Cross Tolerance to Drought and low Phosphorus Stress in Mungbean is Regulated by Improved Antioxidant Capacity, Biological N2-Fixation, and Differential Transcript Accumulation

Surendra Kumar Meena  
ICAR-Indian Agricultural Research Institute

Renu Pandey (✉ renu_iari@rediffmail.com)  
Indian Agricultural Research Institute  https://orcid.org/0000-0002-9244-8579

Sandeep Sharma  
ICAR-Indian Agricultural Research Institute

Gayacharan  
ICAR-National Bureau of Plant Genetic Resources

Krishnapriya Vengavasi  
ICAR-Sugarcane Breeding Institute

Harsh Kumar Dikshit  
ICAR-Indian Agricultural Research Institute

Kadambot H.M. Siddique  
The University of Western Australia

Madan Pal Singh  
ICAR-Indian Agricultural Research Institute

Research Article

Keywords: Drought, Low phosphorus, Osmolytes, Oxidative metabolism, Biological N2-fixation, Relative gene expression, Vigna radiata

DOI: https://doi.org/10.21203/rs.3.rs-226221/v1

License: ☭  This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Aims The mobility of phosphorus (P) depends on availability of water in soil; both are limited resources for crop production. We studied the mechanisms governing cross tolerance in the contrasting mungbean accessions for drought and low P stress.

Methods Tolerant (IC333090 and IC507340) and sensitive (IC488526 and EC397142) mungbean accessions were grown in soil with treatments: control (sufficient P, irrigated), low P (no P, irrigated), drought (sufficient P, irrigation withheld), and combined stress (no P, irrigation withheld) as well as recovery.

Results Drought reduced the relative water content and membrane stability index, affecting overall plant growth. Combined stress (low P and drought) significantly increased root growth, leaf area, and biomass in tolerant accessions, which was attributed to enhanced nutrient uptake and symbiotic N\textsubscript{2}-fixation. Combined stress also increased osmolyte concentration, antioxidative compounds, and scavenging activity of antioxidant enzymes in tolerant accessions while recovery from drought significantly reduced osmolyte concentration. Transcript abundance of candidate genes related to drought and low P was significantly higher in leaves of IC333090 than IC488526. Conversely, low-P-induced genes (VrSPX1, VrPHO1, VrSQD1, VrPEPCase, and VrMDH) in IC488526 were either downregulated or did not significantly change under combined stress. The drought recovery was better in IC333090 due to enhanced expression of stress-responsive genes.

Conclusions Tolerant mungbean accession could be used as potential donor parents in breeding programs. Traits imparting cross tolerance to drought and low P stress may facilitate better varietal selection for increased crop productivity under low P, drought, and the combined stress.

Introduction

Legumes are a major source of protein in the human diet, which significantly influence soil fertility through biological nitrogen (N\textsubscript{2}) fixation. Mungbean \textit{[Vigna radiata] L. (Wilczek)} is a short duration legume crop cultivated by marginal and poor farmers for grain and fodder purposes. It is an active N\textsubscript{2}-fixer, requiring an adequate amount of phosphorus (P) for symbiosis. The dependence on P fertilizers threatens the availability of rock phosphate reserves, which are predicted to be depleted in the next 50 to 80 years (Cordell et al. 2009). Drought is a severe threat to agriculture, with its profound effect on the metabolic and physiological functions of crops. The relationship between P and soil water is well known; soil P movement occurs through mass flow and diffusion, which depends on pores filled with water (Oliveira et al. 2010). P availability decreases with reduced soil moisture content (Hira and Singh 1977). Under water-deficit conditions, P application enhances root growth, nutrient uptake, and water use efficiency, leading to increased yield, thereby ameliorating adverse effects of drought (Waraich et al. 2011). During drought stress, higher relative water content (RWC) maintained in cells and tissues plays an important role in sustaining metabolic activity through osmotic adjustment (Slabbert and Kruger
In legumes, the major constraints to biological N$_2$-fixation include drought (Sinclair et al. 1987), soil acidity, high N fertilization, and nutrient limitations, such as P, whose unavailability under abiotic stress is detrimental to yield (Sulieman and Tran 2015). Further, a reduction in carbon supply to nodules coupled with reduced canopy size is associated with low stomatal conductance under drought stress (Streeter 2003).

Abiotic stress responses include excess production of reactive oxygen species (ROS), such as superoxide (O$_2^{-}$•), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH•) and singlet oxygen (¹O$_2$), causing lipid peroxidation, protein degradation, enzyme inactivation, DNA damage and membrane injury (Sharma et al. 2012). ROS are scavenged by antioxidative defense systems comprising enzymes, such as superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT), and non-enzymatic antioxidants, such as carotenoids, ascorbate, tocopherol and reduced glutathione (Farooq et al. 2016; AbdElgawad et al. 2016). Increased ROS production under low P or drought stress leads to oxidative stress (Tewari et al. 2007; Chen et al. 2015; Farooq et al. 2016). Osmotic adjustment is a key survival strategy for sustained growth to avoid cell damage from dehydration under water deficit stress. Increases in proline content and total soluble sugars are crucial for osmotic adjustment which is associated with drought avoidance and tolerance strategies as reported in alfalfa (Medicago sativa L.) (Zhang et al. 2018).

At the molecular level, transcript abundance of drought stress induced (DSI) genes, such as Δ-pyrroline-5-carboxylate synthetase (P5CS), Ras-related protein-Rab-18 (RAB18), dehydrins (DHNS), dehydration responsive element binding protein (DREB1/DREB2D) and 9-cis-epoxycarotenoid dioxygenase (NCED), have been reported. Proline accumulation was attributed to the upregulation of P5CS under drought (Chen et al. 2009), while RAB18 assumes an important role in tolerance against various stresses, such as drought, cold temperature, sugar, and salinity (Welin et al. 1994; Shinozaki and Yamaguchi-Shinozaki 2000). In ABA biosynthesis, NCED is the rate-limiting enzyme; its overexpression resulted in ABA accumulation, improving drought tolerance in Arabidopsis (Tong et al. 2017). Similarly, P stress induced (PSI) genes and transcription factors are differentially expressed under low P, altering key metabolic pathways and imparting low P stress tolerance to plants. Phosphate transporter 1 (PHO1) and SPX1 are transcription factors with crucial roles in Pi starvation signaling. PHO1 functions as a Pi exporter, mediating the efflux of Pi out of cells (Stefanovic et al. 2011), while SPX1 has a role in Pi homeostasis during Pi loading into xylem vessels (Duan et al. 2008). Scavenging and remobilization of internal Pi is regulated by purple acid phosphatases (PAPs) and sulfolipid sulfoquinovosyl diacylglycerol (SQD1, SQD2) genes under low P stress (Fang et al. 2009). Pi fixed in external media are predominantly mobilized by low molecular weight organic acids exuded by roots under P stress (Vengavasi and Pandey 2016). Genes encoding enzymes of the tri-carboxylic acid (TCA) pathway, such as malate dehydrogenase (MDH) and phosphoenolpyruvate carboxylase (PEPcase), were upregulated under low P in soybean (Vengavasi et al. 2016). Moreover, increased expression of MDH under drought stress was also reported in creeping bentgrass (Agrostis stolonifera) (Merewitz et al. 2011). The expression of candidate genes
has been mostly studied under individual stresses, but their response to combined stress needs further investigation.

With this background, we characterized the response of four contrasting mungbean accessions to drought, low P and the combined stress at the physiological, biochemical and gene level. It was hypothesized that the tolerance to combined stress in mungbean may be governed by an improved antioxidant scavenging system and biological N₂-fixation with an upregulation of PSI and DSI genes. The identified accessions might serve as potential ‘donor’ parent in breeding programs to develop mungbean varieties with tolerance to low P, drought, and the combined stress.

**Material And Methods**

Plant material and experimental conditions

This study was conducted with four mungbean accessions, which were identified in an earlier study involving 1232 accessions, and categorized as low P and drought stress tolerant (IC333090 and IC507340) and low P and drought stress sensitive (IC488526 and EC397142) (Gayacharan et al. 2020). This grouping was done based on extensive physiological studies where the accessions showed tolerance to individual stresses of drought, low P as well as to their combined stress (Meena et al. 2021).

The mungbean accessions were grown in soil and subjected to four treatments: (1) control (sufficient P, irrigated), (2) low P (no P, irrigated), (3) drought (sufficient P, irrigation withheld), and (4) combined stress (no P, irrigation withheld). The experiment was conducted as mentioned in our previous report (Meena et al. 2021). Seeds were sown during the summer season in earthen pots (30 cm diameter, 30 cm height) filled with sandy loam soil (pH 7.9, EC 0.155 mS m⁻¹) with low available soil P (Olsen P 7.8 mg kg⁻¹ soil). Recommended dose of nitrogen (20 kg N ha⁻¹) and potassium (60 kg K₂O ha⁻¹) supplied as urea and muriate of potash, respectively were mixed with soil prior to sowing. In the control and drought treatments, recommended dose of phosphate (40 kg P₂O₅ ha⁻¹) as single super phosphate was also added. Each replicate pot contained three healthy plants. There were ten pots per treatment and accession; three of which were used for destructive sampling. Drought stress was imposed at 35 days after sowing by withholding irrigation for 10 days until soil moisture had declined to 10–11%. The water stress condition was maintained for two more days during which plant samples were collected from all treatments for physiological and biochemical analyses. The recovery treatments included rewatering of drought-imposed pots, addition of P and re-watering of combined stress pots, and addition of P to low P pots. For the recovery treatments, sampling was done 48 h after recovery. The experiment was conducted under natural weather conditions (Suppl. Figure 1).

Physiological traits, biomass partitioning, and yield attributes

Physiological traits, including MSI (Sairam et al., 1997) and RWC (Barrs and Weatherley 1962), were measured on fully opened top trifoliate leaves. Total leaf area per plant was measured with a leaf area
meter (Li-COR 3000, Lincoln Nebraska, USA). Roots and shoots were separated and dried at 80°C to constant weight. Specific leaf weight (SLW) of fully expanded young trifoliate leaves was determined according to Gardner et al. (1985). Root: shoot ratio was expressed on a dry weight basis. For yield, pods were harvested in two pickings as the crop was indeterminate. The yield attributes such as pod number per plant, seed number per pod, seed yield (g plant⁻¹), and 100 seed weight (g plant⁻¹) were recorded by summing up both the picking from individual plant.

Nodule biomass, nitrogenase activity, and leghemoglobin content

Nitrogenase activity was measured as acetylene reduction activity (ARA), based on the ability of nitrogenase to reduce acetylene to ethylene, and expressed as nmol ethylene g⁻¹ nodule FW h⁻¹ (Hardy et al. 1968). Nodules from the roots were detached, counted and expressed as number of nodules plant⁻¹. Fresh weight of nodules was recorded and expressed as g plant⁻¹ nodule FW. To estimate leghemoglobin (LHb) content, the method given by Appleby and Bergersen (1980) was followed. Nodules were homogenized in phosphate buffer (0.1 M, pH 6.5, 1:3 w/v) followed by centrifugation at 20,000 g for 20 min at room temperature. An equal amount of alkaline pyridine reagent was added to the supernatant before dividing into two parts. A few crystals of sodium dithionite were added to one part and potassium hexacyanoferrate to the other, and the absorbances recorded at 556 and 539 nm, respectively. The LHb was calculated from the difference between the absorbance divided by molar extinction coefficient (ε 23.4) and expressed as mmol g⁻¹ nodule FW.

Estimation of oxidative stress markers

Oxidative stress markers, hydrogen peroxide (H₂O₂) and malondialdehyde (MDA), were measured in leaves. The H₂O₂ content was determined by measuring the intensity of the light-yellow colored titanium-hydro-peroxide complex using titanium reagent at 415 nm and expressed as µmol g⁻¹ FW (Rao et al. 1997). The level of lipid peroxidation was estimated by measuring the content of thiobarbituric acid reactive substances, expressed as equivalents of MDA. MDA content, expressed as nmol g⁻¹ FW, was calculated using its extinction coefficient (155 mM⁻¹ cm⁻¹) after subtracting non-specific values (absorption at 600 nm) from specific values (absorption at 532 nm) (Heath and Packer 1968).

Estimation of antioxidant compounds

Ascorbic acid content was estimated in leaves by measuring the intensity of the pink colored complex, formed due to the reduction of dinitrophenyl hydrazine (DNPH) to phenyl hydrazone, using ascorbic acid in acidic medium at 530 nm (Mukherjee and Choudhuri 1983). To estimate reduced glutathione (GSH), leaf tissue was homogenized in TCA buffer and the intensity of the yellow-colored complex measured using Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) or DTNB) at 415 nm. GSH concentration was expressed as nmol of GSH g⁻¹ FW (Moron et al. 1979).

Estimation of osmolytes
Proline and total soluble sugars (TSS) were estimated in leaves as a marker of drought and low P stress. For proline content, fresh leaf tissue was homogenized in 3% aqueous sulphosalicylic acid and centrifuged at 8000 g for 15 min at room temperature. The supernatant was decanted and washed twice with 3% aqueous sulphosalicylic acid followed by 1 h incubation in a boiling water bath with ninhydrin reagents and acetic acid. After toluene extraction, the absorption of the upper organic phase (pink color) was measured at 520 nm, and proline content was expressed as µmol proline g\(^{-1}\) FW (Bates et al. 1973),

To estimate TSS, leaf tissue was homogenized in 80% ethanol followed by 1 h incubation in a boiling water bath. After filtering and re-extraction, the supernatant was mixed with anthrone reagent and incubated in a boiling water bath for 8 min. After cooling, the absorbance of the dark green color was measured at 630 nm (Sadasivam and Manickam 1992).

Antioxidant enzyme activity

To estimate antioxidative enzyme (SOD, CAT, POD, GR, APX) activity, an extraction was carried out in 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA whereas for APX activity, 1 mM ascorbic acid was added in above extraction buffer. SOD activity was estimated according to Dhindsa et al. (1981) by measuring the decrease in absorbance of formazone at 560 nm produced by O\(_2\)\(^{-}\) and nitroblue tetrazolium (NBT). For CAT activity, the decomposition of H\(_2\)O\(_2\) to water and molecular oxygen was monitored by measuring the decrease in absorbance at 240 nm (Aebi 1984). POD activity was assayed according to Castillo et al. (1984) by monitoring the oxidation of guaiacol to tetra-guaiacol at 470 nm. GR activity was assayed by adding oxidized glutathione and NADPH and measuring the decrease in absorbance at 412 nm (Smith et al. 1988). APX activity was estimated by monitoring ascorbic acid oxidation to mono-dehydroascorbic acid and dehydroascorbic acid, and measuring the decline in absorbance at 290 nm (Nakano and Asada 1981).

Tissue phosphorus concentration and uptake

The P concentration in leaves, stems, roots, nodules, and grain was estimated using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (VDV 5110, Agilent Technologies, Singapore) after digesting the dried material with a di-acid mixture (9:4 HNO\(_3\) : HClO\(_4\)). The tissue P concentration was multiplied by the respective tissue's dry weight to obtain P uptake, expressed as mg P plant\(^{-1}\).

Expression analysis of PSI and DSI genes

For expression analysis of candidate genes, contrasting mungbean accessions (tolerant - IC333090, sensitive - IC488526) identified from the above experiment, were grown and exposed to various stress and recovery treatments. The leaf and petiole tissues were sampled from all treatments, while recovery samples were taken 24 h after restoring the stress treatments. The RNA extraction was performed using PureLink RNA MiNi Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. DNA contamination was removed by treating with DNase I (Promega). cDNA was synthesized using an RT-PCR kit (High-Capacity cDNA Reverse Transcription kit, Thermo Fisher Scientific). Real-time PCR was
performed with KAPA SYBR FAST kit (KAPA BIOSYSTEMS) on a Stratagene Mx3005P QPCR System (Agilent Technologies). For normalization, elongation factor 1-α (VrEF1α) and actin (VrActin) were used as reference genes. The relative transcript abundance under experimental and control conditions (ΔΔCT) was calculated using the comparative cycle threshold method (Schmittgen and Livak 2008). The primers used in RT-qPCR for all candidate genes are listed in Suppl. Table 1.

**Data analysis**

The experiment had a completely randomized design with three factors: soil P level (P), soil moisture regime (W), and genotype (G). Procedures for basic statistical calculations and three-way analysis of variance (ANOVA) were carried out using the statistical software R version 3.6.1 (R Core Team, 2019). Graphs were plotted using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA).

**Results**

Influence of drought, low P, and combined stresses on biomass partitioning and yield

P level, moisture regime, genotype, and their interactive effects (P×W, P×G, W×G, and P×W×G) significantly ($P \leq 0.05$) influenced biomass accumulation and partitioning, total leaf area, SLW, and root: shoot ratio (Suppl. Table 2; Fig. 1a–d). The average reduction percentage in leaf dry weight was 43% and stem dry weight by 57% under drought, while in low P treatment, it was by 25% in leaf dry weight and 26% in stem dry weight, relative to the control. Leaf and stem dry matter declined more in sensitive accessions (IC488526 and EC397142) than tolerant ones (IC333090 and IC507340) (Fig. 1a). Combined drought and low P stress aggravated the adverse effect on biomass accumulation, with an average reduction percentage in leaf and stem dry weights by 52% and 65%, respectively, relative to the control. Biomass accumulation in stems was more sensitive to low P and drought stress (individual or combined) than leaves.

Averaged across accessions, root dry weight increased by 25% and 21% under low P and drought stress, respectively, but declined by 20% under combined stress. The tolerant accessions (IC333090 and IC507340) had significantly more root growth than the sensitive genotypes (IC488526 and EC397142) (Suppl. Figure 2). The average root: shoot ratio increased by 152% under drought stress, 89% under combined stress, and 71% under low P stress, relative to the control (Fig. 1b). IC333090 had the highest root: ratio under drought and combined stress. Total dry weight significantly declined in all accessions in all treatments, relative to the control, more so under combined stress (45%) than drought stress (17–54%, avg 26%) or low P stress (5–8%, avg 8%).

Total leaf area declined significantly more in plants grown under combined stress than those grown under drought or low P stress (Fig. 1c). Under combined stress, IC507340 had the smallest reduction in leaf area (38.4%), while EC397142 had the largest (80.3%). The combined stress increased SLW, while it was lowest in the control, suggesting that stress inhibited leaf expansion (Fig. 1d). Accessions IC333090
and EC397142 had significantly higher SLW under combined stress than the control, while IC507340 did not significantly change in any treatment, indicating optimum turgor maintenance for cell expansion and growth.

Yield traits were significantly ($P \leq 0.05$) influenced by $P$, $W$, $G$ and their interactive effects (Suppl. Table 2). However, the effect of $P \times W \times G$ on yield was non-significant. Yield parameters exhibited a significant reduction in all three stress conditions (Fig. 2a–d). Tolerant accessions exhibited lesser reduction due to stress in the number of pods per plant, the number of seeds per pod, and total seed yield per plant compared to sensitive accessions. The adverse effects of stress treatment were visible on size (length) of pods among tolerant and sensitive accessions, with severe pod size reduction under combined stress (Suppl. Figure 2). Among accessions, the least yield reduction was observed in IC507340 (34%) and IC333090 (37%), while the maximum reduction was found in IC488526 (93%) and EC397142 (59%) under combined stress. Among yield traits, seed yield was most adversely affected while 100-seed weight was least influenced by low $P$, drought and combined stress.

**Influence of low $P$, drought and combined stresses on nodule traits and $N_2$ fixation**

$P$ level, moisture regime, genotype, and $W \times G$ and $P \times W \times G$ interactive effects significantly reduced nodule number and fresh weight, leghemoglobin content, and nitrogenase activity (Suppl. Table 2). The combined stress reduced nodule number per plant the most (62%), followed by drought stress (40%), and low $P$ stress (16%) relative to the control (Fig. 3a). Under all stress conditions, IC333090 had the smallest reduction in nodule number (37%), while EC397142 had the largest (81%), relative to the control. Averaged across accessions, nodule fresh weight declined by 42% under low $P$ stress and 63% under the combined stress (Fig. 3b). The low $P$ and drought stresses had similar effects on nodule fresh weight. Under all stress conditions, nodule fresh weight declined more in sensitive accessions than tolerant accessions.

Under all stress conditions, nodule LHb content declined, relative to the control. The combined stress decreased LHb content more in sensitive accessions (82%) than tolerant accessions (61%), relative to the control (Fig. 3c). Low $P$ stress had little effect on LHb content in IC333090. Low $P$ stress had no effect on nitrogenase activity. Drought and combined stress severely impaired nitrogenase activity, relative to the control, more so in sensitive accessions (IC488526 and EC397142) than tolerant accessions (IC333090 and IC507340) (Fig. 3d). The addition of $P$ under drought stress aided biological $N_2$-fixation, which decreased the reduction in nitrogenase activity more than the combined stress.

**Influence of low $P$, drought, and combined stresses on RWC, membrane injury, and oxidative stress markers**

$P$ level, moisture regime, and genotype significantly ($P \leq 0.05$) affected RWC, MSI, and $H_2O_2$ and MDA content. No significant $P \times W$ interactive effect occurred for MSI or $H_2O_2$ (Suppl. Table 3). The combined stress reduced RWC and MSI in tolerant accessions by 19 and 27%, respectively, and sensitive accessions by 24 and 45%, respectively, relative to the control (Fig. 4a, b). Under combined stress, IC507340 had the smallest reduction in RWC, while IC333090 had the least electrolyte leakage, increasing membrane...
stability. The H$_2$O$_2$ and MDA content increased under drought and combined stress but declined during the drought recovery period (Fig. 4c, d). Averaged across accessions, H$_2$O$_2$ content increased under low P, drought, and combined stresses by 33, 67, and 83%, respectively, relative to the control (Fig. 4c) and MDA content increased by 18, 55, and 103%, respectively (Fig. 4d). MDA content more than doubled in sensitive accessions under combined stress, relative to the control.

Effect of low P, drought, and combined stresses on osmolytes and antioxidant metabolites

P level, moisture regime, genotype, and their interactive effects (P×W, P×G, W×G, and P×W×G) significantly (P ≤ 0.05) affected proline, total soluble sugar, GSH, and ascorbate contents, except for P level on total soluble sugar and ascorbate contents (Suppl. Table 3). Averaged across accessions, proline and total soluble sugars increased under low P, drought, and combined stresses, relative to the control (Fig. 4e, f). Proline content increased markedly under drought and combined stresses, but decreased during the recovery. Proline content increased more in tolerant accessions than sensitive accessions under all three stresses. Averaged across accessions, total soluble sugars increased by 25–64% under low P, drought, or combined stress, relative to the control. IC333090 had the highest total soluble sugars under all three stresses. Averaged over accessions, ascorbate content declined while GSH increased under all three stresses, relative to the control (Fig. 4g, h). Ascorbate content declined more in sensitive accessions than tolerant accessions; for example, drought stress decreased ascorbate content by 51% in sensitive accessions and 10% in tolerant accessions, relative to the control. GSH content increased more in tolerant accessions than sensitive accessions under drought and combined stresses.

Effect of low P, drought, and combined stresses on the activity of antioxidant scavenging enzymes

P level, moisture regime, genotype, and their interactive effects (P×W, P×G, W×G, and P×W×G) significantly (P ≤ 0.05) affected the activity of antioxidant scavenging enzymes, including SOD, APOX, CAT, POX, and GR, except for the effect of low P on CAT activity (Suppl. Table 3). Averaged across accessions, SOD, CAT and GR activities increased under all treatments, relative to the controls (Fig. 5a, b, e). SOD activity increased by 16% under low P, 49% under drought, and 81% under the combined stress. Similarly, CAT and GR activities increased the most under combined stress, relative to the control. Recovery from drought and combined stresses decreased SOD, CAT, and GR activities to some extent, but they remained higher than the control. APOX activity significantly declined under low P and combined stresses, relative to the control, but not under drought stress (Fig. 5c). Drought stress increased POX activity more than the combined stress (Fig. 5d). Tolerant accessions (IC333090 and IC507340) had higher SOD and APOX activities under drought and combined stresses, CAT activity under drought stress, and GR activity under the combined stress and during recovery, than sensitive accessions (IC488526 and EC397142).

Effect of low P, drought, and combined stresses on tissue phosphorus concentration and uptake

P level, moisture regime, and genotype significantly (P ≤ 0.05) affected P concentration in different tissues and P uptake (Suppl. Table 4). The combined stress decreased stem and root P concentration but
increased nodule P concentration, relative to the control (Suppl. Table 5). Drought stress reduced leaf and root P concentration but increased nodule P concentration, relative to the control. Among the tissues, nodules had the highest P concentration, equivalent to grain P%. Furthermore, P uptake in leaves, stems, and grains significantly declined in all treatments, relative to the control, more so under the combined stress (Table 1, Fig. 6a, e). Drought stress had no significant effect on P uptake in roots or nodules. P uptake declined more in sensitive accessions than tolerant accessions, particularly in grain and shoots (stem and leaves) of IC488526 and EC397142 under combined stress.

The physiological responses of tolerant and sensitive accessions to low P, drought, and combined stresses revealed two contrasting accessions for molecular characterization - IC333090 (tolerant) had the smallest reductions in most traits and IC488526 (sensitive) had the largest reductions under all treatments.

Relative expression of phosphorus and drought stress induced genes in contrasting mungbean accessions

Transcript abundance of P stress induced (PSI) and drought stress induced (DSI) genes were analyzed in leaves and petioles of contrasting accessions. P level had a differential response on PSI and DSI genes, while genotype and moisture regime significantly altered gene expression (Suppl. Table 6). Leaf tissue of IC333090 (tolerant) had significantly higher relative expression of PSI genes (VrSQD1, VrSPX1, VrPHO1, VrPAP1, VrMDH, VrPEPcase, and VrIFR) and DSI genes under low P, drought and combined stresses than IC488526 (sensitive) (Fig. 7). Moreover, PSI gene expression in IC333090 increased several-fold during the recovery from drought or the combined stress. The expression pattern of PSI and DSI genes in response to low P, drought, and combined stresses and their recovery varied in petioles. IC333090 showed maximum relative expression of PSI genes, VrSQD1 and VrSPX1, under drought stress, and VrPAP1 and VrMDH during recovery from drought and the combined stress, while IC488526 exhibited increased expression of VrPHO1 during recovery from combined stress.

Among the DSI genes, IC333090 had significantly higher relative expression of VrP5CS and VrRAB18 in leaf tissue under stress and during recovery than IC488526 (Fig. 8). In addition, VrDHN3 and VrDREB were upregulated in IC333090 during recovery from drought and combined stresses. Drought and combined stresses and the recovery from drought stress upregulated the expression of VrNCED and VrDHN3, suggesting that these genes are specific to drought stress. In petioles, IC333090 had increased expression of VrP5CS in all treatments and VrRAB18 under combined stress and its recovery. In IC488526, VrDHN3 was significantly upregulated under stress and recovery conditions.

Discussion

Combined stress affects biomass partitioning and yield more than individual stresses of drought and low P
In this study, combined stress decreased biomass and leaf area in mungbean more than low P or drought stress alone. However, the reverse was true for SLW, being higher under the combined stress than the control or individual stresses. Low P stress reduces leaf area by reducing cell division and cell size (Kavanova et al. 2006). Sharma et al. (2020) also reported reduced shoot and root dry weight and root mass ratio in chickpea plants under low P and drought stress as compared to control. Growth inhibition is often related to altered plant water status, decreasing leaf RWC (Dichio et al. 2003). A reduction in leaf expansion is the first line of defense to maintain cell water potential. Baroowa et al. (2015) reported reduced lower leaf areas in mungbean and black gram in response to drought stress. The positive effects of P on plant growth under drought have been attributed to improved water relations and drought tolerance as reported in lentil (Lens culinaris) (Matar et al. 1992), moth bean (Garg et al. 2004), and soybean (Jin et al. 2006). Singh et al. (2000) reported that plants supplied with P could draw more water from soil at low water potential, as seen in white clover (Trifolium repens) and cluster bean (Cyamopsis tetragonoloba) (Burman et al. 2009). Likewise, increased MSI is associated with osmotic adjustment at higher levels of P nutrition in maize (Zea mays) under drought stress (Premachandra et al. 1990), resulting in improved photosynthetic performance which in turn, is tightly coupled to membrane lipid composition. An increase in leaf thickness, as indicated by SLW, suggests that maintaining thicker leaves is correlated with relative seedling growth rate and net assimilation rate, which generally decline under drought stress (Terzi et al. 2010). In our study, total leaf area and dry matter accumulation significantly declined under drought stress, but SLW did not change, which agrees with earlier reports on mungbean (Sangakkaran et al. 2001; Kumar and Sharma 2009).

The genotypic response in terms of dry matter allocation under all stress conditions varied significantly among mungbean accessions. Under the combined stress, tolerant accessions allocated more dry matter to roots than shoots (Fig. 1a). Organ-specific translocation and allocation of dry matter is a key attribute for drought tolerance rather than total biomass production per se. Mungbean allocated more carbon to roots than shoots under drought stress (Sangakkaran et al. 2001), which would contribute to the drought tolerance ability of IC333090 and IC507340. Increased root biomass under drought as well as low P stress will increase water and nutrient acquisition, an important mechanism of drought tolerance in chickpea (Vadez et al. 2008). The effect of low P and drought stress are mostly encountered at the root level. Drought reduces nutrient uptake by decreasing nutrient mobilization in soil and restricting transpiration flow and root growth. Fine roots with small diameters play a crucial role in water and nutrient uptake, especially under water-limited conditions. Shallow root systems with more basal roots acquire P more efficiently from low P soils than deeper root systems, whereas deeper tap root systems can help to overcome terminal moisture stress (Pang et al. 2021).

Low P and drought stress had a negative effect on yield traits (Fig. 2a–d). Mungbean yield significantly declines when subjected to water stress (Baroowa et al. 2015) and low P stress (Pandey et al. 2014). However, the combined stress had a drastic effect on yield attributes, particularly in sensitive accessions (Supplementary Fig. 2). P fertilization and grain yield attributes had a positive linear relationship in chickpea (Neenu et al. 2014) and soybean (Jin et al. 2006) suggesting that P fertilization could mitigate drought effect during the reproductive stage. Positive effects of P application to legumes exposed to
drought stress, as seen in the present study, have been reported for mungbean (Malik et al. 2006) and cowpea (Uarrota 2010).

Drought and combined stress severely impair biological N\textsubscript{2}-fixation and other nodule traits in comparison to low P stress

Nodule fresh weight, nodule number, and LHb content significantly declined under all three stresses, particularly the combined stress, severely impairing nitrogenase activity (Fig. 3a–d). The combined stress reduced the fixation of atmospheric nitrogen (N\textsubscript{2}), reducing biomass accumulation, particularly in sensitive accessions. Drought stress inhibits nodule initiation, growth, development, and function (Streeter 2003) and impairs biological N\textsubscript{2}-fixation in leguminous crops through impaired nitrogenase activity in nodules (Serraj et al. 1999). Reduced nodule numbers and dry weights have been reported in soybean exposed to drought stress (Sinclair et al. 1987).

Low P stress reduced nodule number and biomass, LHb content, and nitrogenase activity less than drought and combined stresses. P nutrition plays a key role in nodule development and N\textsubscript{2}-fixation. Low P stress affected nodule number and biomass more than shoot growth in Medicago and common bean, reducing nitrogenase activity (Hart 1989; Vadez et al. 1996). Legume crops have a high P demand due to its role in energy transfer reactions in nodules during N\textsubscript{2}-fixation. P might be directly involved in the transportation of ureides through xylem, which is significantly affected by nodule mass (Vadez et al. 1997). There are a few reports on the interactive effects of drought and low P stress on nodule traits and nitrogenase activity. Tobita et al. (2010) reported that low P stress substantially decreased nodule biomass in Alnus hirsuta, while drought stress increased biomass allocation to nodules. Rotaru (2010) reported poor nodulation and reduced nodule number and mass in soybean under combined drought and low P stress. Further, drought aggravated the accumulation of ureides in nodules and roots under low P stress, adversely affecting ureide metabolism and translocation. Serraj et al. (2001) observed that ureide accumulation in nodules triggered a feedback mechanism, impairing nitrogen fixation.

Combined stress aggravates oxidative stress while P supply augments a plant’s ability to cope with oxidative damage

In the present study, RWC and MSI declined significantly, more so under combined stress than drought stress, corroborating an earlier report on maize (Premachandra et al. 1990). Higher membrane injury under combined stress in mungbean accessions resulted from marked increases in H\textsubscript{2}O\textsubscript{2} and MDA contents, key factors for oxidative stress (Fig. 4c, d). The recovery from drought and combined stresses reduced oxidative stress markers, but they remained higher than those in the control. Excessive ROS production leads to DNA fragmentation, protein degradation, and cell death. Higher MDA accumulation is an indicator of membrane lipid peroxidation, which damages cell membranes, increasing permeability and the loss of ion selectivity (Sharma et al. 2012). The mechanism through which P nutrition reduces ROS production to maintain membrane stability needs investigation.
In the present study, drought stress accumulated the most osmolytes, proline, and total soluble sugars, when compared to well-watered conditions; a decline in proline content was noted in the recovery from drought and combined stresses (Fig. 4e, f). Osmotic adjustment is a key mechanism contributing to cellular drought tolerance, whereby a variety of solutes accumulate in the cytosol to maintain cellular turgidity, osmotic potential, and membrane stability (Ramanjulu and Bartels 2002; Keunen et al. 2013). We observed significant changes in ROS scavengers, with ascorbate content decreasing and GSH increasing under drought and combined stresses (Fig. 4g, f). However, tolerant accessions had higher ascorbate and GSH contents than sensitive accessions. Amelioration of drought-induced oxidative stress largely depends on ascorbate and glutathione pools in reduced and oxidative states (Anjum et al. 2015; AbdElgawad et al. 2016). Compared to combined stress, drought exhibited increased levels of osmolytes and ROS scavengers, attributed to positive effect of P fertilization on oxidative stress in the combined stress treatment. Similar results were observed in Alnus cremastogyne seedlings, where P application ameliorated the adverse effects of drought through enhanced antioxidant enzyme activities (Tariq et al. 2018). We also observed higher SOD, CAT, and POX activities, but variable APOX and GR activities under drought stress (Fig. 5a–e). Genotypic variability in APOX activity exists in Amaranthus under drought stress (Slabbert and Kruger 2014). Increased SOD activity enables superoxide radical scavenging, while increased CAT and POX activities enable H$_2$O$_2$ scavenging. We found higher APOX and GR activities under drought stress in tolerant accessions in comparison to sensitive accessions, which was due to reduced H$_2$O$_2$ accumulation, thereby enhancing tolerance to oxidative stress. Increased SOD and CAT activities have been reported in mungbean under drought stress (Baroowa et al. 2016). We observed increased SOD, CAT, and GR activities under low P stress, relative to the control, but to a lesser degree than that observed under drought or combined stress. Similar increase in ROS production and antioxidative enzyme activity in response to low P has been reported in peanut (Arachis hypogea) (Patel et al. 2020), Brassica (Chen et al. 2015), and rice (Veronica et al. 2017).

Combined stress reduces P concentration in all plant parts except root nodules

Present study showed that different stresses significantly reduce P concentration and uptake, relative to the control (Fig. 6a, b). Tissue P concentration, biomass accumulation, and P uptake were the most sensitive P-deficiency traits to stress, as reported in mungbean (Pandey et al., 2014) and soybean (Vengavasi and Pandey 2016). Wide genotypic variation in phosphorus use efficiency (PUE) has been reported in mungbean (Pandey et al. 2013; Meena et al. 2020; Reddy et al. 2020), indicating that P-efficient genotypes uptake more P to shoots than roots. This suggests that P translocation from roots to shoots is an adaptive strategy to increase PUE (Krishnappa et al. 2011; Krishnappa and Hussain 2014). Likewise, moisture deficit significantly reduced P uptake in common bean (Santos et al. 2004) and wheat (Epie and Maral 2018). Low soil moisture reduces P diffusion (Hira and Singh 1977), resulting in less available P at the root zone for plant uptake. However, IC333090 and IC507340, belonging to the tolerant group, increased root growth and carboxylate exudation under drought stress to maintain higher P concentrations and uptake than sensitive accessions. Further, the nodules had higher P concentrations than other tissues in all stress treatments. An earlier study confirmed that mungbean nodules concentrate
up to 20% of total plant P (Gunawardena et al. 1992). A positive correlation between nodule biomass and P content suggests tight regulation between biological N\textsubscript{2}-fixation and nodule P requirement (Lazali et al. 2016). P application has a positive effect on symbiotic parameters in legume crops, including nodulation, nodule dry weight, and leghaemoglobin content (Singh and Singh 2011). We observed a similar P application effect under drought stress, with tolerant accessions having higher nitrogenase activity than sensitive accession, which was attributed to higher nodule P concentration.

Upregulation of PSI and DSI genes imparts cross-tolerance to combined stress of low P and drought stress

Under different stress combinations and recovery, we observed relatively higher expression of PSI genes in the leaves of IC333090 (tolerant) (Fig. 7). The regulatory role of PSI genes/transcription factors have been well-studied under low P stress, but not under drought stress. SPX proteins play an essential role in regulating AtPHR1/OsPHR2 under low P stress (Secco et al. 2012). In common bean, overexpression of PvSPX1 changed root architecture and P homeostasis (Yao et al. 2014). Induction of PHR1 suppressed another key gene, PHO1, being a negative transcriptional regulator of high-affinity Pi transporter, Pht1 expressed under low P stress (Gaza et al. 2014). In our study, the expression of VrPHO1 in leaves doubled in IC333090 compared to IC488526 under low P, drought, and combined stress, but in petioles, the expression was higher in IC488526. While the direct role of PHO1 under drought stress has not been reported, ABA significantly enhanced its expression in guard cells of Arabidopsis leaf (Zimmerli et al. 2012).

In leaves, IC333090 had several-fold higher expression of VrSQD1 than IC488526 under stress, indicating enhanced membrane integrity in tolerant accessions. The SQD1 gene plays an important role in the structural stability of photosynthetic membranes during P deficiency. Phospholipids remobilized from membranes are replaced by sulpholipids, such as sulfoquinovosyl diacylglycerol. The SQD1 gene is involved in sulpholipid biosynthesis; an increase in its transcript level has been reported under low P stress (Fang et al. 2009). Present study revealed that SQD1 is also regulated under drought; however, further investigation is needed. Another PSI gene, VrPAP1, was induced in petioles rather than leaves of IC333090 under low P and combined stresses and recovery. The VrIFR gene was upregulated in leaves of IC333090, particularly during recovery. IFRs encode for enzymes involved in the biosynthesis of isoflavonoid phytoalexin, which protects plants from abiotic and biotic stresses through its antioxidative properties (Kim et al. 2009; Rípodas et al. 2013). Upregulation of IFR under low P stress has been observed in soybean (Vengavasi et al. 2017), barley (Long et al. 2019), and Arabidopsis (Wu et al. 2003). In irrigated vs. non-irrigated soybean, total isoflavone content increased 2.5-fold (Bennett et al. 2004), indicating higher isoflavone reductase enzymes. IFR has been identified as a candidate gene for increased root length in response to low P stress in barley (Long et al. 2019). In kidney bean, lateral root elongation and nodule number were related to increased IFR expression (Rípodas et al. 2013). We found a 5-fold increase in VrIFR expression under combined stress due to the cumulative effect of low P and drought stress, increasing root growth in the tolerant accession (Fig. 1a; Supplementary Fig. 1).
*PAP* gene family members are involved in scavenging Pi from organic P sources present in intercellular spaces and external media, such as soil. Enhanced expression of *PAP1* under P starvation is an important strategy for improving plant growth under low P stress (Mehra et al. 2017; Pandey et al. 2018). Similar to low P stress, an increase in *PAP* transcripts was also reported in wheat under drought stress (Sharma and Kaur 2008). Drought reduces soil P bioavailability, enhancing the expression of intercellular *PAP*. However, induction of the *PAP* gene under drought stress needs to be explored at the molecular level. Interestingly, the relative expression of genes, *VrPEPCase* and *VrMDH*, increased several-fold in IC333090 under all treatments. *PEPC* and *MDH* are key enzymes in the TCA cycle; the metabolites of this pathway (citrate, malate, oxalate) are exuded into the rhizosphere under low P stress. We observed enhanced exudation of organic acids in IC333090, which corroborates with the expression pattern of genes regulating their synthesis (Meena et al. 2021). Similar enhancements in the transcript level of *PEPCase* have been reported for white lupin (Uhde-Stone et al. 2003) and soybean (Vengavasi et al. 2016) under low P stress. Likewise, increased expression of *GmMDH* in soybean (Vengavasi et al. 2016) under low P stress increased malate exudation.

Among the DSI genes, *VrP5CS* expression increased in both accessions, in all treatments as compared to control, more so in tolerant accession than the sensitive accession, but significantly decreased during recovery (Fig. 8). Pyrroline-5-carboxylate synthetase (*P5CS*) is a key enzyme involved in proline biosynthesis under drought stress. As seen in our study, drought stress upregulated the *P5CS* gene before downregulating it during recovery in maize landraces (Schafleitner et al. 2007). Increased expression of *P5CS* under low P stress was reported in *Arabidopsis* (Aleksza et al. 2017). The promoter region of *P5CS* contains the P1BS (PHR1 binding site) domain, a transcription factor regulating several PSI genes. This was confirmed in our study where proline accumulation increased in leaf tissue of IC333090 under the individual and combined stresses (Fig. 6). The tolerant accession had 8-fold higher relative expression of the *VrNCED* gene in leaves than the sensitive accession under drought and combined stresses and drought recovery. The *NCED* gene encoded an enzyme catalyzing a rate-limiting reaction during ABA biosynthesis (Xiong et al. 2002). *NCED3* was upregulated under low P stress in Arabidopsis, indicating an ABA-dependent signaling pathway inducing *P5CS* expression and proline accumulation (Aleksza et al. 2017). A similar enhanced transcript level of *P5CS*, resulting in ABA accumulation, imparted drought tolerance in cowpea (Iuchi et al. 2001) and peanut (Wan and Li 2006).

The expression of the DSI gene *VrRAB18* increased more than 10-fold under low P and combined stresses and recovery conditions (Fig. 8). Similarly, *RAB18* was also expressed under P deficiency (Ciereszko and Kleczkowski 2002). Induction of *RAB18* is ABA-dependent, as observed in ABA-deficient (*aba-1*) and ABA-insensitive (*abi1*) Arabidopsis mutants (Welin et al. 1994). The DSI gene, *VrDHN3*, was induced under drought and combined stresses and during drought recovery in leaves of IC333090 and petioles of IC488526. DHNs are unfolded proteins functioning as protective molecular chaperones for enzymes and phospholipids, enhancing plant tolerance to abiotic stresses (Lv et al. 2018). Expression of *DHN3* and *DHN44* were highly correlated with yield in barley under drought stress (Park et al. 2006). However, we report 5-fold higher expression of *DHN3* in petioles of the sensitive accession under low P stress as compared to control. Differential expression of *VrDHN3* in tolerant and sensitive mungbean accessions
suggests that it is tissue-specific when it comes to imparting drought tolerance. Another key DSI gene, \( VrDREB \), was significantly upregulated during recovery from drought and combined stresses in IC333090. \( DREB \) plays a major role in root architecture and tolerance to abiotic stresses (Yang et al. 2017). While there is no direct evidence of \( DREB \) gene expression under P deficiency, Chen et al. (2018) showed that overexpression of \( JcERF035 \) (a member of the DREB subfamily) from \( Jatropha curcas \) responded to P starvation and altered root morphology, biosynthesis, and accumulation of anthocyanin pigments in transgenic \( Arabidopsis \) plants.

**Conclusions**

This study shows that the tolerance to combined stress was regulated at physiological, biochemical and molecular level governing the genotypic variability in mungbean accessions. The tolerant accession (IC333090) was efficient in scavenging the ROS produced under stress conditions of low P, drought or their combination owing to higher antioxidant enzyme activity, relative to sensitive accession (IC488526). The ability of fixing atmospheric nitrogen was higher in IC333090 due to lesser reduction in nodule traits under all stress conditions which led to increased root growth, leaf area, biomass, and nutrient uptake. Further, the networking of low P and drought stress induced genes at the molecular level in tolerant mungbean accession IC333090 was responsible for imparting tolerance and a faster recovery to low P, drought, or combined stresses. The identified accession can be included in the Vigna breeding program to develop mungbean cultivars with improved tolerance for cultivation in the soils with limited P and water availability, or both. The differential expression of P and drought stress induced genes in tolerant and sensitive accessions might pave the way for allele mining to identify and develop functional markers through candidate gene association studies in mungbean.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ARA          | acetylene reductase activity |
| DSI          | drought stress induced |
| GSH          | reduced glutathione |
| LHb          | leghemoglobin |
| MDA          | malondialdehyde |
| MSI          | membrane stability index |
| PSI          | phosphorus stress induced |
| RWC          | relative water content |
| TSS          | total soluble sugars |
Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This study was funded by the ICAR-Indian Agricultural Research Institute, New Delhi under the institute project (CRSCARI-SIL20144047279).

References

AbdElgawad H, Zinta G, Hegab MM, Pandey R, Asard H, Abuelsoud W (2016) High salinity induces different oxidative stress and antioxidant responses in maize seedlings organs. Front Plant Sci 7:276.

Aebi H (1984) Catalase in vitro Methods in Enzymology 105 121-126.

Aleksza D, Horvath GV, Sandor G, Szabados L (2017) Proline accumulation is regulated by transcription factors associated with phosphate starvation. Plant Physiol 175: 555-567

Anjum NA, Umar S, Aref IM, Iqbal M (2015) Managing the pools of cellular redox buffers and the control of oxidative stress during the ontogeny of drought-exposed mungbean (Vigna radiata L.) - role of sulfur nutrition. Front Environ Sci 2:66.

Appleby CA, Bergersen FJ (1980) Preparation and experimental use of leghaemoglobin. In: Methods for Evaluating Biological Nitrogen Fixation, F.J. Bergersen (ed) (Chichester: Wiley) :315-335

Baldwin JC, Karthikeyan AS, Raghothama KG (2001) LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant Physiol 125:728–737

Baroowa B, Gogoi N, Farooq M (2016) Changes in physiological, biochemical and antioxidant enzyme activities of green gram (Vigna radiata L.) genotypes under drought. Acta Physiol Plant 38:219

Baroowa B, Gogoi N, Paul S, Baruah KK (2015) Response of leaf water status, stomatal characteristics, photosynthesis and yield in black gram and green gram genotypes to soil water deficit. Funct Plant Biol 42:1010-1018

Barrs HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust J Biol Sci 15:413-428
Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. Plant Soil 39:205-207

Bennett JO, Yu O, Heatherly LG, Krishnan HB (2004) Accumulation of genistein and daidzein, soybean isoflavones implicated in promoting human health, is significantly elevated by irrigation. J Agri Food Chem 52:7574-7579

Burman U, Garg BK, Kathju S (2009) Effect of phosphorus application on cluster bean under different intensities of water stress. J Plant Nutri 32:668-680

Castillo FI, Penel I, Greppin H (1984) Peroxidase release induced by ozone in Sedum album leaves. Plant Physiol 74:846-851

Chen JB, Yang JW, Zhang ZY, Feng XF, Wang SM (2009) Two P5CS genes from common bean exhibiting different tolerance to salt stress in transgenic Arabidopsis. J Genet 92:461-469

Chen S, Zhao H, Ding G, Xu F (2015) Genotypic differences in antioxidant response to phosphorus deficiency in Brassica napus. Plant Soil 391:19-32

Chen Y, Wu P, Zhao Q, Tang Y, Chen Y, Li M, Jiang H, Wu G (2018) Overexpression of a phosphate starvation response AP2/ERF gene from physic nut in Arabidopsis alters root morphological traits and phosphate starvation-induced anthocyanin accumulation. Front Plant Sci 9:1186

Ciereszko I, Kleczkowski LA (2002) Effects of phosphate deficiency and sugars on expression of rab18 in Arabidopsis: hexokinase-dependent and okadaic acid-sensitive transduction of the sugar signal. BBA Gene Structure Expression 1579:43-49

Cordell D, Drangert JO, White S (2009) The story of phosphorus: global food security and food for thought. Glob Environ Change 19:292-305

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93-101

Dichio B, Xiloyannis C, Angelopoulos K, Nuzzo V, Bufo SA, Celano G (2003) Drought-induced variations of water relations parameters in Olea europaea. Plant Soil 257:381-389

Duan K, Yi K, Dang L, Huang H, Wu W, Wu P (2008) Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant J 54:965-975

Epie KE, Maral E (2018) Shoot and root biomass, phosphorus and nitrogen uptake of spring wheat grown in low phosphorus and moisture content conditions in a pot experiment. J Plant Nutri 41:2273-2280
Fang Z, Shao C, Meng Y, Wu P, Chen M (2009) Phosphate signaling in *Arabidopsis* and *Oryza sativa*. Plant Sci 176:170-180

Farooq M, Gogoi N, Barthakur S, Baroowa B, Bharadwaj N, Alghamdi SS, Siddique KHM (2016) Drought stress in grain legumes during reproduction and grain filling. J Agron Crop Sci 203:81-102

Gardner FP, Pearecer RB Mitchell RL (1985) Growth and Development. *In Physiology and crop plants. The IOWA State University Press*:187-208

Garg BK, Burman U, Kathju S (2004) The influence of phosphorus nutrition on the physiological response of moth bean genotypes to drought. J Plant Nutri Soil Sci 167:503-508

Gayacharan, Tripathi K, Meena SK, Panwar BS, Lal H, Rana JC, Singh K (2020) Understanding genetic variability in the mungbean (*Vigna radiata*L.) genepool. Ann Appl Biol 177:346-357

Gaza LHR, Jost R, Finnegan PM (2014) *Arabidopsis* PHOSPHATE TRANSPORTER1 genes *PHT1;8* and *PHT1;9* are involved in root-to-shoot translocation of orthophosphate. BMC Plant Biol 14:334

Gunawardena SFBN, Danso SKA, Zapata F (1992) Phosphorus requirements and nitrogen accumulation by three mungbean (*Vigna radiata* (L) Welzek) cultivars. Plant Soil 147:267-274

Hardy RW, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N2 fixation: laboratory and field evaluation. Plant Physiol 43:1185-1207

Hart AL (1989) Distribution of phosphorus in nodulated white clover plants. J Plant Nutr 12:159-171

He J, Jin Y, Turner NC, Chen Z, Liu HY, Wang XL, Siddique KH, Li FM (2019) Phosphorus application increases root growth, improves daily water use during the reproductive stage, and increases grain yield in soybean subjected to water shortage. Environ Exp Bot 166:103816

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189-198

Hira GS, Singh NT (1977) Observed and predicted rates of phosphorus diffusion in soils of varying bulk density and water content 1. Soil Sci Soc Am J 41:537-540

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. Plant J 27:325-333

Jin J, Wang G, Liu X, Pan X, Herbert SJ, Tang C (2006) Interaction between phosphorus nutrition and drought on grain yield, and assimilation of phosphorus and nitrogen in two soybean cultivars differing in protein concentration in grains. J Plant Nutr 29:1433-1449
Kavanova M, Lattanzi FA, Grimoldi AA, Schnyder H (2006) Phosphorus deficiency decreases cell division and elongation in grass leaves. Plant Physiol 141:766-775

Keunen ELS, Peshev D, Vangronsveld J, Van Den Ende WIM, Cuypers ANN (2013) Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. Plant Cell Environ 36:1242-1255

Kim SG, Kim ST, Wang Y, Kim SK, Lee CH, Kim KK, Kim JK, Lee SY, Kang KY (2009) Overexpression of rice isoflavone reductase-like gene (OsIRL) confers tolerance to reactive oxygen species. Physiol Plant 138:1-9

Krishnappa R, Hussain IA (2014) Phosphorus acquisition from deficient soil: involvement of organic acids and acid phosphatase in pigeon pea (Cajanus cajan L. mills sp). Indian J Plant Physiol 19:197-204

Krishnappa R, Umesh HR, Hussain IS (2011) Variations in phosphorus uptake and its utilization in pigeonpea genotypes grown under phosphorus deficiency. J Food Legumes 24:96-100

Kumar A, Sharma KD (2009) Physiological responses and dry matter partitioning of summer mungbean (Vigna radiata L.) genotypes subjected to drought conditions. J Agron Crop sci 195:270-277

Lazali M, Bargaz A, Brahimi S, Amenc L, Abadie J, Drevon JJ (2016) Expression of a phosphate-starvation inducible fructose-1, 6-bisphosphatase gene in common bean nodules correlates with phosphorus use efficiency. J Plant Physiol 205:48-56

Long L, Ma X, Ye L, Zeng J, Chen G, Zhang G (2019) Root plasticity and Pi recycling within plants contribute to low-P tolerance in Tibetan wild barley. BMC Plant Biol 19:341

Lv A, Su L, Liu X, Xing Q, Huang B, An Y, Zhou P (2018) Characterization of Dehydrin protein, CdDHN4-L and CdDHN4-S, and their differential protective roles against abiotic stress in vitro. BMC Plant Bio 18: 299

Malik AMJAD, Waheed A, Qadir G, Asghar R (2006) Interactive effects of irrigation and phosphorus on green gram (Vigna radiata L.). Pakistan J Bot 38:1119

Matar A, Torrent J, Ryan J (1992) Soil and fertilizer phosphorus and crop responses in the dryland Mediterranean zone. In Advances in Soil Science, Springer, New York, NY: 81-146

Meena SK, Gayacharan, Singh MP, Pandey R (2020) Photosynthetic and yield traits identified through multivariate analysis in mungbean exhibiting tolerance to the combined stresses of low phosphorus and drought. Indian J Genet Pl Br 80:291-300

Meena SK, Pandey R, Sharma S, Gayacharan, Kumar T, Singh MP, Dikshit HK (2021) Physiological basis of combined stress tolerance to low phosphorus and drought in mungbean core set derived from diverse germplasm. Agron 11:99. https://doi.org/10.3390/agronomy11010099
Mehra P, Pandey BK, Giri J (2017) Improvement in phosphate acquisition and utilization by a secretory purple acid phosphatase (OsPAP21b) in rice. Plant Biotech J 15:1054–1067

Merewitz EB, Gianfagna T, Huang B (2011) Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an IPT gene for cytokinin synthesis. J Exp Bot 62:5311-5333

Moron MS, Depierre JW, Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. BBA-Gen Sub 582:67-78

Mukherjee SP, Choudhuri MA (1983) Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. Physiol Plant 58:166-170

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880

Neenu S, Ramesh K Ramana S, Biswas AK, Rao AS (2014) Growth and yield of different varieties of chickpea (Cicer arietinum L.) as influenced by the phosphorus nutrition under rainfed conditions on Vertisols. Int J Bio-res Stress Manag 5:053-057

Oliveira EMM, Ruiz HA, Alvarez V, Hugo V, Ferreira PA, Costa FO, Almeida ICC (2010) Nutrient supply by mass flow and diffusion to maize plants in response to soil aggregate size and water potential. Rev Bras Cienc Solo 34:317-328

Pandey R, Krishnapriya V, Kishora N, Singh SB Singh B (2013) Shoot labelling with 14CO₂: a technique for assessing total root carbon exudation under phosphorus stress. Indian J Plant Physiol 18:250-262

Pandey R, Lal MK, Vengavasi K (2018) Differential response of hexaploid and tetraploid wheat to interactive effects of elevated [CO₂] and low phosphorus. Plant Cell Rep 37:1231–1244

Pandey R, Meena SK, Krishnapriya V, Ahmad A Kishora N (2014) Root carboxylate exudation capacity under phosphorus stress does not improve grain yield in green gram. Plant Cell Rep 33:919-928

Pang J, Wne Z, Kidd D, Ryan MH, Yu RP, Li L, Cong WF, Siddique KHM (2021) Advances in understanding plant root uptake of phosphorus. In: Gregory P (ed.) Understanding and improving crop root function. Burleigh Dodds Sciences Publishing Limited, Cambridge UK, Pp. 1-53. http://dx.doi.org/10.19103/AS.2020.0075.16

Park SY, Noh KJ, Yoo JH, Yu JW, Lee BW, Kim JG, Paek NC (2006) Rapid upregulation of Dehydrin3 and Dehydrin4 in response to dehydration is a characteristic of drought-tolerant genotypes in barley. J Plant Bio 49:455-462

Patel, M., Rangani, J., Kumari, A., & Parida, A. K. (2020). Mineral nutrient homeostasis, photosynthetic performance, and modulations of antioxidative defense components in two contrasting genotypes of
Arachis hypogaea L. (peanut) for mitigation of nitrogen and/or phosphorus starvation. J Biotech 323:136-158

Premachandra GS, Saneoka H, Fujita K, Ogata S (1990) Cell membrane stability and leaf water relations as affected by phosphorus nutrition under water stress in maize. Soil Sci Plant Nutr 36:661-666

Qin, X, Zeevaart JA (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. PNAS 96:15354-15361

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Ramanjulu S, Bartels D (2002) Drought-and desiccation-induced modulation of gene expression in plants. Plant Cell Environ 25:141-151

Rao MV, Paliyath G, Ormrod DP, Murr DP Watkins CB (1997) Influence of salicylic acid on \( \text{H}_2\text{O}_2 \) production, oxidative stress and \( \text{H}_2\text{O}_2 \) metabolizing enzymes. Plant Physiol 115:137-149

Reddy VRP, Das S, Dikshit HK, Mishra GP, Aski M, Meena SK, Singh A, Pandey R, Singh MP, Tripathi K, Gore PG (2020) Genome-wide association analysis for phosphorus use efficiency traits in mungbean (\textit{Vigna radiata} L. Wilczek) using genotyping by sequencing approach. Front Plant Sci:11

Rípodas C, Dalla Via V, Aguilar OM, Zanetti ME, Blanco FA (2013) Knock-down of a member of the isoflavone reductase gene family impairs plant growth and nodulation in \textit{Phaseolus vulgaris}. Plant Physiol Biochem 68:81-89

Rotaru V (2010) The effects of phosphorus application on soybean plants under suboptimal moisture conditions. Lucrari Stiintifice 53:27-30

Sadasivam S, Manickam A. (1992) Determination of amylose. \textit{In: Biochemical Methods for Agricultural Sciences}. New Delhi: Wiley Eastern Limited: 12–13

Sairam RK, Deshmukh PS Shukla DS (1997) Tolerance to drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. J Agron Crop Sci 178:171-177

Sangakkaran UR, Frehner M, Nosberger J (2001) Influence of soil moisture and fertilizer potassium on the vegetative growth of mungbean (\textit{Vigna radiata} L. Wilczek) and cowpea (\textit{Vigna unguiculata} L. Walp). J Agron Crop Sci 186: 73–81

Santos MGD, Ribeiro RV, Oliveira RFD, Pimentel C (2004) Gas exchange and yield response to foliar phosphorus application in \textit{Phaseolus vulgaris} L. under drought. Braz J Plant Physiol 16:171-179

Schafleitner R, Gutierrez-Rosales RO, Gaudin A, Alvarado-Aliaga CA, Nombeo-Martinez G (2007) Capturing candidate drought tolerance traits in two native Andean potato clones by transcription profiling of field
grown plants under water stress. Plant Physiol Biochem 45, 673–6

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101

Secco D, Wang C, Arpat BA, Wang Z, Poirier Y, Tyerman SD, Whelan J (2012) The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. New Phytol, 193:842-851

Serraj R, Vadez V, Sinclair T (2001) Feedback regulation of symbiotic N\textsubscript{2} fixation under drought stress. Agronomie 21:621–626

Serraj R, Thomas R, Sinclair Larry CP, (1999) Symbiotic N\textsubscript{2} fixation response to drought. J Exp Bot 331:143-155

Sharma AD, Kaur P (2008) Drought-stress induced changes in the expression of acid phosphatases in drought tolerant and susceptible cultivars of wheat. World J Agric Sci 4:471-475

Sharma M, Pang J, Wen Z, De Borda A, Kim HS, Liu Y, Lambers H, Ryan MH, Siddique KH (2020) A significant increase in rhizosheath carboxylates and greater specific root length in response to terminal drought is associated with greater relative phosphorus acquisition in chickpea. Plant Soil https://doi.org/10.1007/s11104-020-04776-x

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot 26

Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol 3:217-223

Sinclair TR, Muchow RC, Bennett JM, Hammond LC (1987) Relative sensitivity of nitrogen and biomass accumulation to drought in field-grown soybean. Agron J 79:986-991

Singh DK, Sale PW, Pallaghy CK, McKenzie BM (2000) Phosphorus concentrations in the leaves of defoliated white clover affect abscisic acid formation and transpiration in drying soil. New Phytol 146:249-259

Singh DP, Singh BB (2011) Breeding for tolerance to abiotic stresses in mungbean. J Food Legumes 24:83-90

Slabbert M M, Kruger GHJ (2014) Antioxidant enzyme activity, proline accumulation, leaf area and cell membrane stability in water stressed Amaranthus leaves. S Afr J Bot 95:123-128

Smith IK, Vierheller TL, Thome CA (1988) Assay of glutathione reductase in crude tissue homogenates using 5, 5'-dithiobis (2-nitrobenzoic acid). Anal Biochem 175 408-413
Stefanovic A, Arpat AB, Bligny R, Gout E, Vidoudez C, Bensimon M, Poirier Y (2011) Over-expression of PHO1 in Arabidopsis leaves reveals its role in mediating phosphate efflux. Plant J 66:689-699

Streeter JG (2003) Effects of drought on nitrogen fixation in soybean root nodules. Plant Cell Environ 26:1199-1204

Sulieman S, Tran LSP (2015) Phosphorus homeostasis in legume nodules as an adaptive strategy to phosphorus deficiency. Plant Sci 239:36-43

Sultana S, Tureckova V, Ho C L, Napis S, Namasivayam P (2014) Molecular cloning of a putative Acanthus ebracteatus-9-cis-epoxycarotenoid deoxygenase (AeNCED) and its overexpression in rice. J Crop Sci Biotech 17:239-246

Tariq A, Pan K, Olatunji OA, Graciano C, Li Z, Sun F, Zhang L, Wu X, Chen W, Song D, Huang D (2018) Phosphorous fertilization alleviates drought effects on Alnus cremastogyne by regulating its antioxidant and osmotic potential. Sci Rep 8:5644

Terzi R, Saglam A, Kutlu N, Nar H, Kadioglu A (2010) Impact of soil drought stress on photochemical efficiency of photosystem II and antioxidant enzyme activities of Phaseolus vulgaris cultivars. Turk J Bot 34:1-10

Tewari RK, Kumar P, Sharma PN (2007) Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency. J Integr Plant Biol 49:313-322

Tobita H, Uemura A, Kitao M, Kitaoka S, Utsugi H (2010) Interactive effects of elevated CO2, phosphorus deficiency, and soil drought on nodulation and nitrogenase activity in Alnus hirsuta and Alnus maximowiczii. Symbiosis 50:59-69

Tong SM, Xi HX, Ai KJ, Hou HS (2017) Overexpression of wheat TaNCED gene in Arabidopsis enhances tolerance to drought stress and delays seed germination. Biol Plant 61:64-72

Uarrota VG (2010) Response of cowpea (Vigna unguiculata L. Walp.) to water stress and phosphorus fertilization. J Agron 9:87-91

Uhde-Stone C, Gilbert G, Johnson JM, Litjens R, Zinn KE, Temple SJ, Vance CP, Allan DL (2003) Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. Plant Soil 248:99-116

Vadez V, Beck DP, Lasso JH, Drevon JJ (1997) Utilization of the acetylene reduction assay to screen for tolerance of symbiotic N2 fixation to limiting P nutrition in common bean. Physiol Plant 99:227-232

Vadez V, Rao S, Kholova J, Krishnamurthy L, Kashiwagi J, Ratnakumar P, Sharma KK, Bhatnagar-Mathur P, Basu PS (2008) Root research for drought tolerance in legumes: quo vadis. J Food Legumes 21:77-85
Vadez V, Rodier F, Payre H, Drevon JJ (1996) Nodule permeability to O$_2$ and nitrogenase-linked respiration in bean genotypes varying in the tolerance of N$_2$ fixation to P deficiency. Plant Physiol Biochem 34:871-878

Vengavasi K, Pandey R (2016) Root exudation index: screening organic acid exudation and phosphorus acquisition efficiency in soybean genotypes. Crop Pasture Sci 67:1096-1109

Vengavasi K, Kumar A, Pandey R (2016) Transcript abundance, enzyme activity and metabolite concentration regulates differential carboxylate efflux in soybean under low phosphorus stress. Indian J Plant Physiol 21:179-188

Vengavasi K, Pandey R, Abraham G, Yadav RK (2017) Comparative analysis of soybean root proteome reveals molecular basis of differential carboxylate efflux under low phosphorus stress. Genes 8:341

Veronica N, Subrahmanyam D, Kiran TV, Yugandhar P, Bhadana VP, Padma V, Jayasree G Voleti SR (2017) Influence of low phosphorus concentration on leaf photosynthetic characteristics and antioxidant response of rice genotypes. Photosynthetica, 55:285-293

Wan XR, Li L (2006) Regulation of ABA level and water-stress tolerance of Arabidopsis by ectopic expression of a peanut 9-cis-epoxycarotenoid dioxygenase gene. Biochem Biophys Res Commun 347:1030-1038

Waraich EA, Ahmad R, Ashraf MY (2011) Role of mineral nutrition in alleviation of drought stress in plants. Aust J Crop Sci 5:764-777

Welin BV, Olson A, Nylander M, Palva ET (1994) Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in Arabidopsis thaliana. Plant Mol Biol 26:131-144

Wu P, Ma L, Hou X, Wang M, Wu Y, Liu F, Deng XW (2003) Phosphate starvation triggers distinct alterations of genome expression in Arabidopsis roots and leaves. Plant Physiol 132:1260-1271

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14:S165-S183

Yang G, Yu L, Zhang K, Zhao Y, Guo Y, Gao C (2017) A ThDREB gene from Tamarix hispida improved the salt and drought tolerance of transgenic tobacco and T. hispida. Plant Physiol Biochem 113:187-197

Yao ZF, Liang CY, Zhang Q, Chen Z J, Xiao BX, Tian J, Liao H (2014) SPX1 is an important component in the phosphorus signalling network of common bean regulating root growth and phosphorus homeostasis. J Exp Bot 65:3299-3310

Zhang C, Shi S, Wang B, Zhao J (2018) Physiological and biochemical changes in different drought-tolerant alfalfa (Medicago sativa L.) varieties under PEG-induced drought stress. Acta Physiol Plant 40:1-
Zimmerli C, Ribot C, Vavasseur A, Bauer H, Hedrich R, Poirier Y (2012) *PHO1* expression in guard cells mediates the stomatal response to abscisic acid in *Arabidopsis*. Plant J 72:199-21