WW (11–22 $\mu$mol m$^{-2}$ s$^{-1}$) and MS (11–18 $\mu$mol m$^{-2}$ s$^{-1}$) than in SS (10–13 $\mu$mol m$^{-2}$ s$^{-1}$) (Table 1, Figure 3a). $Pn$ was higher in Arkaxiya than Longzhong, at 0, 8.4 and 16.8 $\mu$g P g$^{-1}$ dry soil in the WW treatment and 0, 4.2 and 16.8 $\mu$g P g$^{-1}$ dry soil in the MS treatment, but not in the SS treatment where there was no difference between the two genotypes (Table 1, Figure 3a). There was no difference in stomatal conductance ($gs$) between Arkaxiya and Longzhong in all three water treatments (Table 1, Figure 3b). The narrow variation in $gs$ was found in both genotypes and changed little (<20%) with the four applied P treatments; it was <30% in all three water treatments. The responses of transpiration rate ($Tr$) to the different water and P treatments were similar to those seen for $gs$ in both genotypes, varying from 2.1 to 3.6 mmol H$_2$O m$^{-2}$ s$^{-1}$ (Table 1, Figure 3c). The intrinsic water use efficiency (WUE$_i$) for both genotypes increased with applied P in the WW and MS treatments (Table 1, Figure 3d), whereas in the SS treatment, the WUE$_i$ in Longzhong increased to a maximum at 4.2 $\mu$g P g$^{-1}$ dry soil and remained the same as applied P increased further, but in Arkaxiya it was similar at all levels of applied P (Figure 3d). There was no difference in WUE$_i$ between the two genotypes at nil-applied P in all three water treatments, while WUE$_i$ was higher in Arkaxiya than in Longzhong at 16.8 $\mu$g P g$^{-1}$ dry soil in the WW. In contrast, a higher WUE$_i$ in Longzhong than Arkaxiya was observed at 8.4 $\mu$g P g$^{-1}$ dry soil in the SS treatment.

3.4. Water-Use Efficiency and Phosphorus-Use Efficiency

The water-use efficiency (WUE) for both genotypes increased markedly with applied P, but the water treatment had only a small (but significant) effect on WUE (Table 1, Figure 4a). There was no difference between two genotypes in WUE at any soil P applied levels in all three water treatments. Phosphorus-use efficiency (PUE) is the change in plant growth per unit of P applied, which decreased in both genotypes as the level of applied P increased in the WW and MS treatments, but not in the SS treatment (Table 1, Figure 4b). The higher PUE in Arkaxiya than Longzhong was observed at 8.4 and 16.8 $\mu$g P g$^{-1}$ dry soil in the WW treatment, while there was no difference in the MS and SS treatments.

3.5. Relative Oxygen Species (ROS) and Lipid Membrane Peroxidation

$O_2^-$ production and H$_2$O$_2$ content of the leaves in both genotypes increased with a reduction in SWC and applied P (Table 1, Figure 5a,b), reaching maxima at nil-applied P in the SS treatment. The ANOVA showed no difference in $O_2^-$ production ($p > 0.05$), and a significant difference in H$_2$O$_2$ concentration ($p < 0.05$) in the leaves of the two genotypes (Table 1). The $O_2^-$ production was higher in Longzhong than Arkaxiya at nil-P applied soil in the WW treatment (Figure 5a). Meanwhile, the H$_2$O$_2$ concentrations of the leaves of Arkaxiya were similar to those of Longzhong in most cases, and were only higher in Longzhong than Arkaxiya at 4.2 $\mu$g P g$^{-1}$ dry soils in the WW and MS treatments (Figure 5b). Lipid membrane peroxidation, as measured by MDA content (Figure 5c), was lower in Arkaxiya than Longzhong at 0 and 4.2 $\mu$g P g$^{-1}$ dry soils in the MS treatments. Under severe water shortage (SS treatment), there was no difference in MDA concentration between two genotypes.
3.3. Gas Exchange

The net photosynthetic rate ($P_n$) of both genotypes increased with increased applied P, and the increase in $P_n$ was higher with the increase in applied-P in WW (11–22 $\mu$mol m$^{-2}$ s$^{-1}$) and MS (11–18 $\mu$mol m$^{-2}$ s$^{-1}$) than in SS (10–13 $\mu$mol m$^{-2}$ s$^{-1}$) (Table 1, Figure 3a).

$P_n$ was higher in Arkaxiya than Longzhong, at 0, 8.4 and 16.8 $\mu$g P g$^{-1}$ dry soil in the WW treatment and 0, 4.2 and 16.8 $\mu$g P g$^{-1}$ dry soil in the MS treatment, but not in the SS treatment where there was no difference between the two genotypes (Table 1, Figure 3a). There was no difference in stomatal conductance ($g_s$) between Arkaxiya and Longzhong in all three water treatments (Table 1, Figure 3b). The narrow variation in $g_s$ was found in both genotypes and changed little (<20%) with the four applied P treatments; it was <30% in all three water treatments. The responses of transpiration rate ($T_r$) to the different water and P treatments were similar to those seen for $g_s$ in both genotypes, varying from 2.1 to 3.6 mmol H$_2$O m$^{-2}$ s$^{-1}$ (Table 1, Figure 3c). The intrinsic water use efficiency (WUE$_i$) for both genotypes increased with applied P in the WW and MS treatments (Table 1, Figure 3d), whereas in the SS treatment, the WUE$_i$ in Longzhong increased to a maximum at 4.2 $\mu$g P g$^{-1}$ dry soil and remained the same as applied P increased further, but in Arkaxiya it was similar at all levels of applied P (Figure 3d). There was no difference in WUE$_i$ between the two genotypes at nil-applied P in all three water treatments, while WUE$_i$ was higher in Arkaxiya than in Longzhong at 16.8 $\mu$g P g$^{-1}$ dry soil in the WW. In contrast, a higher WUE$_i$ in Longzhong than Arkaxiya was observed at 8.4 $\mu$g P g$^{-1}$ dry soil in the SS treatment.

Figure 3. Changes of photosynthesis ($P_n$) (a), stomatal conductance ($g_s$) (b), transpiration rate ($T_r$) (c) and intrinsic water use efficiency (WUE$_i$) (d) of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars), grown for 10 weeks with four P supply levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS). Values are means ± one standard error of the mean ($n = 3$). Different letters between bars represent significant differences between means at $p < 0.05$. 
moisture and applied P limitations (Figure 6), whereas the CAT activity was lower in the SS than in the Longzhong at nil-P applied soil in the MS treatment (Figure 6). Under severe water shortage (SS treatment), there was no difference in MDA concentration between two genotypes. The ANOVA showed that there were no significant differences between genotypes in the activities of SOD, CAT or GR (Table 1, Figure 5b). Lipid membrane peroxidation, as measured by MDA content (Figure 5c), was lower in Arkaxiya than Longzhong at 0 and 4.2 μg P g⁻¹ dry soil in the WW treatment, while there was no difference in the PUE in Arkaxiya than Longzhong was observed at 8.4 and 16.8 μg P g⁻¹ dry soil in the MS treatment (Figure 5a). Meanwhile, the APX activity was higher in Arkaxiya than Longzhong at 4.2 μg P g⁻¹ dry soil in the MS treatment (Figure 6b). The ANOVA showed no difference in O₂− production and H₂O₂ content of the leaves in both genotypes increased with a reduction in SWC and applied P (Table 1, Figure 5a,b), reaching maxima at nil-applied P in the SS treatment. The water treatment had only a small (but significant) effect on WUE (Table 1, Figure 4a). There was no decrease in both genotypes as the level of applied P increased in the WW and MS treatments, but were only higher in Longzhong than Arkaxiya at 4.2 μg P g⁻¹ dry soil in the WW and 4.2 μg P g⁻¹ dry soil in the MS treatment (Figure 6). Values are means + one standard error of the mean (n = 3). Different letters between bars represent significant differences between means at p < 0.05.

Figure 4. Water-use efficiency (a) and P-use efficiency (b) of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars), grown for 10 weeks with four P supply levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS). Values are means + one standard error of the mean (n = 3). Different letters between bars represent significant differences between means at p < 0.05.

Figure 5. Production rate of reactive oxygen species (O₂−) (a), hydrogen peroxide concentration (H₂O₂) (b) and malondialdehyde (MDA) concentration (c) per gram fresh weight (FW) of leaves of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars), grown for 10 weeks with four P supply levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS). Values are means + one standard error of the mean (n = 3). Different letters between bars represent significant differences between means at p < 0.05.

3.6. Antioxidant Enzyme Activities

The activity of antioxidant enzymes in the leaves of both genotypes was increased by both soil moisture and applied P limitations (Figure 6), whereas the CAT activity was lower in the SS than in the
MS treatment (Figure 6b). The ANOVA showed that there were no significant differences between genotypes in the activities of SOD, CAT or GR ($p > 0.05$), but there was a significant difference in APX ($p < 0.01$) in the leaves (Table 1). In the WW treatment, there were no differences in the activities of antioxidant enzymes between the two genotypes (Figure 6). APX activity was higher in Arkaxiya than Longzhong at nil-P applied soil in the MS treatment (Figure 6b). Under severe water shortage (SS treatment), higher SOD activity at 4.2 and 8.4 $\mu$g P g$^{-1}$ dry soil was observed (Figure 6a).

### Figure 6. Activity of superoxidae dismutase (SOD) (a), catalase (CAT) (b), ascorbate peroxidae (APX) (c) and glutathione reductase (GR) (d) per gram fresh weight (FW) of leaves of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars), grown for 10 weeks with four P supply levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS). Values are means $\pm$ one standard error of the mean ($n = 3$). Values are means $\pm$ one standard error of the mean ($n = 3$). Different letters between bars represent significant differences between means at $p < 0.05$.

### 4. Discussion

The study compared the morphological, eco-physiological and biochemical responses to both soil P and soil moisture limitation between two alfalfa genotypes, both widely grown on Loess Plateau, but preferred by farmers in regions with different annual precipitation. The underground mechanisms, such as root morphology and physiology, in response to low soil P and limited soil water have been
discussed in a separate paper [1]. In this paper, the genotypic variations in aboveground mechanisms are reported. We address the implications and other related findings of this study below.

4.1. Plant Growth, Dry Matter Allocation and Morphology

The growth of both genotypes was significantly restricted by low soil P and reduced soil water supply (Figures 1 and 2), which is consistent with previous studies in grass species [8], trees [14] and crops [31]. A higher total DM was found in Arkaxiya than in Longzhong in the WW and MS treatments, and this difference increased with increased applied P (Figure 1). These results suggest that the introduced genotype (Arkaxiya) is better adapted to high soil moisture conditions, compared with the landrace (Longzhong), and helps to explain why Arkaxiya is preferred by farmers on the Loess Plateau in areas with higher precipitation. This finding partly supports the hypothesis (i) ‘that the landrace will be more resistant in low soil P and low soil moisture compared with the introduced genotype, while the latter will grow better in soils with higher levels of available P and soil water’, but the landrace (Longzhong) did not have a growth advantage in the SS treatment. We speculate that, in low soil P and limited water supply, the productivity potential of the landrace in this study may be limited by its short growth duration (only 70 days). We suggest that the landrace (Longzhong) will show greater growth in low applied P and limited soil moisture if the growth duration is extended. Our previous field study showed that Longzhong was well adapted to low soil P when the annual precipitation was 323 mm [4], possibly because of its greater resource exploration and capture in the field. We evaluated the plant growth of alfalfa in response to limited soil water and P condition, and P application increased the plant height and branches, thus alleviating the effects of drought on plant growth. We speculate that P application significantly increased alfalfa productivity [4], potentially because of increased plant height and branches. Thus, it is important to understand the plants’ responses to both soil water and P limitations, which reflect the multiple environmental limits in the field and natural system [9,31].

The alfalfa allocated proportionally more DM to roots than leaves or stems in the MS and SS treatments, while DM was distributed proportionally among roots, leaves and stems in the WW treatment (Figure 1). This means that the plants allocated more DM to roots when water was limited in order to increase root exploration of the soil volume [15]. However, when the amount of P applied was reduced, the biomass allocated to roots did not change markedly (Figure 1), which is in line with results of Wouterlood et al. [32] but contradicts the findings of Suriyagoda et al. [8]. According to optimal allocation theory, plants should allocate biomass to increase their uptake of the resource that is most limiting growth [33]. Our findings indicate that alfalfa roots were more sensitive to limited water than limited P, and thus allocated more assimilates to roots in response to water limitation rather than limited P (Table S1, Figure 1). In our previous study, we found that the shoot P concentration of the alfalfa plant was more stable in responding to soil moisture and P applied, while the root P concentration was dramatically increased with soil P applied [1]. Alternatively, the root P concentration of alfalfa plant was more sensitive to limited P than limited water, and decreased with reduced soil P [1]. It is too early to conclude here that the pattern of dry mass allocated to the roots is not governed by root P concentration just on two alfalfa genotypes. Further, research on P concentration and dry mass allocation with more genotypes under limited soil water and limited P is required in future. Previous studies have demonstrated that more dry mass is allocated to roots of alfalfa under drought [19] or P shortage [34] alone. Chen et al. [9] demonstrated that dry mass allocation to roots depends on the duration of the P shortage. Thus, the interaction of both water and P limitation with dry mass allocation is complex. The low moisture and low applied P delayed flowering to different extents in the two genotypes (Figure S1), indicating that flowering progress is determined by both endogenous genetic components and environmental factors [35]. Future studies on the underlying regulatory mechanisms of flowering progress in alfalfa under different soil water and P would be beneficial.

All shoot morphological parameters in both genotypes, such as plant height, branch number, total leaf area and stem diameter at the base, were reduced by low water supply and low applied P
(Figure 2), which is line with a previous study in *Phoebe zhennan* [14]. A consequence of long-term adaptation to both low water supply and low soil P is reduced total leaf area and reduced water loss by transpiration [8]. There was no difference in total leaf area between the two genotypes in this study (Figure 2c), but obvious genotypic variation in plant height and branching was observed. The different shoot morphologies of the two alfalfa genotypes in term of plant height and branching may be related to their adaptation to environments with different annual rainfalls, but it is too early to conclude from this investigation based on just two genotypes. In the same low rainfall region in which the landrace (Longzhong) is grown, Fang et al. [36] found that the plant height of the resprouting shrub *Caragana korshinskii* Kom. was restricted by limited hydraulic conductance [36], implying that the restricted plant height growth of branching shrubs is the result of adaptation to low rainfall conditions. Previous studies have shown that hydraulic conductance was reduced by P deficiency [37] and drought stress [38]. Whether there are linkages between plant height and hydraulic conductance in different alfalfa genotypes has not been documented to date; this is worth investigating in future. Similar to shoot morphology in the shrub of Fang et al. [36], we speculate that the shorter shoot length and greater branching of Longzhong facilitates an adaptation to severe drought conditions. Thus, our findings potentially support the hypothesis (ii) that ‘the different shoot morphological traits in the two genotypes are involved in their adaptation to soil P and soil moisture’.

### 4.2. Photosynthesis and Water- and P-Use Efficiency

Previous studies have shown that the net photosynthetic rate (*Pn*) and stomatal conductance (*gs*) were reduced by drought stress [15,19,20] and P deficiency [8,14]. Stomatal closure is considered a key factor underlying the reduction in *Pn* under stress conditions [14], and that *gs* is mediated by endogenous abscisic acid (ABA) accumulation and dehydration in leaves [15].

The intrinsic water use efficiency (WUEi) is considered to be an important component of plant adaption to drought, which can be mediated by soil P supply [14]. In this study, WUEi was higher in the introduced genotype than the landrace in the WW treatment with highest applied soil P (16.8 µg P g\(^{-1}\) dry soils), and was lower in the SS treatment at the intermediate applied level of 8.4 µg P g\(^{-1}\) dry soil (Figure 3d). The higher WUEi and PUE in Arkaxiya in the WW and MS treatments compared to Longzhong helps explain the better adaptation of Arkaxiya to higher rainfall environments. Thus, our findings basically supported the hypothesis (iii) that ‘genotypic variation in plant growth performance will be associated with different rates of photosynthesis, water-use efficiency and P-use efficiency’.

### 4.3. ROS Production, Lipid Peroxidation, and Antioxidative Defense System

The over-production of ROS can be induced by drought [15,20] and P deficiency [18], as well as when both limitations are imposed at the same time [14], and may cause oxidative damage to the photosynthetic apparatus, lipids, proteins and DNA [16]. The ROS production and MDA content were found to increase with limited water supply and low applied P (Figure 6). Interestingly, the introduced genotype (Arkaxiya) led to lower ROS production and MDA content than the landrace (Longzhong) in the WW and MS treatments, indicating genotypic variation in stress-induced ROS and lipid peroxidation in alfalfa. However, in the SS treatment, the ROS production and MDA content in the leaves of Arkaxiya were similar to those of Longzhong (Figure 5). A recent study showed that the drought tolerance of alfalfa genotypes, at least in part, depended on enhanced antioxidative protection and a decrease in lipid peroxidation [20]. When the plants were subjected to both soil P and soil moisture limitation, the activities of SOD, CAT, APX and GR were found to increase in both genotypes (Figure 6), suggesting an effective antioxidative defense system for alfalfa in response to limited water and P supply. Our findings revealed higher APX activity in Arkaxiya at nil-applied P in the MS treatment, and higher SOD and CAT in Longzhong than Arkaxiya at 4.2 and 8.4 µg P g\(^{-1}\) dry soils in the SS treatment (Figure 6). These findings partly support the hypothesis (iv) that ‘differences in antioxidative defense will be induced by low soil moisture and low soil P in the two genotypes that
can explain their different growth’. In this study, plants adjusted their shoot morphology in adaptation to water and P limitations to partly alleviate the sharp accumulation of ROS and MDA (both < 2-fold). Considering to the dramatic response of antioxidant defenses to drought alone [19,20], we need to better understand the antioxidant defenses of plants under multiple stressors.

4.4. Whole Plant Responses to Both Water and P Limitations

The adaptative mechanisms in response to both water and P limitation are complex [1,8,9,30]. There are no genotypic differences in the relative total dry mass in most treatments (Figure S2a), except for relative total dry mass at 4.2 and 8.4 µg P g⁻¹ dry soil in the SS treatment. Meanwhile, no genotypic difference in relative plant height and relative branch numbers were observed (Figure S2b,c). We found a similar inherent response (i.e., total dry mass, plant height and branches number) to soil water and P deficiency between Arkaxiya and Longzhong. In addition to the aboveground responses, the plant has evolved underground responses to optimize the water and P uptake [1,9]. In our previous study, we found that increased specific root length and carboxylates in the rhizosphere contribute to P uptake and growth in alfalfa [9]. The colonization with arbuscular mycorrhizal (AM) fungi has been found to enhance plant P uptake under drought [9]. The morphological and physiological plasticities in responses to both water and P limitation are linked to each other through the carbon economy. Thus, the potential tradeoff or cooperation among the adaptive mechanisms in response to environmental stress should be emphasized [1,39].

5. Conclusions

The differences between the introduced genotype (Arkaxiya), with its higher photosynthetic rate (Pn) and higher plant growth compared to the landrace (Longzhong), have enabled us to better understand the alfalfa’s response and adaptation to both water and P deficits. We conclude that the introduced genotype produced more shoot and root dry mass than the landrace when water and available P were plentiful. The greater height and number of branches, that is, the shoot morphological traits, are associated with the greater growth in the introduced genotype than the landrace, and the different adaptations of the two genotypes to water and P deficits. However, physiological traits, such as leaf photosynthesis, transpiration and WUE, did vary between the two genotypes, but were not affected by P deficits and water shortage to the same degree as the morphological traits. Finally, we conclude that differences in antioxidative defense induced by low soil moisture and low soil P in the two genotypes did not completely explain their different growth performances. At the severe water stress level there were no differences in plant growth between the two genotypes, but the landrace (Longzhong) had enhanced antioxidative defenses (mainly SOD and CAT) compared with the introduced genotype (Arkaxiya). As the different shoot morphologies, namely the short height with more branches in the landrace and tall height with fewer branches in the introduced genotype, are linked to their adaptation to soil moisture and P supply, the short, high-branching habit may provide a simple selection tool for alfalfa for low rainfall and low available P environments.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/10/1534/s1, Figure S1: Changes in leaf (a), stem (b) and flower (c) dry mass of two alfalfa cultivars, Arkaxiya (white bars) and Longzhong (grey bars) grown for 10 weeks with four applied P levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS); Figure S2: Relative total dry mass (a), relative plant height (b) and relative branch number (c) of two alfalfa genotypes, Arkaxiya (white bars) and Longzhong (grey bars) grown for 10 weeks with four applied P levels and three water treatments: (i) well-watered (WW); (ii) moderate water shortage (MS); and (iii) severe water shortage (SS); Table S1: The dry mass fraction of roots, leaves, stems and flowers of two alfalfa cultivars, Arkaxiya and Longzhong grown for 10 weeks with four applied P levels, ranging from 0, 4.2, 8.4 and 16.8 µg P g⁻¹ dry soil and three water treatments: (i) maintained at 75-90% of pot capacity (PC) [well-watered (WW)]; (ii) maintained at 45-55% PC [moderate water shortage (MS)]; and (iii) maintained at 30-35% PC [severe water shortage (SS)].

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**References**

1. Fan, J.W.; Du, Y.L.; Turner, N.C.; Wang, B.R.; Fang, Y.; Xi, Y.; Guo, X.R.; Li, F.M. Changes in root morphology and physiology to limited phosphorus and moisture in a locally-selected cultivar and an introduced cultivar of *Medicago sativa* L. growing in alkaline soil. *Plant Soil* 2015, 392, 215–226. [CrossRef]
2. Jia, Y.; Li, F.M.; Wang, X.L.; Xu, J.Z. Dynamics of soil organic carbon and soil fertility affected by alfalfa productivity in a semiarid agro-ecosystem. *Biogeochemistry* 2006, 80, 233–243. [CrossRef]
3. Jiang, H.M.; Jiang, J.P.; Jia, Y.; Li, F.M.; Xu, J.Z. Soil carbon pool and effects of soil fertility in seeded alfalfa fields on the semi-arid Loess Plateau in China. *Soil Biol. Biochem.* 2006, 38, 2350–2358. [CrossRef]
4. Fan, J.W.; Du, Y.L.; Wang, B.R.; Turner, N.C.; Wang, T.; Abbott, L.K.; Stefanova, K.; Siddique, K.H.M.; Li, F.M. Forage yield, soil water depletion, shoot nitrogen and phosphorus uptake and concentration, of young and old stands of alfalfa in response to nitrogen and phosphorus fertilisation in a semiarid environment. *Field Crop Res.* 2016, 198, 247–257. [CrossRef]
5. Gu, Y.J.; Han, C.L.; Fan, J.W.; Shi, X.P.; Kong, M.; Shi, X.Y.; Siddique, K.H.M.; Zhao, Y.Y.; Li, F.M. Alfalfa forage yield, soil water and P availability in response to plastic film mulch and P fertilization in a semiarid environment. *Field Crop Res.* 2018, 215, 94–103. [CrossRef]
6. Turner, N.C.; Molyneux, N.; Yang, S.; Xiong, Y.C.; Siddique, K.H.M. Climate change in south-west Australia and north-west China: Challenges and opportunities for crop production. *Crop. Pasture Sci.* 2011, 62, 445–456. [CrossRef]
7. Cordell, D.; Drangert, J.O.; White, S. The story of phosphorus: Global food security and food for thought. *Glob. Environ. Chang.* 2009, 19, 292–305. [CrossRef]
8. Suriyagoda, L.D.; Ryan, M.H.; Renton, M.; Lambers, H. Multiple adaptive responses of Australian native perennial legumes with pasture potential to grow in phosphorus- and moisture-limited environments. *Ann. Bot.* 2010, 105, 755–767. [CrossRef]
9. Suriyagoda, L.D.; Ryan, M.H.; Renton, M.; Lambers, H. Plant responses to limited moisture and phosphorus availability: A meta-analysis. *Adv. Agron.* 2014, 124, 143–200. [CrossRef]
10. Jiang, M.; Zhang, J. Role of abscisic acid in water stress-induced antioxidant defense in leaves of maize seedlings. *Free Radic. Res.* 2002, 36, 1001–1015. [CrossRef]
11. Desai, S.; Naik, D.; Cumming, J.R. The influence of phosphorus availability and *Laccaria bicolor* symbiosis on phosphate acquisition, antioxidant enzyme activity, and rhizospheric carbon flux in *Populus tremuloides*. *Mycorrhiza* 2014, 24, 369–382. [CrossRef]
12. Asada, K. The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1999, 50, 601–639. [CrossRef] [PubMed]
13. Plaxton, W.C.; Tran, H.T. Metabolic adaptation of phosphate-starved plants. *Plant Physiol.* 2011, 156, 1006–1015. [CrossRef] [PubMed]
14. Tariq, A.; Pan, K.; Olatunji, O.A.; Graciano, C.; Li, Z.; Sun, F.; Sun, X.; Song, D.; Chen, W.; Zhang, A.; et al. Phosphorous Application Improves Drought Tolerance of Phoebe zhennan. *Front. Plant Sci.* 2017, 8, 1561. [CrossRef] [PubMed]
15. Du, Y.L.; Wang, Z.Y.; Fan, J.W.; Turner, N.C.; Wang, T.; Li, F.M. β-Aminobutyric acid increases abscisic acid accumulation and desiccation tolerance and decreases water use but fails to improve grain yield in two spring wheat cultivars under soil drying. *J. Exp. Bot.* 2012, 63, 4849–4860. [CrossRef]
16. Apel, K.; Hirt, H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 2004, 55, 373–399. [CrossRef]
17. Noctor, G.; Foyer, C.H. Ascorbate and glutathione: Keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1998, 49, 249–279. [CrossRef]
18. Chen, S.; Zhao, H.; Ding, G.; Xu, F. Genotypic differences in antioxidant response to phosphorus deficiency in Brassica napus. *Plant Soil* 2015, 391, 19–32. [CrossRef]

19. Du, Y.L.; Wang, Z.Y.; Fan, J.W.; Turner, N.C.; He, J.; Wang, T.; Li, F.M. Exogenous abscisic acid reduces water loss and improves antioxidant defence, desiccation tolerance and transpiration efficiency in two spring wheat cultivars subjected to a soil water deficit. *Funct. Plant Biol.* 2013, 40, 494–506. [CrossRef]

20. Zhang, C.; Shi, S.; Liu, Z.; Yang, F.; Yin, G. Drought tolerance in alfalfa (*Medicago sativa* L.) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation. *J. Plant Physiol.* 2019, 232, 226–240. [CrossRef]

21. Jia, Y.; Li, F.M.; Wang, X.L. Soil quality responses to alfalfa watered with a field micro-catchment technique in the Loess Plateau of China. *Field Crop Res.* 2006, 95, 64–74. [CrossRef]

22. Xu, H.Z.; Yang, Z.P.; Xian, C.Z.; Li, S.Z.; Hu, Y.R.; Yu, H.Y.; Yu, J.Y. Research report of an alfalfa introduction trail. *Pratac Sci.* 2005, 22, 47–52.

23. Olsen, S.R.; Cole, C.V.; Watanabe, F.S. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. *USDA Circ.* 1954, 939, 1–19.

24. Elstner, E.F.; Heupel, A. Inhibition of nitrite formation from hydroxylammoniumchloride: A simple assay for superoxide dismutase. *Anal. Biochem.* 1976, 70, 616–620. [CrossRef]

25. Brennan, T.; Frenkel, C. Involvement of hydrogen peroxide in the regulation of senescence in pear. *Plant Physiol.* 1977, 59, 411–416. [CrossRef] [PubMed]

26. Zhao, S.; Xu, C.; Zou, Q.; Meng, Q. Improvements of method for measurement of malondialdehyde in plant tissues. *Plant Physiol. Commun.* 1994, 30, 207–210.

27. Giannopolitis, C.N.; Ries, S.K. Superoxide dismutase: I. Occurrence in higher plants. *Plant Physiol.* 1977, 59, 309–314. [CrossRef]

28. Aebi, H. Catalase in vitro. In *Methods in Enzymology*; Packer, L., Ed.; Academic Press: London, UK, 1984.

29. Amako, K.; Chen, G.X.; Asada, K. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant Cell Physiol.* 1994, 35, 497–504.

30. Schaedle, M. Chloroplast glutathione reductase. *Plant Physiol.* 1977, 59, 1011–1012. [CrossRef]

31. He, J.; Jin, Y.; Du, Y.L.; Wang, T.; Turner, N.C.; Yang, R.P.; Siddique, K.H.M.; Li, F.M. Genotypic variation in yield, yield components, root morphology and architecture, in soybean in relation to water and phosphorus supply. *Front. Plant Sci.* 2017, 8, 1499. [CrossRef]

32. Wouterlood, M.; Cawthray, G.R.; Turner, S.; Lambers, H.; Veneklaas, E.J. Rhizosphere carboxylate concentrations of chickpea are affected by genotype and soil type. *Plant Soil* 2004, 261, 1–10. [CrossRef]

33. Weiner, J. Allocation, plasticity and allometry in plants. *Perspect. Plant Ecol.* 2019, 331, 207–215. [CrossRef]

34. Pang, J.; Ryan, M.H.; Tibbett, M.; Cawthray, G.R.; Siddique, K.H.M.; Bolland, M.D.; Denton, M.D.; Lambers, H. Variation in morphological and physiological parameters in herbaceous perennial legumes in response to phosphorus deficiency. *Plant Soil* 2010, 331, 241–253. [CrossRef]

35. Cho, L.H.; Yoon, J.; An, G. The control of flowering time by environmental factors. *Plant J.* 2017, 90, 708–719. [CrossRef] [PubMed]

36. Fang, X.W.; Turner, N.C.; Xu, D.H.; Jin, Y.; He, J.; Li, F.M. Limits to the height growth of *Caragana korshinskii* resprouts. *Tree Physiol.* 2013, 33, 275–284. [CrossRef] [PubMed]

37. Shangguan, Z.P.; Lei, T.W.; Shao, M.A.; Xue, Q.W. Effects of phosphorus nutrient on the hydraulic conductivity of sorghum (*Sorghum vulgare* Pers.) seedling roots under water deficiency. *J. Integr. Plant. Biol.* 2005, 47, 421–427. [CrossRef]

38. Irvine, J.; Perks, M.P.; Magnani, F.; Grace, J. The response of Pinus sylvestris to drought: Stomatal control of transpiration and hydraulic conductance. *Tree Physiol.* 1998, 18, 393–402. [CrossRef] [PubMed]

39. Wen, Z.; Li, H.; Shen, Q.; Tang, X.; Xiong, C.; Li, H.; Pang, J.; Ryan, M.H.; Lambers, H.; Shen, J. Tradeoffs among root morphology, exudation and mycorrhizal symbioses for phosphorus-acquisition strategies of 16 crop species. *New Phytol.* 2019, 223, 882–895. [CrossRef]