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Article

Arbuscular Mycorrhizal Fungi and Biofilm Forming Bacteria Act Synergistically to Modulate Proline Metabolism, Antioxidant Defense System and Aquaporin Genes Expression Under Drought Stress

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Abstract

Use of rhizosphere microorganisms provides an alternative or supplement to conventional plant breeding to improve water deficit tolerance of tomato plants. Experiment was carried out to explore the effect of two microbial species, AMF (*Rhizophagus irregularis*) and *Bacillus subtilis*, in single and co-application, on growth, colonization, and molecular aspects of tomato plants under drought stress. Co-inoculated plants showed less reduction in growth traits, photosynthetic pigments, colonization rate, and increased compatible solutes like proline which help in sustaining relative water content than non-inoculated plants. Inoculation considerably enhanced proline dehydrogenase activity, and significantly reduced both Δ_1-pyrroline-5-carboxylate reductase Δ_1-pyrroline-5-carboxylate synthetase activity causing lower proline accumulation in inoculated plants under drought stress. Co-inoculated plants showed obvious upregulation of antioxidant system, thus facilitating amelioration of oxidative stress through exclusion of reactive oxygen species. No inoculation under drought stress upregulated abscisic acid related genes expression but have no effect in plants inoculated either sole or mixed inoculation. Expression of aquaporin genes was upregulated in plants co-inoculated and with AMF alone under normal condition. However the expression of aquaporin genes were decreased or unaffected in plants inoculated with *Bacillus subtilis* but increased in non-inoculated plants. Co-applied AMF and *bacillus subtilis* substantially increase drought tolerance by upregulating proline

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metabolism, antioxidant enzymes and aquaporin genes. Therefore our results suggest that co-inoculation mediated drought tolerance is linked with increased proline accumulation, enhanced antioxidant enzyme activities and differential regulation of ABA biosynthetic and aquaporin genes, which is vital for osmotic adjustment of host plant.

Keywords: Abscisic acid biosynthetic genes; aquaporins; Biofilm forming bacteria; growth traits; oxidative injury; Tomato

1. Introduction

Plants are exposed to numerous environmental stresses among which drought stress is a major constraint that reduces plant productivity and growth by effecting biochemical, morpho-physiological, molecular and metabolic processes (Brodersen et al., 2019, Abbasi & Abbasi, 2010; Massonnet et al., 2007; Zu et al., 2017). Drought stress limits the plant development and growth (Ansari et al., 2019). Drought stress is a major threat to plant survival. One of the unavoidable consequences of water deficiency is an oxidative burst which acts an alarming signal to start acclimation (Kaur & Asthir, 2017). Plants develop different strategies to avoid such stress conditions by osmotic adjustments and different adaptive mechanisms such as reduction in transpiration, increment in root length, hormonal regulation, enzymatic and non-enzymatic antioxidant systems and expression of certain genes responsive to water deficit conditions (Seleiman et al., 2021).

One of the striking responses to mitigate drought stress is the accumulation of osmo-protectants such as sugars, polyols and proline etc. (Per et al., 2017). Among these osmo-protectants proline, a non-protein amino acid is known to have ROS scavenging activity (Banu et al., 2009; Reddy et al., 2015; Szabados & Savouré, 2010). This organic osmo-protectant accumulates in stress exposed tissues of plant and enhances water potential of cell, improves cell turgor and plant growth (Gonzalez et al., 2010, Hayat et al., 2012). The main pathway involved in the synthesis of proline is glutamate pathway (Szabados & Savouré, 2010). Increased accumulation of proline under drought stress is an indication of better osmoprotective activity (Ortiz et al., 2015). In recent years scientists' interest has turned to transport of water across cell membrane aided by aquaporins and their role in abiotic stress. Five subfamilies of aquaporins are known i.e., tonoplast intrinsic

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proteins (TIP), plasma membrane intrinsic proteins (PIP), noduline-26 like intrinsic proteins (NIP), small basic intrinsic proteins and uncharacterized intrinsic proteins (XIP). Aquaporins gene expression and regulation is a part of adaptive mechanism of plants under stressful conditions. These proteins belong to superfamily Major Intrinsic Protein (MIP) are considered as significant channels maintaining water homeostasis through stomatal regulation (Chaumont et al., 2001; Kapilan et al., 2018).

One possible way to enhance drought tolerance in plants is to use different useful microorganisms as inoculants. The role played by microorganisms to provide stress tolerance to plants is an area of great interest for scientists. Microorganisms with their potential genetic and intrinsic genetic capabilities can adapt themselves to various environmental conditions to provide tolerance to abiotic stresses including drought (De Zelicourt et al., 2013; Ruiz-Lozano & Azcón, 2000; Vivas et al., 2003). AMF and bacterial colonization in plant roots improve drought tolerance by enhancing osmotic adjustments, improving antioxidant defense mechanisms and regulating stress responsive genes expression (Zou et al., 2021). Symbiotic association formed by AMF with plant roots is helpful in absorbing water and nutrients from surrounding environment (Raklami et al., 2019; Smith & Read, 2010). Proline metabolized enzyme activities are regulated by different AMF species resulting in melioration of proline metabolism (Wu et al., 2017). Drought tolerance is improved in plants co-inoculated with plant growth promoting rhizobacteria and AMF (Bharti et al., 2016; Marulanda et al., 2009). Microbes enhance plant drought tolerance by various mechanisms such as, balancing plant hormonal level, reduction in stress ethylene production, improve nutrients uptake and increase in antioxidant enzymes (Meena et al., 2017).

Tomato *(Solanum lycopersicum)* belongs to family Solanaceae, is an important vegetable crop rich in antioxidants, nutrients and vitamins (Khanna et al., 2019). All around the world tomato production is greatly affected by drought. Therefore, to develop drought tolerant tomato varieties it is crucial to deeply study the drought responsive mechanisms of tomato while maintaining yield (Cammarano et al., 2020). This will require novel technologies to counterpart traditional approaches, which often fail to prevent yield losses. Studies on improving plant drought tolerance by rhizosphere microorganisms are relatively few and co-inoculation of

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AMF and extracellular polymeric substances producing bacteria is not studied yet. Therefore, this study aimed to investigate the physiological, biochemical and molecular aspects of tomato plant adaptation to drought stress in the presence of AMF and extracellular polymeric substances producing bacteria. They can be used as an eco-friendly and sustainable agricultural strategy that can replace or supplement the need for genetic modification of plants and decrease the use of inorganic fertilizers

2. Results

2.1. Co-applied Bacillus subtilis and AMF ameliorate deleterious effects of drought on growth and colonization in tomato plants

Drought stress significantly reduced dry weight of shoot, root, relative water content, plant height and mycorrhizal colonization (F, M and A) by 21.53%, 0%, 26.29%, 0.57% and 0%, 0%, 0% relative to normal condition (Fig. 1A-D). In contrast, microbial inoculation ameliorate deleterious effects of drought and enhanced dry weight of shoot, root, relative water content, plant height and mycorrhizal colonization (F, M and A) under drought stress (Fig. 2A-C). Regarding microbial inoculation, *Bacillus subtilis* (BS) enhanced shoot dry weight, root dry weight, relative water content and plant height by 34.88%, 21.34%, 27.56% and 44.42% with the same trend observed with AMF application (32.79%, 22.18%, 26.48% and 15.48%) under drought stress as compared to no inoculation treatment (Fig. 2A-C). Conversely, co-application of BS and AMF enhanced mycorrhizal colonization (F, M and A) by 0%, % and % followed by AMF sole application (%, % and %) compared to other treatments under drought stress. These finding shows that microbial inoculation (specifically co-application) is essentially required for reducing harmful effects of drought on growth and mycorrhizal colonization traits under drought stress. So, BS and AMF co-jointly played a role in inducing drought tolerance in tomato plants.

2.2. Bacillus subtilis and AMF joint application enhances photosynthetic pigments and proline contents in drought stressed tomato plants

Total chlorophyll and carotenoids contents were remarkably reduced by 62.06% under drought stress as compared to normal condition. However, microbial inoculation dramatically increased total chlorophyll and

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carotenoid contents under drought stress. Co-applied *Bacillus subtilis* and AMF and sole applied *Bacillus subtilis* considerably increment total chlorophyll and carotenoid contents by 69.93%, 22.34% and 65.73%, 20.21% under drought stress relative to no inoculation under drought condition (Fig. 2D-F). Same trend was observed under normal condition for both treatments. In contrast, drought stress increase proline content by 235.71% in tomato plants as compared to normal condition (Fig. 2D-F). However, microbial inoculation remarkably increased the proline content in drought stressed plants as compared to normal condition. Under drought stress, joint application of *Bacillus subtilis* and AMF along with sole applied *Bacillus subtilis* considerably increased proline content by 131.91% and 129.78% as compared to no inoculation in drought stressed plants.

2.3.Integration of Bacillus subtilis with AMF enhances proline metabolized enzyme activities under drought stressed tomato plants

Proline metabolized enzymes (P5CR and P5CS) showed an upregulated behavior under drought stress and this upregulation was 42.33% and 54.68% as compared to normal condition. However, microbial inoculation either by BS, AMF or combination showed no significant effect under drought stress while under drought stress with no inoculation, these enzymes upregulated by 17.79% and 31.31% as compared to normal condition (Fig. 3A-D). Conversely, drought stress reduced activity of OAT enzyme by 7.28% as compared to normal condition, however, ProDH activity was not significantly affected by drought stress (Fig. 3A-D). Under drought stress, only BS application upregulated OAT enzyme activity by 21.83% which is non-significant with co-application of BS and AMF (12.22%) and AMF (12.22%) compared to no inoculation treatment (Fig. 3A-D). On the other hand, activity of ProDH enzyme was upregulated by 41.93% under drought stress when inoculated with BS as compared to no inoculation treatment. Under normal condition, co-application of BS and AMF enhanced both enzymes as compared to other treatments. These results suggests that a decrease in proline accumulation in inoculated plants is potentially linked with microbe-modulated decrease of glutamate synthetic pathways and an increment of proline catabolism.

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2.4.Combined application of Bacillus subtilis and AMF reduces oxidative stress and upregulates antioxidant enzyme activities under drought stress

When tomato plants were exposed to drought stress, the SOD and CAT activities were elevated by 43.80% and 141.25% as compared to control. Combined application of *Bacillus subtilis* and AMF remarkably increased SOD and CAT activities by 28.61% and 50.60% followed by *Bacillus subtilis* (20.61% and 13.29%) and AMF treatments (21.84% and 10.18%) under drought stress relative to untreated plants (Fig. 4A-D). POD and GR activities also elevated under drought stress by 103.39% and 14.76% as compared to normal condition. Joint inoculation with *Bacillus subtilis* and AMF increased POD and GR activities by 28.63% and 50.60% followed by *Bacillus subtilis* (29.11% and 13.29%) and AMF (26.25% and 70.76%) as compared to no inoculation treatments Fig. 4A-D). The drought stress led to significant increase MDA and $H_2O_2$by 42.4% and 159.20% as compared to normal condition. On the other hand, combined application of *Bacillus subtilis* and AMF reduced MDA content and increased H_2O_2 content under drought stress by 27.02% and 54% as compared to no inoculation in drought stressed plants (Fig. 4A-D). This suggests that co-applied *Bacillus subtilis* and AMF triggers the production of antioxidant enzyme activities under drought stress, thereby protecting the plants the oxidative damage.

2.5.Drought stress upregulates expression of aquaporin and ABA biosynthesis genes in tomato plants

Drought stress considerably upregulated the expression of all three aquaporin genes (*SlPIP2;1*, *SIPIP2;5* and *SlPIP2;7*) and this upregulation was by 303.33%, 322.66% and 198.33% as compared to normal condition. In contrast, sole application of AMF significantly reduced upregulation of *SlPIP2;1*, *SIPIP2;5* and *SlPIP2;7* genes by 91.15%, 64.90% and 47.37% under drought stress as compared to sole application of AMF and their combination (Fig. 4A-E). Under normal condition, co-application of BS and AMF upregulated the expression of *SlPIP2;1* and *SIPIP2;5* while the expression of *SlPIP2;7* gene was upregulated in plants inoculated with only AMF (Fig. 4A-E). There was a significant effect of drought on ABA biosynthesis gene, Le NCED1, encoding 9-cis-epoxycarotenoid dioxygenase (Thompson et al., 2000) and drought stress upregulated this gene by 216.66% as compared to normal condition. Under drought stress, plants inoculated with only *Bacillus*

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subtilis elevated upregulation of LeNCED1 by 21.36% as compared to other treatments, however there was no significant difference in gene expression of plants inoculated with only AMF and their combination with BS (Fig. 4A-E). Under normal condition, sole application of AMF triggered the upregulation of this gene, however, the other treatment effect was non-significant. Regarding the expression of ABA responsive marker gene, Le4, encoding dehydrin (Kahn et al. 1993), drought stress significantly upregulated this gene by 1203.33% as compared to normal condition. There was no significant difference observed on expression of Le4 gene between sole application of *Bacillus subtilis* and AMF, however they downregulated their expression 76.78% and 77.74% as compared to combined treatment under drought stress. However under normal condition, mixed application of BS and AMF and only AMF enhanced upregulation of Le4 gene expression as compared other treatments.

2.6.Principal component hierarchical cluster analysis

Principal component analysis (PCA) was performed using all traits analyzed. Percent contribution of different factors under normal condition was differed from drought stress condition as observed in PC1 and PC2 (Fig. 5). The first component (PC1) accounted for 41% of the variance explained, while second component (PC 2) accounted for 27.2% variance explained (Fig. TableS2). Under normal condition, growth traits and photosynthetic pigments were the main contributor in PC1 while oxidative stress markers, antioxidant activities, proline metabolized enzymes, aquaporin and ABA biosynthesis gene expression were the main contributor under drought stress (TableS3). Similarly, in PC 2 only aquaporin and ABA biosynthesis gene expression were the main contributor under drought stress. In overall map, the microbial inoculants were far from non-inoculants under both drought and normal conditions (Fig. 5).

The hierarchical cluster analysis (HCA) along with heatmap confirmed the PCA results and exhibited a clear separation of the control and drought stressed plants in two main clusters. In addition the cluster topology was similar under two condition (Fig. 6). Under drought stress, the antioxidant activities, oxidative stress parameters, and proline metabolized enzymes, aquaporin and ABA biosynthesis gene expression were strongly correlated with drought stress compared to normal condition. Regarding microbial inoculation

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treatments, proline metabolized enzymes, oxidative stress markers, aquaporin and ABA biosynthesis gene expression were strongly correlated while photosynthetic traits, growth parameters and colonization were negatively correlated in non-inoculated plants under drought stress. However under co-application of Bacillus subtilis and AMF, antioxidant activities and colonization were positively correlated as compared to other traits under drought stress.

3. Discussion

Drought stress has become a disquieting stress factor worldwide. Well organized plan of action needs to be devised to prevent immoderate agricultural loss. During last decade, researchers have turned their focus towards executing and validating the tools and different techniques to prevent productivity and yield loss caused by drought. One of these mechanisms, which is an indispensable factor to facilitate plant survival under drought stress is to establish symbiotic relationship and colonization with *Bacillus subtilis* and AMF (Augé, 2004). In the present study, we have explored the influence of *Bacillus subtilis*, AMF and their combination in enhancing drought tolerance mechanisms in tomato plants. Inoculation with *Bacillus subtilis* significantly enhanced growth traits and alleviate the reduction to a considerable extent. Conversely, mycorrhizal colonization (F, M and A) was enhanced in the co-inoculation (BS+AMF) treatment under drought stress. Other researchers (Wu et al. 2017, Quiroga et al. 2017, and Shah et al. 2020) have also described the growth increases in plants under drought stress mediated by co-inoculation. Less water availability affects the cell division and proliferation by lowering genes expression such as, tubulin and cyclin (Setter and Flannigan, 200). Recently, Zhang et al. 2019 and Shah et al. 2020 also signified that AMF inoculation improves decline in height, growth and biomass accumulation caused by drought in rapeseed, *Zenia insignis* and clover. In the current study, drought stress declined the colonization potential (F, M and A) of AMF, which can be linked to decreased availability of soil moisture. The deleterious effects of drought stress of root AMF colonization might be due to spores germination and the spread of hyphae in soils being inhibited by drought stress. This result is accordant with the earlier work done using *Erythrina variegate* (Manoharan et al. 2010) and *Poincianella pyramidalis* (Frosi et al. 2016). Improvement of growth, biomass and higher leaf RWC in co-

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inoculated plants might be due to enhanced water and nutrient uptake helped by mycorrhizal hyphae and biofilm formation by *Bacillus subtilis* (Manoharan et al. 2010, Frosi et al. 2016 and Shah et al. 2020). In present study, co-inoculation might have ameliorated root morphology resulting in increased absorption of mineral elements (N, P and K). Enhanced growth in co-inoculated tomato plants is directly modulated by their photosynthetic functioning. However, drought considerably decreased the pigment contents, and co-inoculation showed helpful to inhibit the photosynthetic inhibition. Enhanced pigment contents in AMF and BS-colonized tomato plants positively impacted the photosynthetic rate over the drought-stressed and un-inoculated plants. Analogous to our results reduction in chlorophyll content and photosynthesis caused by drought has been described by other researchers (Ahanger et al. 2017; Begum et al. 2019; Shah et al. 2020). Low photosynthesis rate under drought stress is the consequence of abatement of coupling factor and ATP production (Tezara et al. 1999). Co-inoculation-mediated increments in photosynthetic efficiency might be due to enhanced N uptake, which might result in superior Rubisco synthesis. The authors of Abdel-Salam et al. 2018 also reported the increase in chlorophyll content and photosynthetic rate in damask rose caused by AMF inoculation under drought stress conditions. Stress conditions trigger decline in photosynthesis by bringing down the synthesis of Rubisco (Fatma et al. 2014) and upregulating the activity of chlorophyllase (Dalal and Tripathy, 2012). The intensity of these effects caused by drought are increased by impede mineral nutrition, enzyme stability and membrane functioning.

The results of the current study exhibited a lesser accumulation of proline in leaves from co-inoculated tomato plants than non-inoculated plants under drought stress. This result analogous to the previous observations under drought stress in soybean (Porcel et al. 2004), *Erythrina variegate* plants (Manoharan et al. 2010) and *Ocimum gratissimum* (Huang et al. 2014). Low proline accumulation attributable to mycorrhization may be due to minimal strain by drought stress, owing to high water status in plants with AMF. The current study shown drought stress induced greater proline concentrations with an increment of P5CR and P5CS activity, a reduction of OAT activity and no difference of ProDH in tomato leaves, signifying that proline accumulation in co-inoculated and non-inoculated tomato plants was resulting from the improvement of the glutamate

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synthetic pathway of proline but not the ornithine synthetic pathway of proline. This is in conformity with earlier studies of Zou et al 2013. In this study, co-inoculated seedlings exhibited considerably lesser P5CR and P5CS activity but markedly greater ProDH activity than non-inoculated seedlings, regardless of normal or drought conditions. This shows that a reduction in proline accumulation in co-inoculated seedlings is possibly linked with a co-inoculation modulated decline of glutamate synthetic pathways and an upsurge of proline catabolism, which will be still considered by checking the appropriate gene expression. However, the underlying molecular mechanisms in co-inoculated plants still need to be further studied by RNA-seq.

Drought stress cause oxidative stress in plants due to unrestrained reactive oxygen species (ROS) production, which are noxious molecules having ability to cause oxidative damage to DNA, proteins, and lipids (Miller et al., 2010). Increase in antioxidant levels is mandatory for ROS scavenging (Smirnoff, 1993). It has been suggested that protection from oxidative stress through enhanced antioxidant levels may be a mechanism by which plant tolerance to drought stress is enhanced by AMF symbiosis and *Bacillus subtilis* colonization (Ruiz-Sánchez et al., 2010). In this study, lipid peroxidation and H_2O_2 levels were described as indicators of oxidative stress. Malondialdehyde (MDA) formed by the breakdown of polyunsaturated fatty acids acts as a suitable index to estimate the extent of lipid peroxidation. Both oxidative stress markers are found to be increased by drought stress in root and leaf. It was found that under drought stress, compared to control plants AMF and *Bacillus subtilis* inoculated plants showed low H_2O_2 and MDA levels while mixed inoculation resulted in the increase of both stress markers. This shows that, compared to non-inoculation treatment, the co-inoculation of tomato plant is more effective to protect the plants against oxidative stress. Plants have developed antioxidant defense mechanisms to circumvent oxidative damage caused by drought stress. This mechanism includes the catalase enzyme, responsible for converting H_2O_2 to H_2O and O_2 in peroxisomes (Noctor & Foyer, 1998; Miller et al., 2010; Scheibe & Beck, 2011). In the current study, catalase activity was examined in the leaf of tomato plants exposed to drought stress and it was noticed that co-inoculated plants exhibited considerably maximum catalase activities over non-inoculated plants. Highest catalase activity was observed in co-inoculated plants. It was the same treatment with low levels of oxidative stress markers. This

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indicates that co-inoculation of plants improves the catalase activity and protects the plant from oxidative stress caused by drought. It was found in earlier studies that, compared to non-AMF control plants, AMF lettuce plants have improved antioxidant enzyme (SOD) activity under drought stress (Ruiz-Lozano et al., 1996). This response at transcriptional level has confirmed by molecular analysis (Ruiz-Lozano et al., 2001). Relative to non-AMF plants, SOD activity was enhanced by 50% and 138% by co-inoculation during drought stress conditions (Ruiz-Lozano et al., 2001). This increment in SOD activity and expression of gene were associated with enhanced drought stress tolerance. AMF symbiosis has also been reported to augment the glutathione reductase (GR) activity in root and nodules of soybean plants under drought stress (Porcel et al., 2003).

Abscisic acid is an important plant hormone which respond to abiotic stresses in plant including drought (Christmann et al., 2006). Under drought conditions the biosynthesis of abscisic acid is rapidly increased (Hong et al., 2013; Osakabe et al., 2014). It is synthesized in plant roots and then translocated to the leaves where it starts plant adaptation to drought by closing of stomata (Wilkinson & Davies, 2010). The improved drought stress tolerance in AMF plants has been linked to changes in ABA balance. It is reported in several studies that the plant ABA level changes when AMF symbiosis is established (Ruiz-Lozano et al., 2012). ABA regulates water status of plant through regulation of transpiration rate, root hydraulic conductivity and induction of genes encoding enzymes and proteins involved in tolerance against dehydration (Zhang et al., 2006; (Hirayama & Shinozaki, 2007). This study shows, in non-inoculated plants the expression of ABA biosynthesis gene *LeNCED1* was augmented by drought stress and gene expression was downregulated in AMF plants by drought stress. Similarly, in non-inoculated plants the expression of *Le4*, ABA responsive marker gene was increased by drought stress while in AMF plants it remained unaffected. This could indicate that response of AMF to drought stress is not dependent on ABA. It can also be possible that down-regulation of ABA biosynthesis gene is a mechanism by which AMF symbiosis keep up plant growth during drought stress conditions, by the prevention of ABA-mediated plant response to drought stress, which includes closure of stomata, CO2 uptake prevention, low photosynthesis and growth rates. It has been proposed that ABA is

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mandatory for root colonization by AMF (Fester & Hause, 2007) to enhance symbiotic capability under drought stress (Ruiz-Lozano et al., 2009). Transcript level of the ABA biosynthesis gene was measured in this study but not hormone level itself. Correlation between LeNCED1 gene expression and ABA levels in lettuce was reported by RuizLozano et al., (2015), but it is not reported in tomato which indicates that it is not necessary that low transcript level translate to reduced hormone levels. AMF has an ability to transfer water to host plant during symbiotic relationship and it is anticipated that host permeability for water increases and should result in upregulation of aquaporin genes. Under well-watered conditions the aquaporin genes upregulation is expected as observed through this study. Under drought conditions contrasting results were obtained. Porcel et al. (2006) described that under drought stress aquaporin genes under study were downregulated and this downregulation was acute in AMF plants compared to non-inoculated plants. The impact of AMF symbiosis downregulating aquaporin gene may have physiological significance to aid AMF plant to survive in drought stress. The reduction in expression of plasma membrane aquaporin genes in AMF plants under drought stress cab be a regulatory mechanism to restrict the loss of water from cells (Porcel et al., 2006). In a study by Aroca et al. (2007), in AMF and non-inoculated plants under drought stress, expression of four aquaporin genes was analyzed from which three showed distinctive regulation by AMF. Under drought stress, *PIP1;1* was a bit inhibited by AMF while expression pattern of non-inoculated plants remained unchanged. *PIP1;2* was restrained by drought stress analogously in AMF and non-AM plants. *PIP1;3* under drought stress was inhibited in AMF plants but induced in non-AM plants. Thus, AMF symbiosis effect on aquaporin genes expression regulation varies under drought and it dependent on specific aquaporin genes and AMF. The upregulation or downregulation of specific aquaporin genes by AMF symbiosis should improve water status of plant and contribute to global plant tolerance against drought stress, as indicated by their improved growth and water status under drought (Jang et al., 2004). In summary, the aquaporin genes expression inhibition by AMF symbiosis in plants under drought is strategy to conserve water in host plant, to allow such plants to maintain leaf relative water content and higher shoots (Ruiz-Lozano et al., 2009).

4. Materials and Methods

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4.1.Plant materials and experimental setup

Experiment was conducted at the glasshouse of Agricultural Research Station, Charsadda, Pakistan. The top layer soil (0-20cm) having sandy loamy structure, pH 7.5, electrical conductivity 0.73 m/S, carbon 9600 mg kg^{-1}, nitrogen 65.98, phosphorous 18.78 and potassium 80.67 mg kg^{-1}, respectively was taken from the near fields of the research station. Soil was autoclaved at 121^0C for two hours, and autoclaved soil was used to fill pots (21 cm height, 20 cm diameter, 7 kg soil in each pot). AMF inoculum (*Rhizophagus irregularis*) in the form of tomato root fragments and spores was added seven days before sowing at the rate of 50 g (equivalent to ~700 spores). AMF inoculum consisted of tomato infected roots and spores was obtained from the research station. Pots were transferred to chamber having photoperiod of 16 h day/8 h night, light intensity was 300 μmol^{-2} s^{-1}, day/night temperature was $22/18^0$C and relative humidity was 60-70%.

Tomato (var. Riogrande) seeds were procured from National Agricultural Research Center, Islamabad, Pakistan. Healthy and mature seeds were surface sterilized with sodium hypochlorite (0.1% for 15 minutes), and then washed with Milli-Q water (20 minutes). The existence of AMF spores in inocula was verified by wet sieving and sucrose density centrifugation for spores extraction (Pacioni, 1992) and staining of root fragments with ink/vinegar solution (Vierheilig et al., 1998) using microscopic evaluation. Endophytic bacterium *Bacillus subtilis* (extracellular polymeric substances producing strain, Shah et al. 2020) previously used in our study were also used in this experiment (Shah et al. 2020). Nutrient broth medium (CM0001; OXOID) was used for growing strain, then incubated at 28^0C for 3 days till the culture density reached 108 cfu/mL. Seedling dipping method in the bacterial culture was used to inoculate tomato plants, and one ml inocula was poured around the tomato seedling to avoid failure of inocula. The treatments combination were: (a) control (b) *Bacillus subtilis* (c) AMF (d) *Bacillus subtilis* + AMF applied under severe drought stress (20 %±5) and control (70%±5). The percentage of the soil moisture of each pot was determined using a MiniTrase time domain reflectometer (TDR, Soil moisture Equipment Corp, California, USA) and noted as volumetric water content (%). The experimental pots were arranged in completely randomized design having three replicates. Pots were frequently observed and irrigated (70%±5 for normal condition and rest according to the

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treatment), while half-strength Hoagland solution was given once a week to avoid nutrient deficiency. Twenty four days after seedling establishment, the plants were exposed to drought stress for 36 days. After 36 days of drought stress, plants were carefully uprooted and bring to laboratory for experimentation. Root tissues were fixed in formalin: acetic acid: alcohol till further use.

4.1.1. Measurement of growth characters

In each treatment tomato plants were uprooted from three different pots and washed with distilled water. Dry weight of shoot and root was determined. Data for shoot and root dry weight was collected instantly after harvest. To acquire the dry weight, samples were dried at 70ºC. Fresh leave samples from each plant were collected to calculate leaf relative water content (LRWC). To acquire fresh weight, leaves were weighed on digital balance. Leaves were then kept in 5mM CaCl2 solution for 24hrs to regain turgidity and again weighed to get turgid weight. Leaves were then oven dried at 70°C for 72hrs to get dry weight. LRWC was calculated by using formula described by Shah et al. (2020):

$$LRWC = (Fresh\ weight - Dryweight \div Turgid\ weight - Dry\ weight)x100$$

4.1.2. Measurement of photosynthetic pigments

Chlorophyll (a and b) and carotenoid contents were estimated in fully expanded leaves using the method proposed by Arnon (1949). The leaf samples (0.3 g) were incubated in 10mL acetone aqueous solution (80%) and centrifuged for 10 min at 12000g. the extracts absorbance was measured at 663, 645 and 470 nm. Chlorophyll a, b and carotenoids were estimated by UV-spectrophotometer (Kyoto Shimadzu, Japan).

4.1.3. Measurement of hydrogen peroxide (H2O2) malondialdehyde (MDA) contents

H_2O_2 concentration was measured by the method proposed by Najafi Kakavand et al. (2019). Leaf samples (0.5g) were extracted with trichloroacetic acid and centrifuged for 15 min at 12000g. Then, enzyme supernatant (0.5 mL) was added to phosphate buffer (0.5 mL, pH 7.0) and potassium iodide (1 mM). With H_2O_2 as standard, absorbance of reaction mixture was set down at 390nm. MDA content in tomato leaves was estimated as stated by Heath and Packer (1968). Briefly, it involved the homogenization of 0.5g leaf sample

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with 10 mL ethanol and centrifugation at 10,000g for 10 min. 1 mL enzyme extract was then mixed with thiobarbituric acid (TBA, 0.65%) mixture (2 mL) and trichloroacetic acid (TCA, 20%). The mixture was cooled immediately after boiling for 30 minutes and centrifuged again for 5 min at 10000g. MDA content was estimated by nonspecific absorption difference at 600 nm and 532 nm.

4.1.4. Determination of antioxidants enzymes

To determine enzymes, extraction of fresh leaves of B. rapa was done with 5 mL phosphate buffer (50 mM, pH 7.8) containing EDTA (0.2 mM) and polyvinylpyrrolidone (PVP-40, 2%). Homogenate was centrifuged (12,000 g) for 10 min at 40C. The supernatant was used for enzyme activities analysis. Enzyme extracts that were similar were used to determine soluble proteins.

Method used by Giannopolitis and Ries (1977), was used to measure Superoxide dismutase (EC 1.15.1.1, SOD) activity. It is based on photochemical reduction of nitroblue tetrazolium. An enzyme unit was defined as the quantity of enzyme having ability of hampering 50% photoreduction of nitroblue tetrazolium at 560nm. The catalase activity (EC1.11.1.6, CAT) was determined by earlier method proposed by Aebi (1984). H_2O_2 decomposition rate was monitored at 240mm for 3 min and absorbance change was recorded.

The glutathione peroxidase (EC 1.11.1.9, GPX) activity was estimated using H_2O_2 as a substrate (Ahmad et al., 2018).

4.1.5. Mycorrhizal colonization

Per plot two root systems were collected and pooled together. Root material was rinsed and prepared for staining as mentioned by Vierheilig et al. (1998). Roots were cleared in 10% KOH for 5 to 10 minutes at 90°C. For coloration put in black ink (5% in acetic acid) at 90°C for 5 min, then washed with water and kept at room temperature in 8% acetic acid for 25 min. Roots were again washed with water, covered with pure glycerol and kept at 4°C. For microscopic observation, roots were cut into 1 cm segments and 15 segments were put in glycerol on slide and covered with cover slip four times per sample. Mycorrhization rates were estimated by the method used by Trouvelot et al. (1986) and expressed as mycorrhizal colonization such as, root

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segments frequency with mycorrhizal structures at root system scale (F), mycorrhizal colonization intensity at root system scale (M) and arbuscule abundance at root system scale (A).

4.1.6. Total RNA extraction and gene expression assay

MiniBEST Plant RNA Extraction Kit (Takara, Japan) was used to extract total RNA from leaf samples of 40 days old tomato plants (0.1g) according to protocol stated by manufacturers. gDNA eraser was used to digest contaminated DNA. cDNA was synthesized from 1 microgram of RNA using PrimeScript™RT reagent Kit (Takara, Japan). qRT-PCR was performed in triplicates using SYBR®Premix Ex Taq II (Takara, Japan) in accordance with the protocol provided by manufacturers in CFX96™Real-Time System (Bio-RAD, USA). mRNA transcriptional level was estimated by using the 2-ΔΔCT method described by Livak and Schmittgen (2001). Primers used in this study are listed in supplementary data (Table S1).

4.1.7. Proline metabolized enzymes protocol

Ninhydrin method of Troll and Lindsley, 1955 was used to assess concentration of proline in leaves. Leaf P5CS, OAT, and ProDH activities were checked using method stated by Zou et al. 2013. Leaf P5CR activity was assessed according to the method used by Chilson et al.1992 with some minor changes. Fresh leaf samples (0.2g) was homogenized with 5mL 100mM Tris-HCl buffer (pH 7.5) containing 10mM $MgCl_2$, 10mM β-mercaptoethanol, 1mM EDTA, 2% (w/v) polyvinylpolypyrrolidone and 2mM phenylmethanesulfonyl fluoride. The homogenized mixture was centrifuged at 20,000× g for 20min at 4°C. The activity of enzymes was assessed with 1mL volume of reaction mixture containing supernatant, 20mM proline, 200mM glycine buffer (pH 10.3) and 15mM NAD^+. The absorbance at 340nm was taken down and a unit of P5CR was defines as amount of enzyme of 1µmol NADH during 1min (U/g FW).

4.2.Statistical analysis

The statistical analysis of the data was performed in Rstudio software (RStudio Team, 2021). Using Shapiro and Bartlett test in Rstudio default functions, normality and homoscedasticity of the data was evaluated. Normally distributed data were analyzed using construction of two way ANOVA model (drought stress,

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microbial inoculation and their interaction as three sources of variation) and the significance was evaluated using the D'Agostino test of skewedness on the residual variance (Komsta and Novomestky, 2015, package '*moments*'), along with post-hoc Tukey's Honest Significant Detection test (De Mendiburu, 2017, Tukey's HSD, $p<0.05$, package '*agricolae*'). Principal component analysis and hierarchical clustering analysis were perform on all the measured variables under drought stress, normal condition and microbial inoculation using the same software.

5. Conclusion

From the results of the current study, it can be concluded that co-inoculation of *Bacillus subtilis* and AMF have a positive effect on the growth, development and colonization of tomato plants by strengthening vital tolerance mechanisms under drought stress, than inoculation singly with either of them. Co-inoculation (*Bacillus subtilis* and AMF) up-regulated the antioxidant system, leading to alleviation of oxidative damage to membrane function and photosynthetic performance. Furthermore, the elevated accumulation of osmolytes revealed in preserving tissue water and further strengthening the antioxidant defense system, contributing to the stability of the whole plant structure and function. The abscisic acid biosynthesis gene and aquaporin genes differential regulation may contributed in tomato plant drought tolerance improvement by inhibiting ABA-mediated plant response to drought stress, which might decrease plant growth, and by hindrance of water loss from the plant. Co-inoculation-mediated holistic integrated tolerance approaches for enhancing growth of tomato under drought stress condition is recommended.

Data availability statement

The data that support this study will be shared upon reasonable request to the corresponding author.

Author contributions

TS conceived the study and perform data analysis. IS conducted experiment and data collection. TS and IS drafted the manuscript. AA and IA participated in the experiment. FM helped in data visualization. RD and FM reviewed and edited the manuscript. All authors read and approved the manuscript.

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Declaration of competing interest

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Fig. 1 Interactive effects of AMF and *Bacillus subtilis* on (A) dry shoot weight (B) dry root weight (C) relative water content and (D) plant height under drought stress in tomato plants. Mean values ($n=3$) followed by

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different letters above the bars among different treatments showed significant differences at *p<0.05*. *p<0.05, **p<0.01, ***p<0.001*. Significance was checked with ANOVA along with Tukey's HSD post-hoc test for normally distributed data. Kruskal-Wallis test under False Discovery Rate post-hoc correction (FDR, *p<0.05*) were used to analyze non-parametric data. DS=drought stress, MS=microbial inoculation, DS x MS=interaction of drought stress and microbial inoculation.

Fig. 2 Interactive effects of AMF and *Bacillus subtilis* on (A) frequency of mycorrhizal colonization in root system (B) intensity of mycorrhizal colonization in root system (C) arbuscule abundance in root system (D) Total chlorophyll content (E) carotenoids and (F) proline under drought stress in tomato plants. Mean values (*n=3*) followed by different letters above the bars among different treatments showed significant differences at *p<0.05*. *p<0.05, **p<0.01, ***p<0.001*. Significance was checked with ANOVA along with Tukey's HSD post-hoc test for normally distributed data. Kruskal-Wallis test under False Discovery Rate post-hoc correction (FDR, *p<0.05*) were used to analyze non-parametric data. DS=drought stress, MS=microbial inoculation, DS x MS=interaction of drought stress and microbial inoculation.

Fig. 3 Interactive effects of AMF and *Bacillus subtilis* on proline metabolized enzymes (A) P5CR (B) P5CS (C) OAT and (D) ProDH under drought stress in tomato plants. Mean values (*n=3*) followed by different letters above the bars among different treatments showed significant differences at *p<0.05*. *p<0.05, **p<0.01, ***p<0.001, ns=non-significant*. Significance was checked with ANOVA along with Tukey's HSD post-hoc test for normally distributed data. Kruskal-Wallis test under False Discovery Rate post-hoc correction (FDR, *p<0.05*) were used to analyze non-parametric data. DS=drought stress, MS=microbial inoculation, DS x MS=interaction of drought stress and microbial inoculation.

Fig. 4 Interactive effects of AMF and *Bacillus subtilis* on (A) SOD activity (B) CAT activity (C) POD activity (D) GR activity (E) MDA activity and (F) H_2O_2 activity under drought stress in tomato plants. Mean values (*n=3*) followed by different letters above the bars among different treatments showed significant differences at *p<0.05*. *p<0.05, **p<0.01, ***p<0.001*. Significance was checked with ANOVA along with Tukey's HSD post-hoc test for normally distributed data. Kruskal-Wallis test under False Discovery Rate post-hoc

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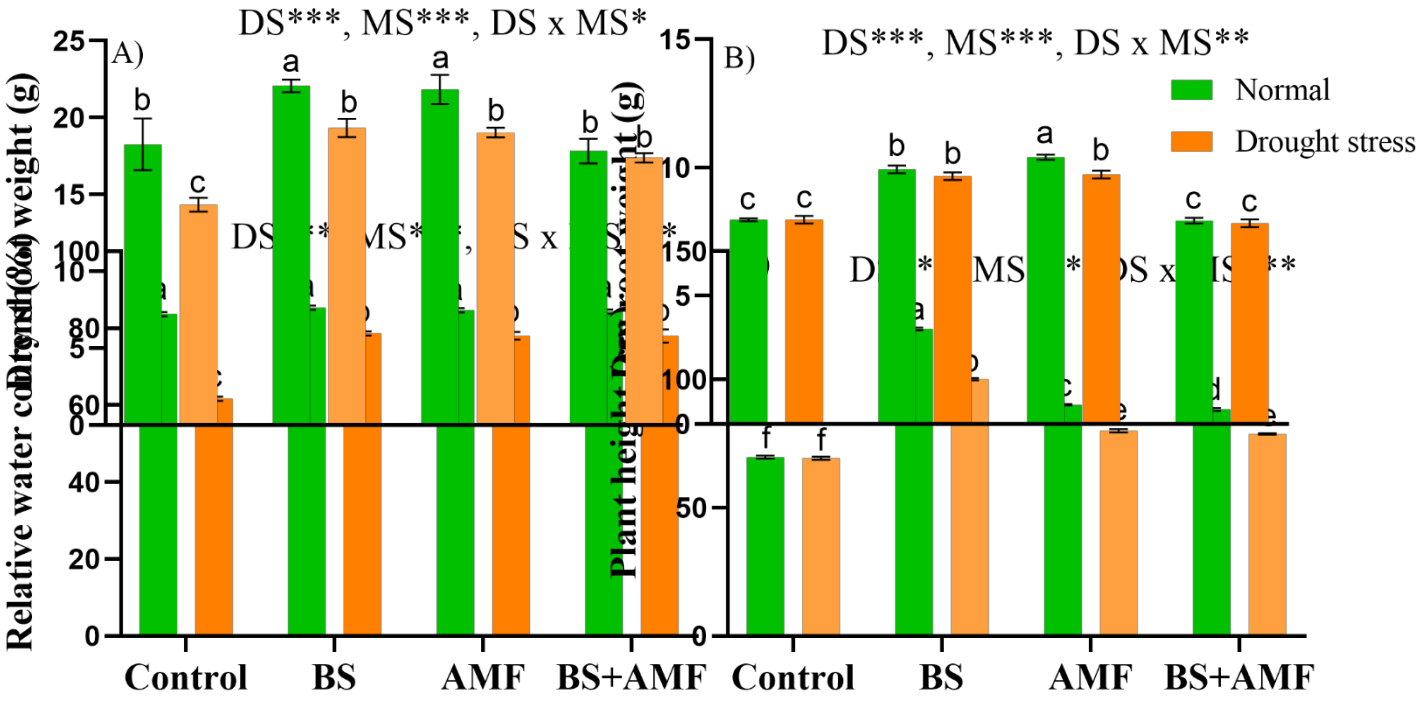
correction (FDR, $p<0.05$) were used to analyze non-parametric data. DS=drought stress, MS=microbial inoculation, DS x MS=interaction of drought stress and microbial inoculation.

Fig. 5 Interactive effects of AMF and *Bacillus subtilis* on relative expression of aquaporin genes (A) *LeSlPIP2;1* (B) *LeSlPIP2;5* (C) *LeSlPIP2;7* (D) *LeNCED1* and (E) *Le4* under drought stress in tomato plants. Mean values ($n=3$) followed by different letters above the bars among different treatments showed significant differences at $p<0.05$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *ns=non-significant*. Significance was checked with ANOVA along with Tukey's HSD post-hoc test for normally distributed data. Kruskal-Wallis test under False Discovery Rate post-hoc correction (FDR, $p<0.05$) were used to analyze non-parametric data. DS=drought stress, MS=microbial inoculation, DS x MS=interaction of drought stress and microbial inoculation.

Fig. 6 Principal component analysis of drought stress, normal condition and microbial inoculation based on different quantitative variables. WW= well-watered, DS= drought stress, BS= *Bacillus subtilis*, AMF=arbuscular mycorrhizal fungi.

Fig. 7 Hierarchical clustering analysis of drought stress, normal condition and microbial inoculation on different quantitative variables. WW= well-watered, DS= drought stress, BS= *Bacillus subtilis*, AMF=arbuscular mycorrhizal fungi



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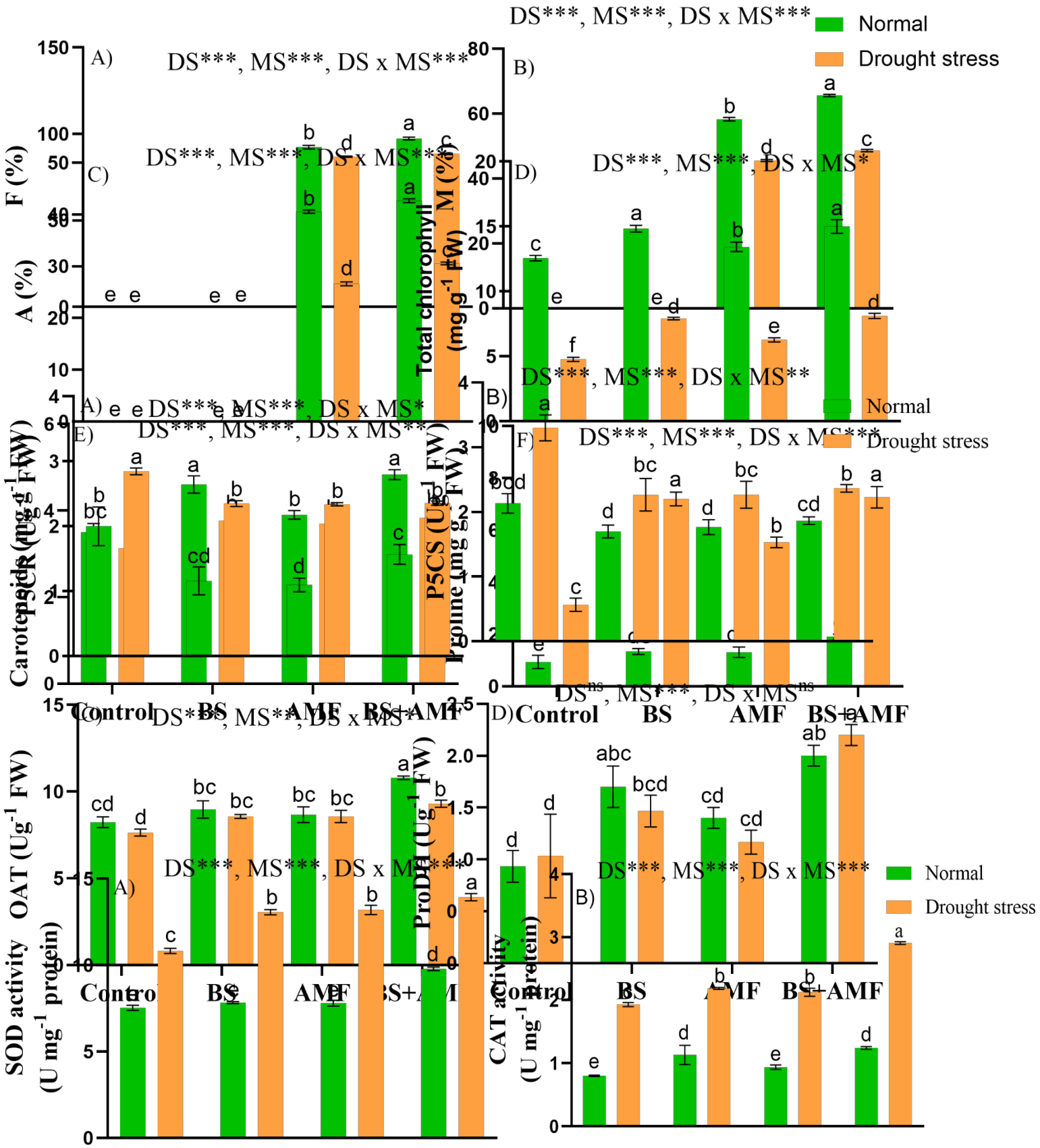

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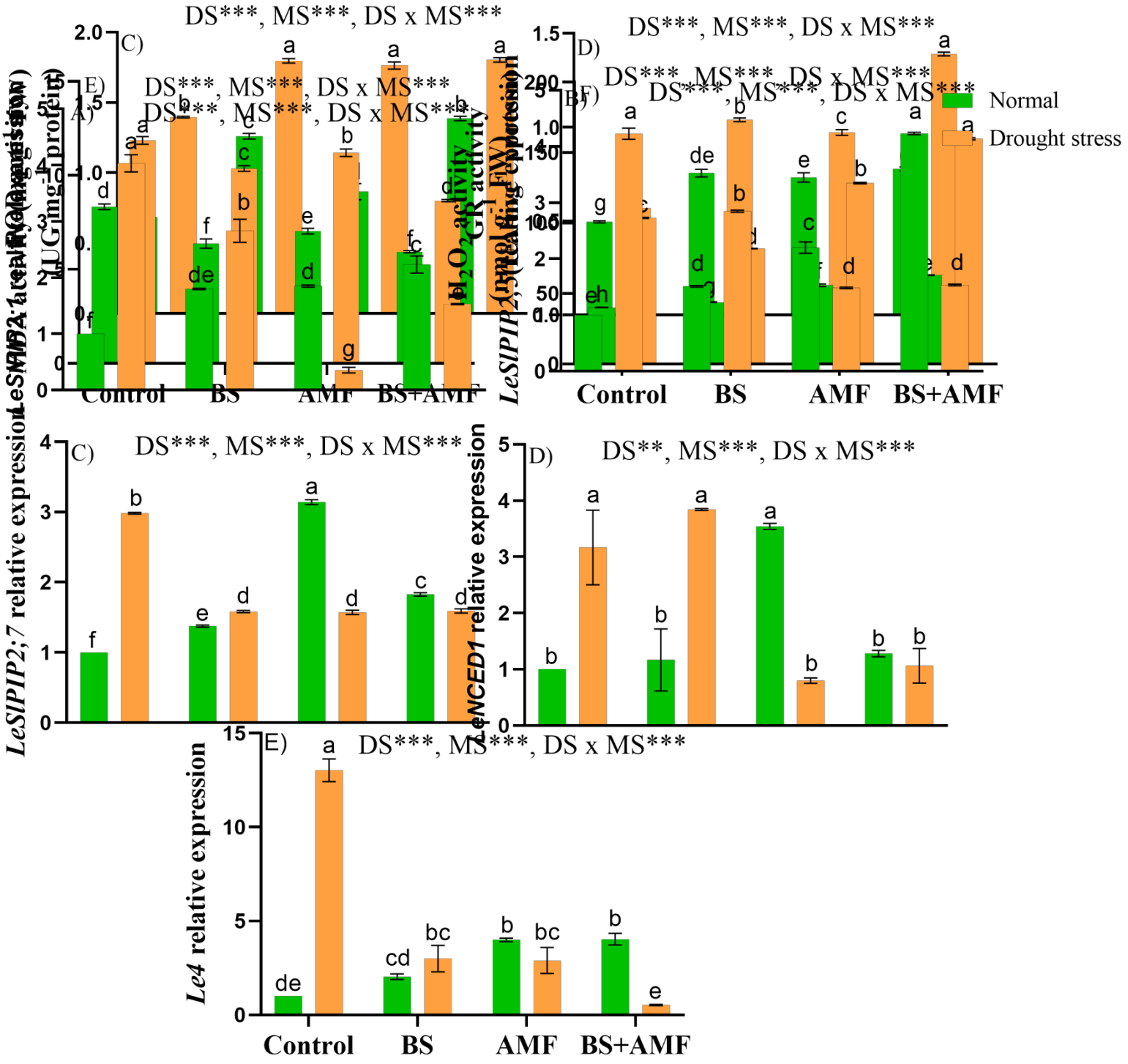

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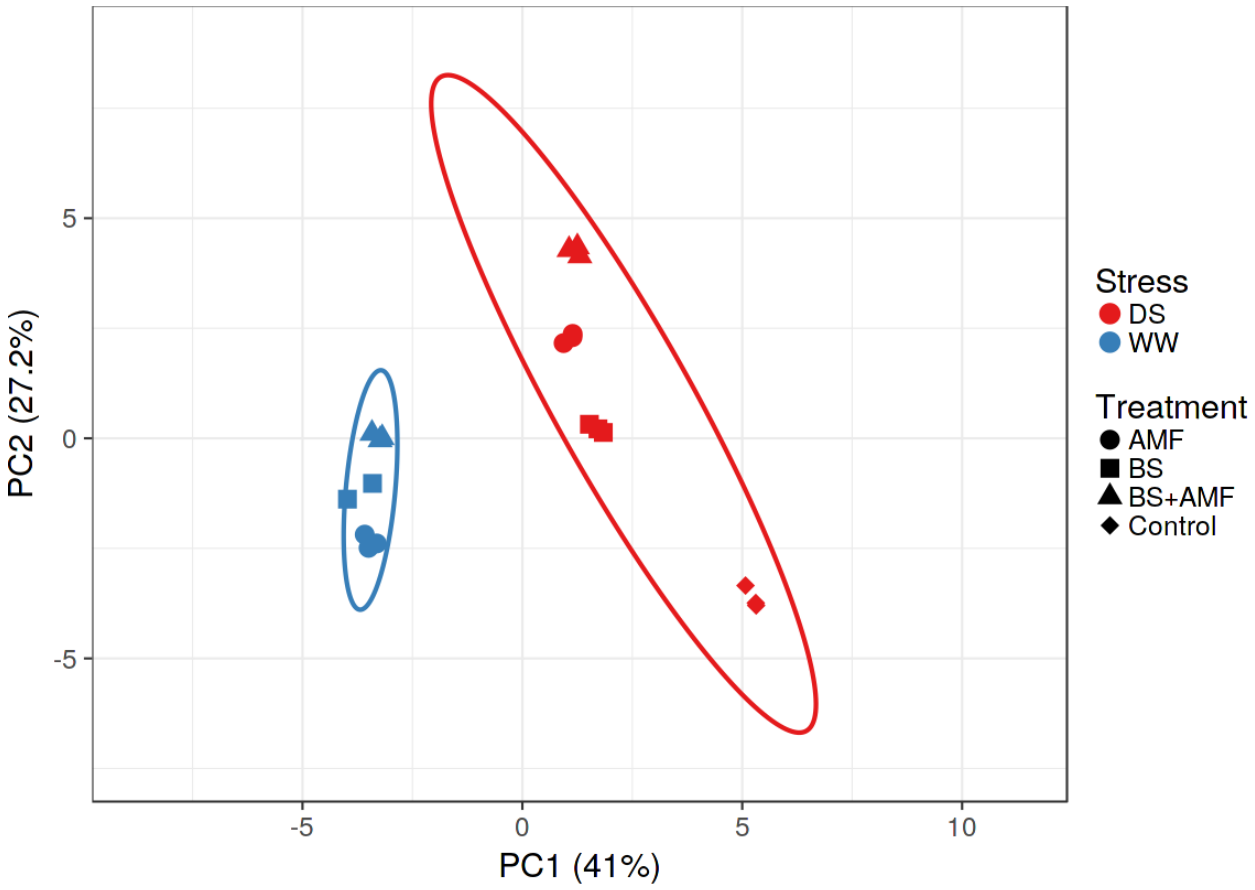

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