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

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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_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_2) fixation. Mungbean [*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_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

2014). 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 ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) 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 (*DREB*/*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 N_2-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 K_2O 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 P_2O_5 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 (H_2O_2) and malondialdehyde (MDA), were measured in leaves. The H_2O_2 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_2O_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-∝ (*VrEF1*∝) and actin (*VrActin*) were used as reference genes. The relative transcript abundance under experimental and control conditions ($^{\Delta\Delta}$CT) 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×W×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×G and P×W×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×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_2O_2 and MDA content increased under drought and combined stress but declined during the drought recovery period (Fig. 4c, d). Averaged across accessions, H_2O_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 ($P \leq 0.05$) 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 ($P \leq 0.05$) 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 ($P \leq 0.05$) 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_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_2), reducing biomass accumulation, particularly in sensitive accessions. Drought stress inhibits nodule initiation, growth, development, and function (Streeter 2003) and impairs biological N_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_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_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_2O_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_2O_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_2O_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_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 sulfoquinivosyl 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. *IFR*s encode for enzymes involved in the biosynthesis of isoflavanoid 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 *PAP1*. 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 Kleczkowsk 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

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.

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Figures

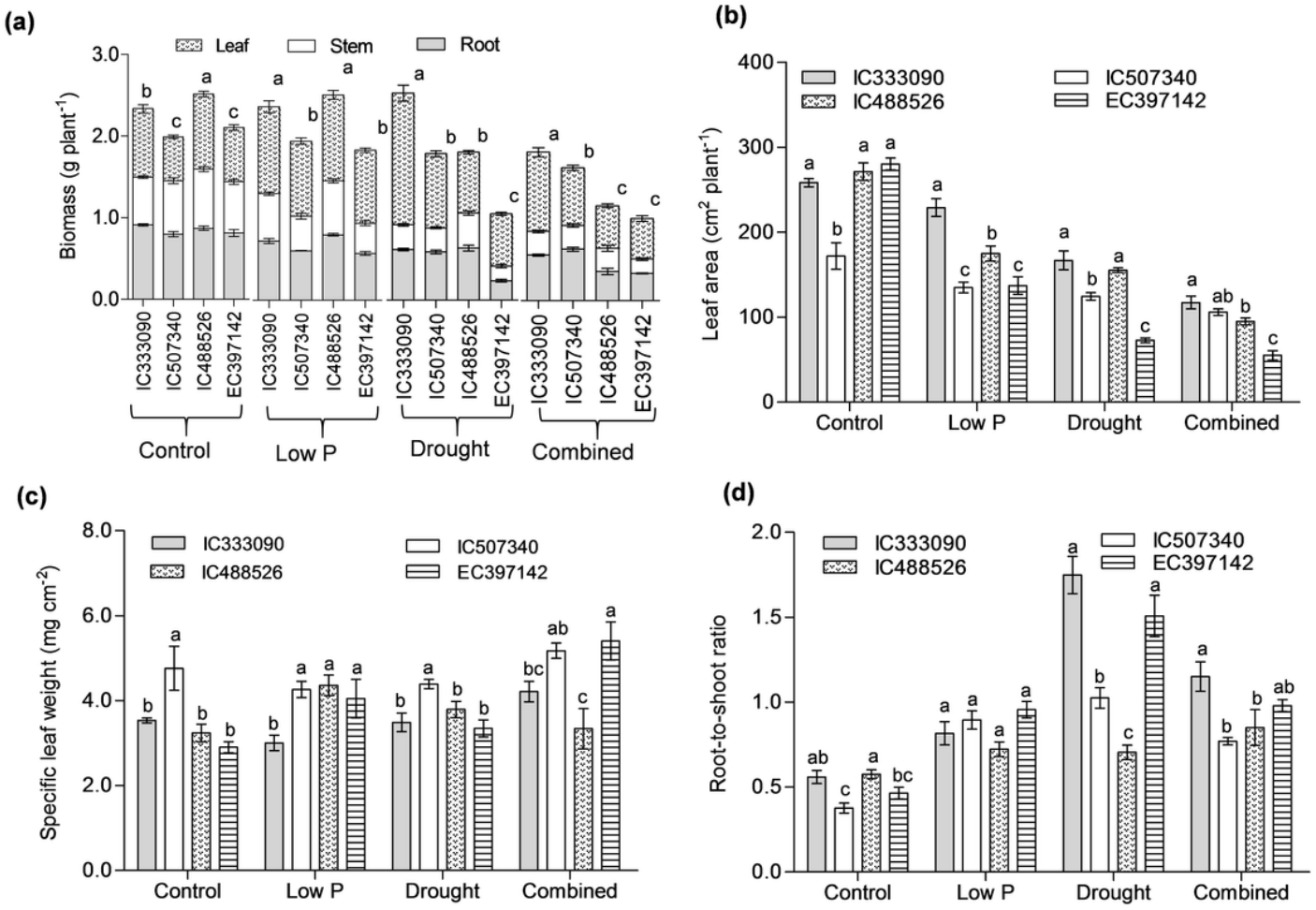

Figure 1

Effect of low P, drought and combined stress on (a) biomass, (b) leaf area, (c) specific leaf weight and (d) root to shoot ratio in four contrasting mung bean accessions. Bar represents the mean of four replications ± SEm, different letters denote significant differences between treatments.

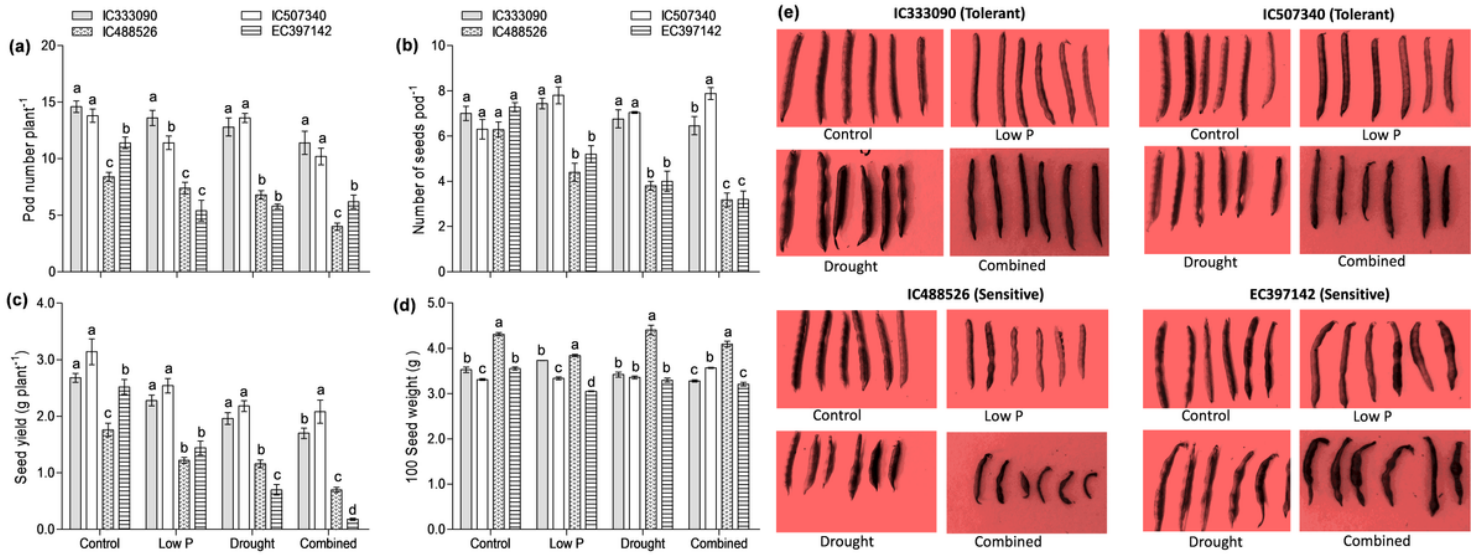

Figure 2

Effect of low P, drought and combined stress on (a) number of pods plant-1, (b) number of seeds pod-1, (c) seed yield plant-1 and (d) test weight in four contrasting mung bean accessions. (e) represents the pod size in response to various treatments. Bar represents the mean of four replications ± SEm, different letters denote significant differences between treatments.

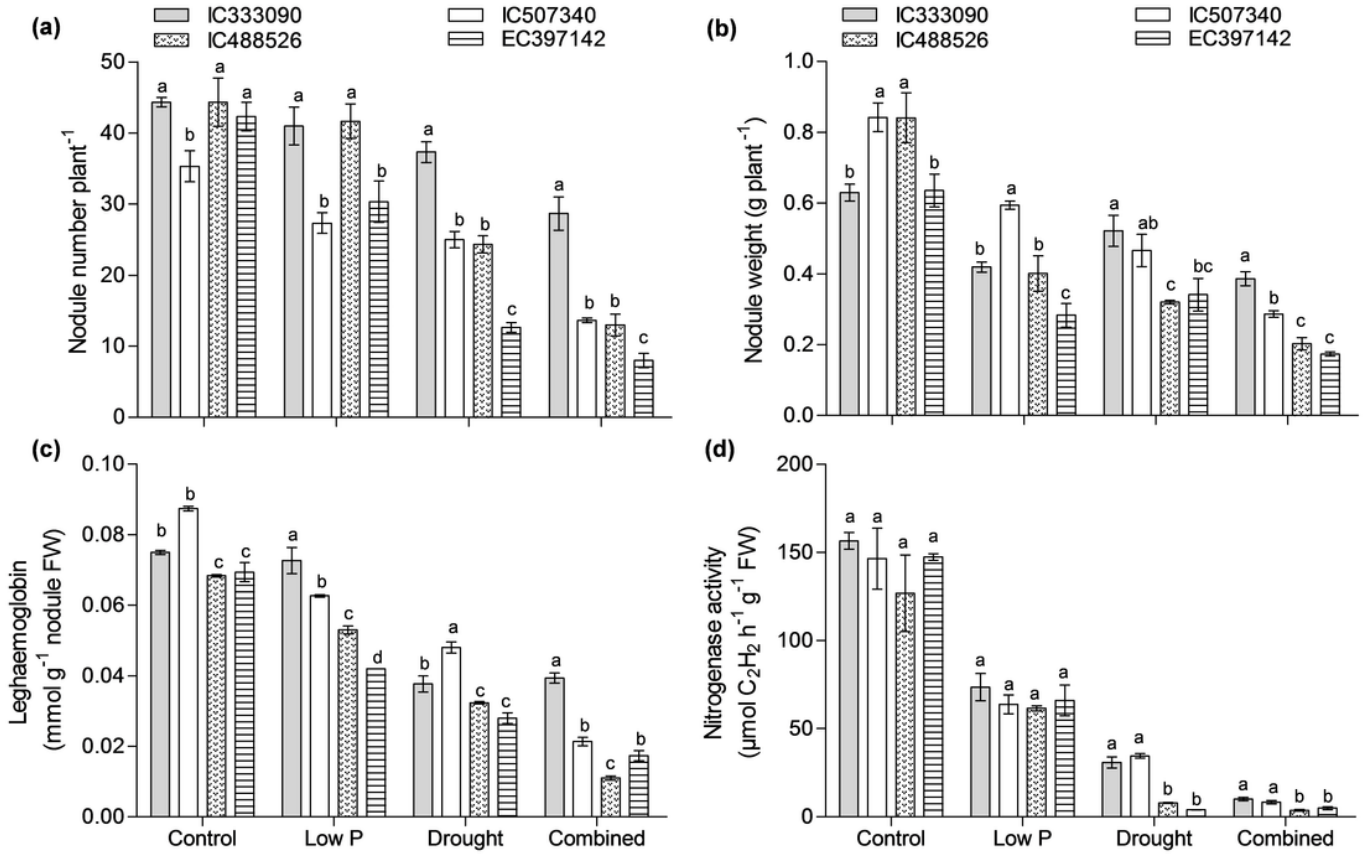

Figure 3

Effect of low P, drought and combined stress on (a) number of nodules plant-1, (b) nodule weight, (c) leghaemoglobin content, and (d) nitrogenase activity (acetylene reduction activity) in four contrasting mungbean accessions. Bar represents the mean of four replications±SEm, different letters denote significant differences between treatments.

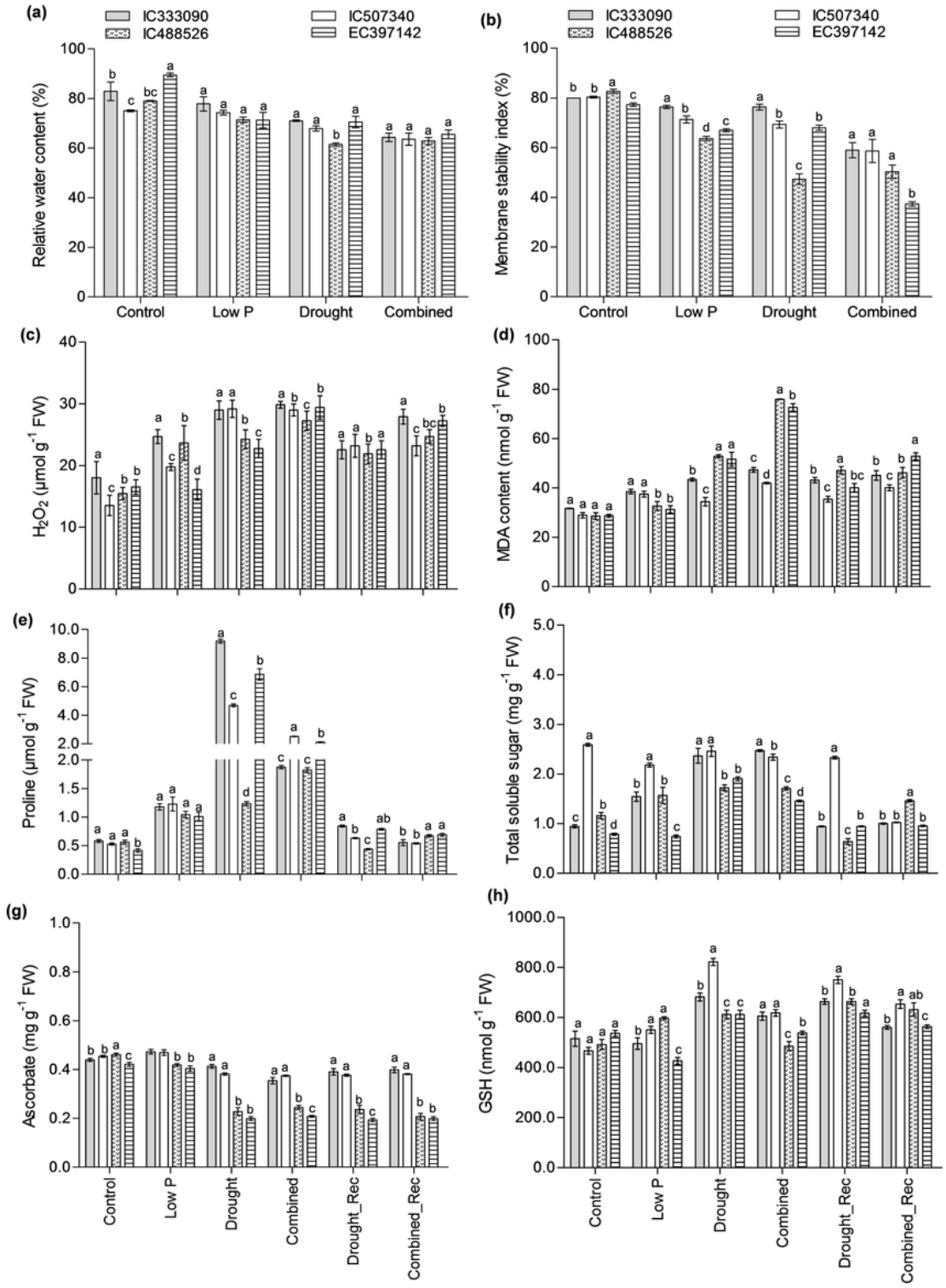

Figure 4

Effect of low P, drought and combined stress and its recovery on (a) relative water content, (b) membrane stability index, (c) H2O2 content, (d) MDA content, (e) proline, (f) total soluble sugar, (g) ascorbate content, and (h) GSH content in four contrasting mungbean accessions. Bar represents the mean of four replications ±SEm, different letters denote significant differences between treatments.

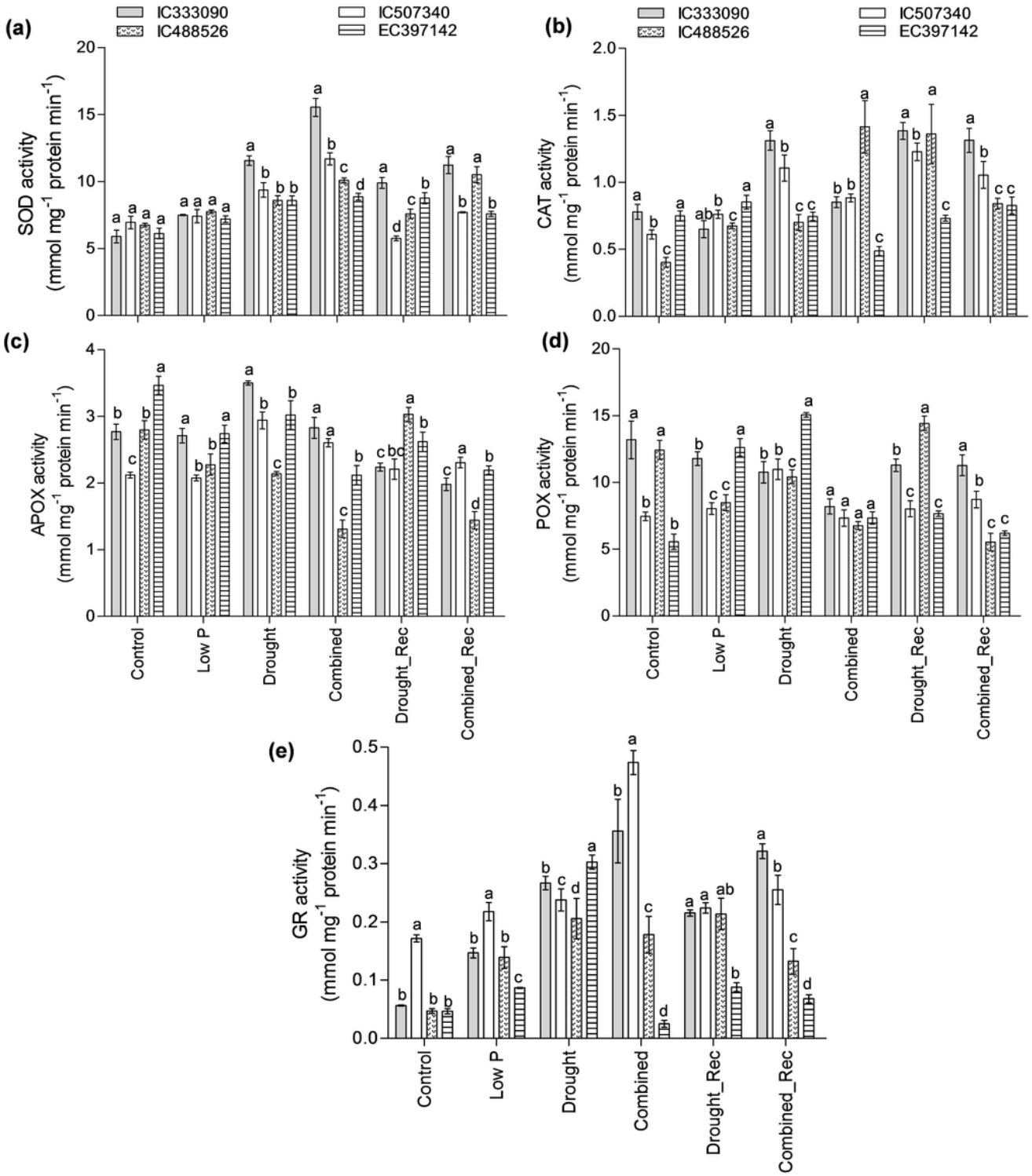

Figure 5

Effect of low P, drought, combined stress and its recovery on activity of (a) super oxide dismutase, (b) catalase, (c) ascorbate peroxidase, (d) peroxidase and (e) glutathione reductase in four contrasting mungbean accessions. Bar represents the mean of four replications ±SEm, different letters denote significant differences between treatments.

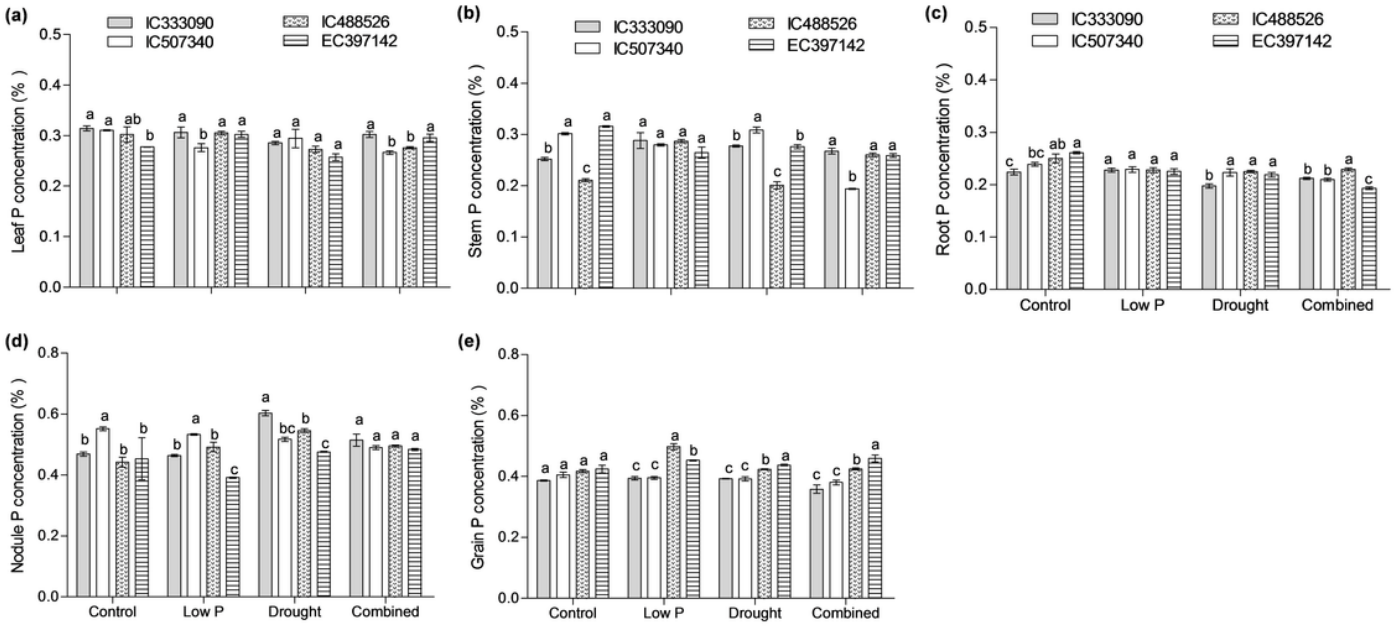

Figure 6

Effect of low P, drought and combined stress on P concentration in (a) leaves, (b) stem, (c) root, (d) nodules and (e) grains in four contrasting mungbean accessions. Bar represents the mean of four replications ±Sem, different letters denote significant differences between treatments.

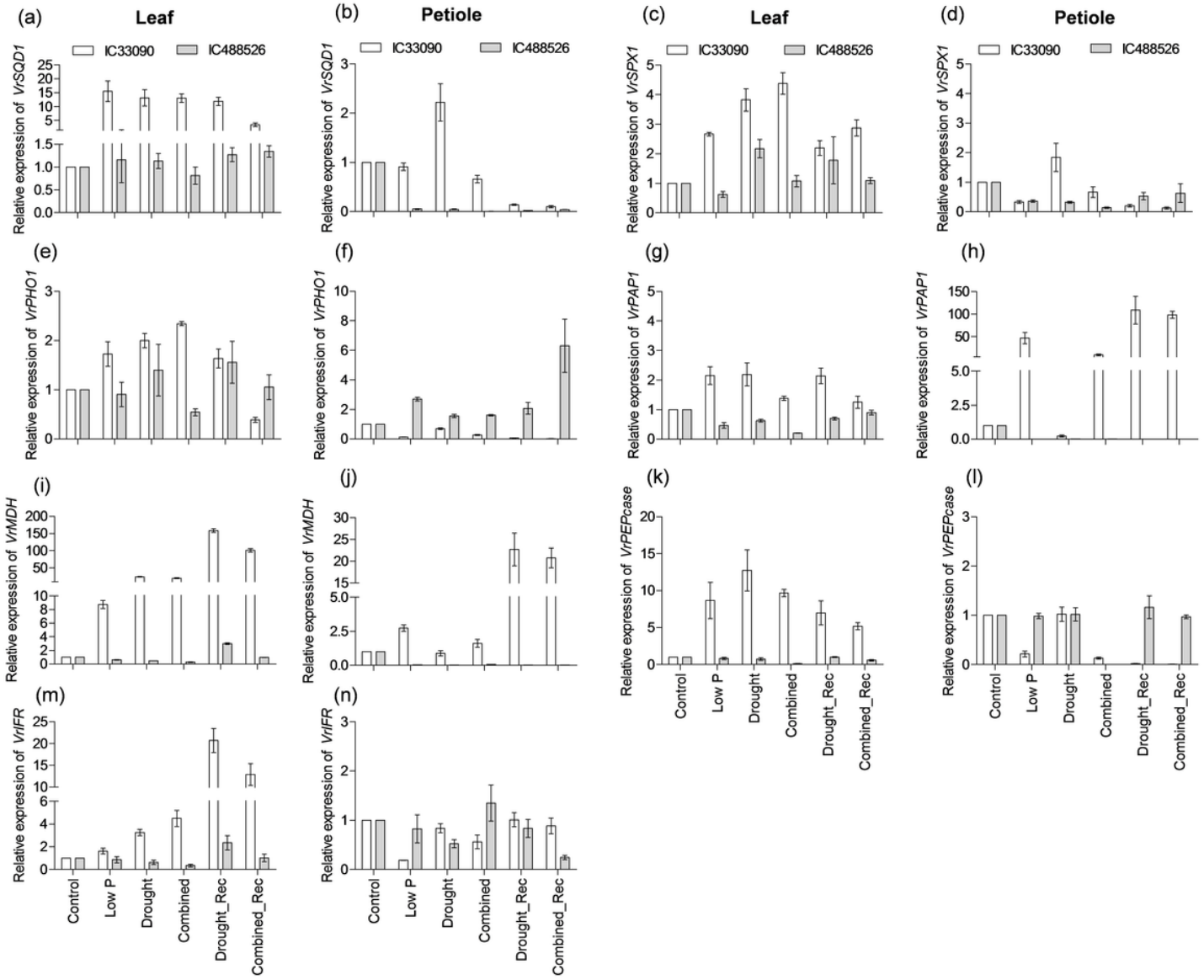

Figure 7

Effect of low P, drought, combined stress and its recovery on the relative expression of phosphorus stress induced (PSI) genes in leaves and petiole of two contrasting mungbean accessions. (a, b) VrSQD1 – sulfoquinovosyl diacylglycerol 1; (c, d) VrSPX1 - yeast Syg1, Pho81, and the human XPR1 proteins; (e, f) VrPHO1 – phosphate transporter 1; (g, h) VrPAP1 - purple acid phosphatase 1; (i, j) VrMDH - malate dehydrogenase; (k, l) VrPEPcase – phosphoenol pyruvate carboxylase; (m, n) VrIFR - isoflavone reductase. Bars represent mean ± SEm, n = 3.

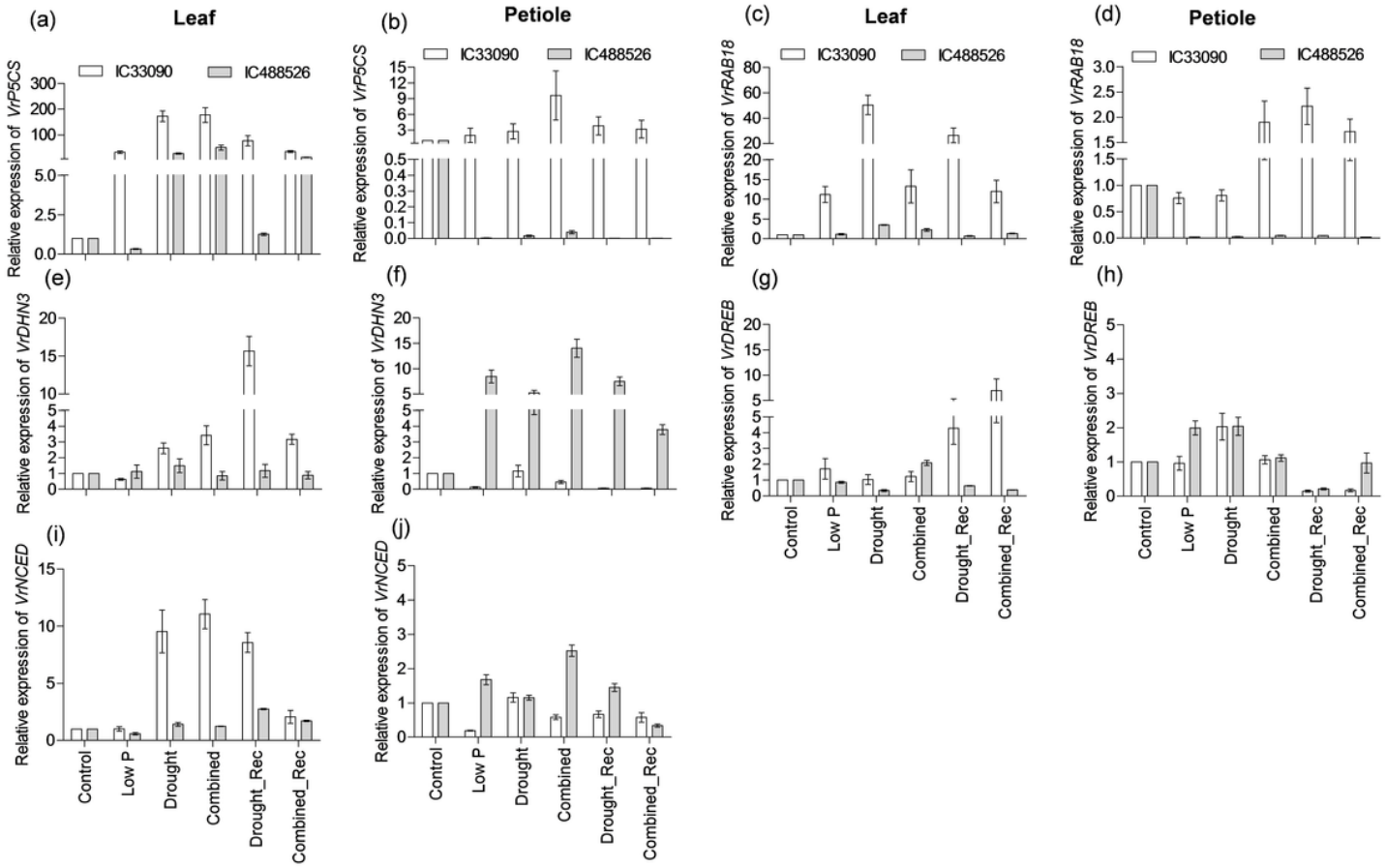

Figure 8

Effect of low P, drought, combined stress and its recovery on the relative expression of drought stress induced (DSI) genes in leaves and petiole of two contrasting mungbean accessions. (a, b) VrP5CS – Δ-pyrroline-5-carboxylate synthetase; (c, d) VrRAB18 - Responsive to ABA 18; (e, f) VrDHN3 – Dehydrin 3; (g, h) VrDREB - Dehydration responsive element binding protein; (i, j) VrNCED - 9-cis-epoxycarotenoid dioxygenase. Bars represent mean ± SEm, n = 3.

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