Farmyard manure regulated the defense signalling network in mash bean by countering stress responses of inglorious couple of charcoal rot fungus and copper

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Article

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Abstract

In the era of global warming, stress combinations instead of individual stresses are realistic threats faced by plants, which affect the metabolic activities in an inimitable mode unlike individual stress. In the current study, charcoal rot disease stress caused by notorious fungal pathogen viz., *Macrophomina phaseolina* (Tassi) Goid coupled with toxic levels of heavy metal copper (Cu) was investigated on morpho-physio-biochemical and molecular responses in mash bean [*Vigna mungo* (L.) Hepper] plants. Soil application with 2% Farmyard manure (FYM) was also used as a warfare agent against the stress/s responses in the plans. Therefore, soil spiked Cu (50 and 100 mg/kg) was inoculated with the pathogen, amended with 2% FYM was sown with mash bean seeds. The individual stress of MP or Cu resulted in more drastic changes in biological (growth, biomass, and yield), and physio-biochemical [(total chlorophyll content, carotenoids, reducing sugar total protein content, and total phenolic, catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO)] attributes with the greater translocation factors and bioaccumulation factors as compared to stress combination. The expression levels of catalase, ascorbate peroxidase, cytokinin-resistant gene as well as protein profiling and other metabolic changes (activity of CAT, POX, and PPO) were more up regulated under single stress conditions by mash bean plants. Alteration in studied parameters in mash bean plants provided the basis of cross-tolerance (hormesis) induced by Cu against the pathogen under stress combination. Nonetheless, 2% FYM in soil encounters the negative effect of stress responses provoked by the pathogen, Cu or both by decreasing Cu uptake by the plants. FYM worked better at lower concentrations (50 mg/kg) of Cu than at higher ones (100 mg/kg), hence could be used as a suitable option to reclaim soil health and better plant productivity.

Introduction

Pakistan is the second largest importer of pulses in the world, while mash bean [*Vigna mungo* (L.) Hepper] is the third largest and most highly praised pulse crop in the country after chickpea and lentil [1]. In addition to being a major source of least expensive protein (22–24%) and energy, mash bean also provides substantial amounts of oil (2.1%), fats (1–2%), raffinose oligosaccharide (31–76%), carbohydrates (50–60%) and vitamins (A and B)[2]. Being a short-duration and drought-tolerant crop, it can be cultivated as emergency vegetation, especially in the rainfed area, which can make significant economic benefits to the farmers[1]. By and large, mash beans can be seeded after exhaustive crops e.g. wheat and rice to restore soil natural matter owing to its nitrogen-fixing ability, hence are considered as fairly profitable from a most economical point[3]. Regardless of good nutritive value and reasonable cost for the consumers, the worldwide yield of mash bean including in Pakistan is very poor, which has decreased its cultivation area in the country[1,2]. Among others, fungal diseases and heavy metals are undoubtedly the two most important stresses having a huge impact on the growth and productivity of crops. In this context, the charcoal disease causes by soil-borne, necrotrophic fungus *Macrophomina phaseolina* (Tassi) Goid is the highest threat for pulses, especially in arid to tropical regions of the world, where it may cause 100% yield losses[4–6]. *M .phaseolina* is a notorious pathogen of over 500 economically valuable crops, causing various dry-weather wilts and rots, exhibits heterogeneous nature, adaptable to diverse environmental conditions, and forms abundant microsclerotia which makes disease control challenging[7–9]. Up till now, there is no known vertical resistance (R-gene based) to *M. phaseolina*, and no systemic fungicides available to combat the menace[10,11].

Nonetheless, routinely practiced and other human-dependent activities often containing heavy metals have increased the risk of fungal pathogen evolution and the jeopardy posed to them[12]. The soil surface is a fertile place for storing heavy metals, and then transferring them to the plants when they are highly concentrated, subsequently incorporated into the food chain and causing liver and brain disorders[13]. Among different metals, copper (Cu) mostly as $CuSO_4$, is the most common naturally occurring compound accumulating in the soil through Cu-based fungicides, agricultural waste, agrochemicals, wood preservatives, tanning, sewage sludge, and other anthropogenic activities. High soil Cu (20–100 mg/kg) concentrations are known to impart toxic effects to soil microorganisms as well as plants and hinder the mineralization of macronutrients (N, P, and K) and micronutrients (Fe and Zn)[14]. In soil, 5–30 mg/kg and in plant tissue 3–10 mg/kg of Cu are regarded as normal[15], where it contributes to hormone signalling, structural strengthening, photosynthesis process, and electron transport chain process in the plants[16]. However, higher Cu converts itself into complexes inside plant tissues, showing a high affinity for cell wall phenolic, carbonylic, carboxylic, sulfhydryl groups, nitrogen, oxygen, and sulphur atoms[17]. Excess Cu reduces plant growth and yield, decreases plant capacity to explore the soil for water and nutrient, alters root system design, while causing chlorosis, and necrosis, and induces lipid peroxidation and protein oxidation by acting as pro-oxidant[3,18]. So far, *M. phaseolina* can tolerate Cu toxicity due to higher osmotic pressure in the cell structure, while mycelial growth inhibition and morphological disorders were also recorded with increasing Cu concentration from 25–100 ppm, which transformed its viability and sporulation[19]. It is worth to mention at elevated concentrations, heavy metals not only alter the activities of harmful soil microorganisms but also affect beneficial flora which may also contribute to a reduction in soil fertility[20]. Thus, the concurrent occurrence of Cu with *M. phaseolina* either aggravates or inhibits the effect of latter leading to either enhanced or reduced susceptibility to pathogens[21]. In this connection, 5–20 ppm of Cu has been reported to show the hormetic effect, while ≥ 40 ppm induces a toxic effect on plants under biotic stress[22]. Therefore, it is important to address the effect of both stress/s on plants simultaneously along with effective countermeasures to address both stresses[23,24].

Organic soil amendments, including Farmyard manure (FYM), are generally utilized as a cheaper and easily available fertilizer to supply macro- and micro-nutrients and restore the soil's physical and chemical qualities in Pakistan[4]. FYM is a decomposed mixture of dung, urine, litter, and leftover materials from roughages and fodder fed to animals. A well-decomposed FYM contains 0.5–1.5% N, 0.2–0.4% P_2O_5, and 0.5–1.0% K_2O, thus act as a rich source of many nutrients (N, P, K, Ca, Mg, S, Zn, Fe, and Mn) in soil and plants. FYM helps to manage soil-borne disease by improving the natural suppressiveness of the soil through generating microclimates unfavorable for pathogens, releasing fungitoxic compounds (e.g. ammonia nitrous acid), increasing competition against pathogens for resources, reducing the sclerotial number, and minimizing the inoculum contact with plant roots[25]. Furthermore, FYM acts as a sink while reducing the mobility of metal in soil and its uptake by plants through their effect on the adsorption, complexation, reduction, and volatilization of metals[26]. Apart from the role of FYM in disease and heavy metal stress tolerance in plants, FYM-mediated defense responses against stress combinations still need to be explored.

Recent shreds of evidence suggest that plants evolved multiple and interconnected signaling pathways to regulate different sets of stress-responsive genes resulting in diverse physiological and metabolic responses to confer tolerance to environmental stresses[24]. These include pathogenesis-related (PR) proteins, transcription factors, and enzymes involved in producing metabolites and hormones. Many efficient antioxidative systems

work against reactive oxygen in coordination. Enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO) are known to act against stress/s[6,8]. These enzymes may be manipulated, overexpressed or down-regulated depending on the strength of stress, duration, enzyme type, plant species etc. Various workers have reported increased activities of many antioxidant defense system enzymes in plants to overcome the oxidative stress induced by biotic or abiotic stresses[27]. In all cases, these enzymes are encoded by multiple gene families and are present in several compartments of the cells. Few of the genes including catalase (CAT), ascorbate peroxidase (APX), and cytokinin resistant 1 (CYR1) are expressed as a result of cellular responses in plants against stress[28]. CAT gene (heme peroxidases) translates the hydrogen peroxide to water and reduces the ROS levels to shelter the cells' death by inducing a hypersensitive response under biotic stress[29] and enhancing tolerance under metal-induced oxidative stress in the plants[30]. APX genes (heme peroxidase and copper oxidase family) are also involved in several stresses while acting against hydrogen peroxide in ascorbic acid and glutathionine cycles, when it is overexpressed, the sugarcane plants show significant disease and Cu tolerance[31,32]. CYR1 (cryptochrome family) is involved in cytokinin signal transduction and activates R gene promoters in developing MYMIV-resistance in susceptible *Vigna* sp.[33] and cytokinin modulate patterning also helps to escape from heavy metal stress[34]. Based on reports, it is crucial to assess alteration in activities of stress-related metabolizing antioxidant enzymes and their gene expression profiles to elucidate tolerance levels of in mash bean under charcoal rot disease and Cu stress.

The current study aimed to investigate the *in vivo* polygonal interaction of plant-pathogen-heavy metal along with the role of FYM under Cu stress on charcoal rot disease, physiological and biological attributes of mash bean plants. This is a new area of research and so far few investigations have been carried out in this field. The findings of the present study will be helpful in the management of charcoal rot disease of mash bean by natural, easily available, cheap sources especially under abiotic stress of Cu.

Results

The effect of MP (positive control: T_2), Cu (T_3 and T_4), and Cu + MP (T_5 and T_6) on the disease, metal toxicity, growth, and yield of mash bean plants were compared with each other as well as with negative control (T_1). Likewise, the effect of soil amendment in T_7 (2% FYM), T_8 (2% FYM + MP), T_9 (2% FYM + 50 mg/kg Cu), T_{10} (2% FYM + 100 mg/kg Cu), T_{11} (2% FYM + 50 mg/kg Cu + MP), and (2% FYM + 100 mg/kg Cu + MP) was assessed with respect to corresponding control (T_1-T_6).

The mash bean plants in the negative control treatments (T_1) were healthy and asymptomatic. The infected plants in T_2 exhibited extensive necrotic lesions on roots, stems, and spikes. The most striking symptom was the sudden wilting and drying of the whole plant, and the infected plants were covered with black bodies that were giving the charcoal and ashy appearance of dead plants (plant mortality 86%). Typical Cu toxicity symptoms (i.e. chlorosis, reduction in leaf area, plant height with less branching, reduced root area and branching, reduction in pod size, and number with more immature pods) were observed in T_3 and T_4, however, no mortality was recorded in the plants. Under combined stress of Cu + MP (T_5 and T_6), the mash bean plants showed fewer symptoms of disease or Cu toxicity, but mortality (31 and 58%, respectively) was recorded (Fig. 1).

Cross-section of mash bean root showed in comparison to untreated control roots where all the epidermal cells were intact cortex was homogeneous, stele included the central core of vascular tissue and the root hairs were

turgid, infection of *M. phaseolina* and Cu either alone or in combination rendered abnormalities. Roots infected with *M. phaseolina* (T_2) only clearly presented thick brown bulky hyphae inside cortical cells, these structures were also found in the deterioration of the pith of the root, xylem, and phloem. Exposure to increasing concentrations of Cu in T_3 and T_4 resulted in a reduction in absence of root hairs. Damage to the epidermal cells and the cortex was manifested by losing cell shape with signs of shrivelling and disintegration. Further, as compared to the control roots where the stele was in a tetrarch condition, there was a lack of complete differentiation and pith formation in response to the Cu. Under the combined stress of Cu + MP, anatomical features were less severely disintegrated as compared to the individual effect of metal or pathogen (Fig. 1).

The inoculated plants in T_2 exhibited a significantly ($p \leq 0.05$) greater disease severity index (DSI: 100%) and plant mortality (PM: 86%) as compared to healthy plants in T_1. Soil amendment with 2% FYM significantly ($p \leq 0.05$) managed disease by reducing DSI and PM to 25% in T_8 (2% FYM + MP). Cu toxicity in T_3 and T_4 induced chlorosis in 33% and 60% of the foliage, respectively, while 2% FYM alleviated the Cu toxicity stress by reducing symptoms to 3% (T_9) and 17% (T_{10}), respectively. Under combined stress of Cu (50 mg/kg) + MP in T_5, the DSI and PM of 61 and 41%, respectively, and 2% FYM alleviated both stresses completely so the plants were healthy and asymptomatic in T_{11}. The DSI (49%), PM (38%) and Cu toxicity symptoms (38%) in T_6 (100 mg/kg Cu), significantly reduced to 13-15% in T_{12} (2%FYM + Cu + MP) (Table 3).

Generally, the growth and yield attributes of mash bean plants were more severely affected due to the separate effects ofMP or Cu as compared to their combined stress (Fig. 2). The separate effect of pathogen or metal was statistically ($p \leq 0.05$) at par with each other, therefore, all six growth attributes (length, fresh and dry weight of shoot and root) significantly ($p \leq 0.05$) declined by 40-70%, 20-50%, and 40-60% in T_2 (MP), T_3 (50 mg/kg Cu), T_4 (100 mg/kg Cu), respectively as compared to T_1. When the excess Cu(50 mg/kg) and MP were given together in T_5, the shoot attributes were insignificantly affected but root attributes decreased significantly by 30% over T_1. Moreover, the plants in T_5 had 2-fold ($p \leq 0.05$) greater growth attributes over T_2, while insignificantly differing with respect to the corresponding metal treatment (T_3). When a higher Cu dose (100 mg/kg) was combined with the pathogen in T_6, the plant growth attributes were 20-40% less than T_1, while 30-40% (shoot) and 70-100% (root) more than T_2, and 30-60% greater than corresponding metal treatment (T_4).

Nonetheless, soil amended with 2% FYM significantly enhanced all attributes of growth either in the presence or absence of stress/s. Therefore, the length, fresh and dry biomass of the shoot, as well as roots were improved significantly ($p \leq 0.05$) by 30-50% with 2% FYM in T_7 (without pathogen or metal) as compared to T_1 (without 2% FYM/pathogen/metal). Likewise, in T_8 (2% FYM + MP), the said attributes of mash bean plants were considerably enhanced up to 2-3 folds as compared to T_2 (MP). In T_9 (50 mg/kg Cu + 2% FYM), T_{10} (100 mg/kg Cu + 2% FYM), T_{11} (50 mg/kg Cu + 2% FYM + MP), T_{12} (100 mg/kg Cu + 2% FYM + MP), the stress of inglorious couple (Cu + MP) was alleviated, and all growth attributes improved to the extent, that the difference between their mean values was statistically same to T_1 (Fig. 2).

T_I calculated by taking growth attributes acts as an indicator to determine the capability of a plant to grow in a stressful environment. Results revealed that mash bean plants had the minimum T_I (T_2: 0.44) when flourishing under the biotic stress of *M. phaseolina* (MP) followed by 0.53 (T_4) and 0.64 (T_3) with abiotic stress of 100 mg/kg and 50 mg/kg Cu, respectively as compared to the negative control (T_1: 1). So far, the T_I of mash bean

plants under combination stress of Cu + MP in T_5 (0.78) and T_6 (0.67) was less than the negative control (T_1), but greater as compared to their corresponding metal treatments. However, soil manuring with 2% FYM elevated T_I in all treatments with the highest T_I of 1.37 in T_7 (2% FYM but without MP or Cu), followed by 1.13, 1.03, 1.01, 0.99, and 0.92 in T_8, T_9, T_{10}, T_{11}, and T_8, T_{12}, respectively (Fig. 3).

The number of pods and pod weight were significantly decreased by 50%, 45%, 60%, 30%, and 50% in T_2 (MP), T_3 (50 mg/kg Cu), T_4 (100 mg/kg Cu), T_5 (50 mg/kg Cu + MP), and T_6 (100 mg/kg Cu + MP), respectively as compared to T_1. Like growth assays, the yield parameters were insignificantly different in T_5 and T_6 with respect to T_2, while these attributes were 20% more with respect to corresponding metal treatments in T_3 and T_4. Soil application with 2% FYM significantly ($p \leq 0.05$) enhanced the number of pods and pod weight up to 2-fold in all treatments (T_7-T_{12}) in comparison to their corresponding control. Moreover, a rise of 100, 30, 60, 40, 75 and 50% were noticed in yield traits in T_7 (2% FYM), T_8 (2% FYM + MP), T_9 (2% FYM + 50 mg/kg Cu), T_{10} (2% FYM + 100 mg/kg Cu), T_{11} (2% FYM + 50 mg/kg Cu + MP), and T_{12} (2% FYM + 100 mg/kg Cu + MP), respectively as compared to T_1 (Fig. 4).

Separate and combined effects of MP inoculation and Cu-spiking caused a significant reduction of 50, 40-50, and 30-40% in the total chlorophyll content (TCC), carotenoids (CR), reducing sugar (RS) and total phenolics (TPC) at 45th day of sowing over negative control. Soil amendment with 2% FYM significantly ($p \leq 0.05$) and variably improved the said attributes by 30-40%, 80-130%, 50-70%, 60-90%, 30-70%, and 50-60% in T_7 (2% FYM), T_8 (2% FYM + MP), T_9 (2% FYM + 50 mg/kg Cu), T_{10} (2% FYM + 100 mg/kg Cu), T_{11} (2% FYM + 50 mg/kg Cu + MP), and T_{12} (2% FYM + 100 mg/kg Cu + MP), respectively as compared to their corresponding control treatments. Furthermore, the effect of 2% FYM in T_7-T_{12} was statically equal or greater than T_1 (Fig. 5).

Biotic stress of MP and levels of Cu (50 and 100 mg/kg) either alone or in combination significantly increased the biochemical parameters like total protein content and antioxidant enzymes (POX and PPO) 2-3 folds as compared to the negative control after 45 days of sowing, while these attributes decreased significantly after 90 days of sowing. However, the application of 2% FYM significantly enhanced these attributes variably up to 2-folds after 45 days, and comparatively less profoundly after 90 days of sowing under stress conditions as compared to their corresponding control as well as over negative control treatments (Fig. 6). CAT activity decreased significantly by 30-60% after 45 days in the treatments provided with the Cu or MP, while the enzyme activity decreased more drastically by 40-80% after 90 days of sowing. So far, in Cu + MP treatments, the activity of CAT improved significantly. Moreover, the 2% FYM either significantly improved the CAT activity variably (20-60%) in different treatments as compared to the respective control and negative control (Fig. 6).

The findings of real-time PCR indicated, that the expression levels of CAT and CYR1 genes were significantly decreased in the positive control of mash bean plants by 40% and 70%, respectively, while that of ascorbate peroxidase substantially improved to many folds as compared to the negative control. In treatments provided with 50 and 100 mg/kg Cu, the expression level of CYR1 changed insignificantly, however, the expression levels of CAT improved significantly by 22 and 89%, respectively and that of ascorbate peroxidase improved by 95% and 250%, respectively relative to the negative control. The simultaneous effect of Cu + MP changed the expression level of CAT by insignificantly decreasing it, and for CRYI by significantly decreasing, and for ascorbate peroxidase by significantly improving it over negative control. The maximum expression level of the CYR1 gene was recorded in negative control after soil amendment with 2% FYM. In comparison, it was

significantly decreased in the rest of the treatments supplemented with 2% FYM. Likewise, 2% FYM also reduced expression level of CAT and acrobate peroxidase in all treatments as compared to negative control (Fig. 7).

Relative to the negative control, treatment inoculated with the MP only (positive control) and provided with abiotic stress of Cu (50 and 100 mg/kg) induced considerable changes in the electrophoretic profiles of mash bean leaves the in range of ~ 10 kDa to 65 kDa. Protein bands at ~ 65 kDa were observed in all treatments. However, the maximum intensification was observed due to the effect of MP (T_2), 50 mg kg^{-1} Cu(T_3) and 100 mg kg Cu(T_4). Likewise, it was up-regulated due to amendment with 2% FYM in Cu-spiked + MP-inoculated soil (T_5 andT_6). Likewise, band intensification at ~ 55 kDa was more pronounced under the separate effect of MP or Cu than in their combined stress. After amendment with 2%, FYM expression was up-regulated 4-5 times in T_{10} andT_{11} while decreased in remaining treatments. The expression of ~ 44 kDa protein was up-regulated in treatments provided with the MP or metal stress (T_2 – T_7). After the amendment, band expression was intensified 3-4 times in T_9 and T_{10} and remained the same in others as in the negative control. Bands at ~ 25-35 kDa were up-regulated after biotic or abiotic stress while down-regulated after adding 2% FYM in these treatments compared to the negative control. New bands at ~ 13 kDa were observed due to biotic stress of *M. phaseolina* and abiotic stress of Cueither present alone or in combination (Fig. 8).

Soil fortification with Curesulted in an increase in Cu accumulation by different plant parts in order of root > stem > leaves > grains in a concentration-dependent manner (Fig. 9). Therefore, when Cu (50 and 100 mg/kg) alone was added to soil, roots accumulated 53-65% of Cufollowed by stem (30-33%), leaves (16-18%) and grains (1-2%). In Cu + MP, the plant parts accumulated less Cu, and the accumulation rate ranged between 28-30%, 16-17%, 8-10%, and 0.8-1% by root, stem, leaves and grain, respectively. After mixing of 2% FYM in soil, the plant accumulated half time less Cu (Fig. 9). TF and BAF values (0.88-1.18 and 3.56-5.32, respectively) revealed an increase in accumulation and subsequent translocation of Cu to above-ground parts from the root under Cu stress, while Cu + MP treatments caused a reduction in TF and BAF values (0.79-1.11 and 1.48-2.36, respectively) indicating limited accumulation and later translocation to shoot and grains from the underground part. Over and above, FYM caused more translocation of the metal in the soil as indicated by low values of TF and BAF as compared to their respective control treatments (Table 4).

There was negative relationship ($r = -0.69$, $p > 0.05$) between Cu accumulation and 50 mg/kg of soil Cu in the soil, while the positive relationship ($r = 0.99$, $p < 0.01$) was observed due to effect of 100 mg/Kg of soil Cu. Rest of the treatments showed significant ($p < 0.01$) negative correlation ($r = -0.96$, $p < 0.01$) between the Cu concentrations and Cu accumulation by the mash bean plants. Likewise, a negative significant correlation ($r = \sim -0.99$, $p < 0.01$) was found between Cu accumulation and FYM effect on soil metal levels. Correlation for Cu accumulation with the yield (pod weight) revealed that this parameter was strongly and highly significantly correlated ($r = -0.99$, $p < 0.01$) with yield. The inversely proportional relation showed that yield was significantly decreased with increased metal accumulation in mash bean plants.

A PCA was performed to identify the association of variables with each other and their effect on the treatments. PC1 and PC2 accounted explained 79.24% of the variability in the data among all traits tested in this study (Fig. 10). PC1 mainly correlated all growth attributes, and PC2 described the correlation of metal accumulation, translocation factor, and bioconcentration factor, but the vectors of PC1 and PC2 pointed in the opposite directions, demonstrating a negative correlation. Four groups are made on the basis of the response to treatments. Group I, consisted of control (T_1) along with treatments that received 2% FYM (T_8-T_{12}) with

significantly greater growth attributes. The moderately tolerant treatments (T_3, T_5, & T_6) are placed in group II in the middle of the biplot as compared to the control (group I). Highly sensitive treatments towards MP (Group III: T_2) and Cu (Group IV: T_4) are present on the extreme left side of the biplot as sensitivity increases as treatments are placed away from the origin (Fig. 10).

Discussion

The contemporary study was conducted to ascertain the impact of charcoal rot on mash beans under abiotic stress of excess Cu providing FYM as soil amendments against stress/s. Generally, morpho-growth, as well as, physio-molecular traits of mash bean plants were more prone to individual stress of *M. phaseolina* (MP) and excess Cu, than under their simultaneous action.

Infection caused by MP has declined the growth, and yield of mash bean plants significantly by 30–70%, inciting 100% disease incidence and 86% plant mortality. The infected plants exhibited necrotic lesions, wilting, and drying of the whole plant. Anatomical features of mash bean root also revealed the occurrence of fungal hyphae in epidermal cells passing through intercellular spaces of the cortex, causing the disintegration of vascular bundles possibly through the production of toxic compounds like phaseolinone by embedded sclerotia[11]. The current findings are in harmony with many previous reports, where MP has deteriorated plant health by inciting charcoal rot disease in them[3,4,8] due to the successful establishment of the pathogen within host tissue along with the production of different toxins and cell wall degrading enzymes for disruption of the vascular system[11]. The host susceptibility to MP may also be ascribed to suppression of the auxin response and alteration in the jasmonic acid and ethylene pathways by the pathogen[4]. MP-induced reduction in the chlorophyll concentration is likely to be associated with a decrease in the stomatal conductance and photosynthesis, while the increase in respiration rate and other metabolic pathways involved in defense mechanisms along with the enhanced movement of metabolites to the fungal cells[4]. Likewise, the reduction in TPP, TPC, and RS has been associated with low levels of resistance in the host plants[11]. So far, changes in the activity of CAT, POX, and PPO might also be linked with the over-accumulation of ROS due to disturbance in electron flow at the membrane-bound organelles, however, the enhancement in the enzyme activity might not be enough to encounter the pathogen-induced stress resulting increase in the plant susceptibility to mortality[5–8].

Cu-excess (50 and 100 mg/kg) condition strongly impaired mash bean morpho-physiological responses by reducing these attributes (30–60%) and induced foliar chlorosis, reduced leaf area, shoot branching, size, and the number of pods, however, no mortality was recorded in the plants. A reduction in the number and length of root hairs, and damage to the root cuticle, followed by damage to the meristem, thickening, and cracking of the root cuticle were also observed[35]. Similar findings have been reported earlier due to Cu phytotoxicity, where excess Cu is likely to imbalance uptake of essential elements (N, P, K, Ca, Mg, Fe, and Zn), affecting the cell membrane of the root cuticle, altering the auxin homeostasis, cell division, cell expansion and elongation, ultimately thickening and hardening of the cell wall decreases cell elasticity, decreases growth and biomass production in the plants [36,37]. The simultaneous use of Cu + MP led to fewer symptoms of disease or Cu toxicity, along with only a 20–50% reduction in the growth and yield attributes but plant mortality ,was recorded. High Cu interacting with MP antagonistically may reduce fungal burden as copper sulfate has been used through the ages as a potent antimicrobial agent [19]. The other possibility might be associated with the mobilization of Cu during a fungal infection at the pathogen-host interaction axis, as MP may acquire Cu homeostasis machinery that brings into

play to succeed in establishing an infection in the host as indicated by 50% reduction in the yield attributes with plant mortality[38]. Exposure to Cu in soil fosters the production of ROS, which possibly by reducing the efficiency of the photosynthetic process interfere with the chlorophyll organization and functionality, causing the reduction of electron transport and PSII activities hence decreasing chlorophyll a, b, carotenoids, photosynthetic gas exchange, photosystem II and PSII quantum yield [36]. Below a certain value of Cu, the synthesis of low molecular weight stress proteins reinforced the action of the antioxidant enzymes. Adrees et al.[39] reported that the increase of $CuSO_4$ in the growth medium caused a dose-dependent increase in ROS generation due to enhancing electrolyte leakage which boosted activities of SOD, CAT, and POX. Liu et al. [31] in another study indicated that a toxic level of Cu for 24 h triggers the MAPK (mitogen-activated protein kinases) pathway in *Zea mays* causing an enhancement in the activities of SOD, CAT, and APX. In another study, Younis et al.[40] exhibited enhancement in SOD and CAT activities under low doses of Cu, but their activities declined under high levels of Cu in *Phaseolus vulgari*. Liu et al.[32] also documented increase in SOD and POX activities at 5 mg/L concentration of Cu, while declining in the CAT activity in *Salvinia natans*.

The tolerance indices of the mash bean plants in terms of growth and yield parameters were 0.4, 0.5–0.6, and 0.7–0.8 with MP, Cu, and Cu + MP, respectively, which may reveal that the plants experienced stress with T_I values < 1. However, under combined stress mash bean plants probably developed tolerance due to hormesis effect[41], which could lead to adaptive responses of organisms to moderate environmental challenges, improving their functionality and/or tolerating stronger challenges in the future resulting in improvement in the activity of enzymes[20]. Hence, it is plausible to suppose that, high Cu not only affects plants but also pathogens under stress combinations involving Cu and MP, which was further evident by the reduction in the translocation factors and bioaccumulation factors under combined stress. Moreover, greater accumulation of Cu in the root followed by the stem, leaves, and grains could be ascribed to efficient metal efflux through the plasma membrane, chelation of Cu with organic molecules, stimulation of phytochelatins, metallothioneins and heat shock proteins in roots may restrict upward movement from roots to aerial parts[42].

Soil mixing with 2% FYM substantially improved growth and yield in mung beam plants under stress and unstressed conditions, which resulted in high T_1 values (< 1) in the different treatments[4]. The nutritional profile of sandy loam soil (sand: 42%, silt: 32% and clay: 25%) indicated it contained a sufficient amount of organic matter (6.14%) and other nutrients (N: 0.29%, P: 0.03%, and K: 0.21%, respectively), which were deficient in bare soil (organic matter: 0.4%; N: 0.01%, P: 0.001% and K: 0.01%, respectively). The nutrients in FYM possibly furnished abounding organic matter for the growth and development of beneficial microorganisms, hence improving root architecture, supporting dissolution and nutrient availability to crop plants[18]. Therefore, the FYM possibly by increasing the resources for the self-protection of plants which may lead to restoration in chloroplast-to-nucleus communication, therefore accounting for improvement in crop yield. Therefore, the mash bean plants, exposed to single or simultaneous stress in the presence of 2% FYM exhibited an increase in TCC, CR, RS, TP, TPC, and RS as well as the activities of CAT, POX, and PPO presenting metabolic cost for limiting the adverse effects of MP, Cu, and both[4]. Over and above, the plant accumulated half-time less Cu in the presence of 2% FYM due to the strong sorption of Cu to the soil organic matter.

Changes in the expression of redox homeostasis enzymes viz., catalase (CAT), ascorbate peroxidase (APX), and cytokinin-resistant genes (CYR1) are responsible for morpho-physio-biochemical responses to Cu, MP, and an inglorious couple of Cu and MP in the mash bean plants. The enhancement in the genes of expression of CAT

and APX under separate and simultaneous stress of MP and Cu may indicate the antagonistic action of CAT and APX against the overproduction of H_2O_2[43]. The reduction in the expression of CAT and APX after soil amendment with 2% FYM may reveal CAT and APX as important and highly regulated for induction of compensatory mechanisms in mash beans against stress/s environment[44]. Mash bean plants respond positively to 2% FYM under stress conditions by showing the highest expression of the CYR1 gene as compared to the rest of the treatments. One function of CYRI is to regulate genes involved in root growth indicating an important function of genes in regulating root growth[45]. It seems that the mash bean plant responds to this gene in 2% FYM amended soil probably due to the higher uptake of nutrients from the soil with the highest increase in root length as compared to the rest of the treatments as evident in the present study. The reduction in CYRI expression in the rest of the treatments could be due to the reprogramming of the molecular machinery after stress.

An increase in total protein content may specify mash bean plant could withstand a stressed environment[24]. Protein profiling acquired through SDS-PAGE reflected several changes in the 45-day mash bean leaf relative to the negative control. Many bands at approximately 13 kDa, 25 kDa, 35 kDa, 44 kDa, 65 kDa, 55 kDa and 44 kDa were observed with greater intensity in the treatments containing MP or Cu. Kieffer et al.[46] documented a marked increase in the abundance of PR proteins class I chitinases (27–28 KDa; PR-3 family), several β-1,3-glucanases (PR-2 family), and thaumatin-like protein (PR-5 family) in cadmium exposed poplar leaves. Likewise, an increase in the expression of PR proteins (10–40 kDa) against heavy metal stress was correlated with adaptation to stressful environments[47]. Furthermore, greater intensity of protein bands at 37–50 kDa (PR-2 family) in plants is responsible for inhibiting fungal growth through the disintegration of cell wall chitin and glucagon[24]. By contrast, the expression level of the said bands decreased in Cu + MP might be due to the cross-tolerance mechanisms, and normalized in 2% FYM + Cu + MP possibly due to the enhancement of a plant's resistance against stresses.

PCA explained 79% of the data variability[8]. Factor-loading matrix extracted from biplot analysis of all PCA derived from growth, yield, physio-biochemical, molecular (gene expression), T_1, TF, and BCF responses indicated, a positive correlation of the studied growth and yield attributes of mash bean plants with 2% FYM soil amendments. Moreover, the translocation factor and bioconcentration factor are positively correlated with the treatments in the highly sensitive to moderately tolerant groups. Besides, all treatments in group I are located near the control group, which presented the significance of 2% FYM as a soil amendment in alleviating MP and Cu stress. Therefore, mixing 2% FYM could be utilized to alleviate the charcoal rot disease in mash bean plants growing under the toxic concentration of Cu (50 and 100 mg/kg).

Materials And Methods

Experiment

The experiment was conducted during the period of May–July (average temperature: 40 ± 5°C and average relative humidity 50 ± 5%) in pots kept in a tunnel in the Experimental Station of the Faculty of Agricultural Sciences, University of the Punjab Lahore, Pakistan. For the greenhouse assay, the soil was then sterilized by fumigation[8]. The Cu solutions (50 and 100 mg/kg) for spiking were prepared with the soil's saturation/water holding capacity from $CuSO_4$. $5H_2O$, and the soil was spiked by spraying an aqueous solution of Cu with the continuous turning of the soil and left for 15 days for drying[24]. Decomposed 2% FYM was mixed in the measured amount of sterilized, sieved metal spiked soil, filled in pots (7 × 6 h × w, 5 kg/pot), and left for another 4 days.

Later soil was artificially infected by pouring 100 ml of cultural suspensions (2.0 x 10^5 sclerotia/ml) of *M. phaseolina* ((FCBP-0751) in each pot's upper 2–6 inch layer. The inoculated soil in each pot was left for 3 days under natural environmental conditions to establish the pathogen. Certified surface-sterilized seeds of mash bean var. Maikhaldia 6066 (provided by Ayub Agriculture Research Institute, Pakistan) were surface-sterilized with 1% Clorox for 5–10 minutes prior to washing with sterilized distilled water 3 times[4]. After drying, seeds were sown (10 seeds per pot), and 7 were maintained after a successful stand. The pots were placed in a completely randomized design with six replications (Table 1). All plants were kept inside a transparent plastic chamber to facilitate the infection process, and the experiment was intended for 90 days.

Disease And Cu Toxicity Measurements

After 90 days of sowing, the mash bean plants were analysed for Cu toxicity symptoms (percentage chlorosis or yellowing in foliage), disease severity index, plant mortality, and tolerance index (T_{index}).

$$\mathrm{Diseaseindex} = [\mathrm{R}(\,\mathrm{rating} \times \mathrm{numberofplantsrated})/\mathrm{Totalnumberofplants} \times \mathrm{highestrating}] \times 100$$

$$\mathrm{Mortality}\,(\backslash\%) = \frac{\mathrm{No.\,ofplantsdied}}{\mathrm{Totalno.\,ofplantassessed}} \times 100$$

$$\mathrm{G_i} = \left(\frac{\mathrm{G_x}}{\mathrm{G_{max}}}\right) \mathrm{T_{index}} = \sum \left(\frac{\mathrm{G_x}}{\mathrm{G_{max}}}\right) \mathrm{n^{-1}}$$

Where, Gi = normalized growth parameter; Gx = individual growth parameter; Gmax = maximum value; Tindex = Tolerance index

Anatomical Assays

To examine anatomical changes in roots, the sections of the treated as well as control samples the roots were cut and prepared by several washing with 0.3, 0.5, 0.9, and 1% of alcohol and stained with 0.25% (w/v, dissolved in 50% ethanol) safranin for tissue differentiation. These sections were mounted in 20% glycerin to prepare temporary mounts and observed under a compound microscope and photographed with a digital imaging system[48].

Physio-biochemical Assays

Physiological attributes like total chlorophyll content (TCC), carotenoids (CR), reducing sugar (RS), and total phenolics (TPC) were assessed in the leaf samples of 45 days old plants, while the total protein content (TPP) and activities of enzymes were assays in the leaf of 45 and 90-days-old mash bean plants.

Total Chlorophyll Content (Tcc), Carotenoids (Cr), Reducing Sugar (Rs), And Total Phenolic Content (Tp)

The concentration of TCC (Chl a, Chl b, and CR), was assayed using the Lichtenthaler method[49] against blank of acetone at 646, 663 and 470 nm for Chl a, Chl b and CR, respectively. RS was measured in the leaf sample homogenized in ethanol (80%), centrifuged at 800 rpm for 10 min followed by the addition of arsenomolybdate reagent and measurement of the samples at 620 nm[50] and using Folin–Ciocalteu method[51], the TPC of the samples was measured at 760 nm against a blank using gallic acid as a standard.

Total Protein Content (Tpp) And Antioxidant Enzyme Activities

TPP along with the catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO) activity were estimated after 45 and 90 days of seed sowing. Homogenized leaf samples in chilled sodium phosphate buffer (100 mM, pH 6.8) were analysed for TPP and enzyme activities. Leaf extract containing reagents [(Reagent A: sodium carbonate (Na_2CO_3) + NaOH (0.1 N) + sodium potassium tartarate) (Reagent B: copper sulfate) (Reagent C: mixing 50 mL reagent A with 1 mL of reagent B)] was finally mixed with the Folin-phenol reagent, incubated for 30 min, and quantified for TPC at 620 nm using as standard the bovine serum albumin[52]. The assay mixture for CAT activity (EC: 1.11.1.6) was measured 240 nm, which contained buffer B (0.05 M sodium phosphate buffer (pH 7.0) and 0.036% hydrogen peroxide) and the enzyme extract. For POX activity (EC 1.11.1.7), reaction mixture contained phosphate buffer (pH 7.0), enzyme extract, 5.33% pyrogallol, and 0.5% H_2O_2. The samples at 420 nm after incubation for 5 min at 25°C[53]. PPO activity (EC 1.14.18.1), was determined at 495 nm in the reaction mixture made with 0.1 M sodium phosphate buffer (pH 7.0), 10 mM catechol and enzyme extracr[54].

Gene Expression By Rt-pcr

For the gene expression study, the ground leaf powder was homogenized in extraction buffer, followed by shaking in a shaker incubator at 42°C for 1.5 hours. Later on, the samples were mixed with potassium chloride, centrifuged and the collected supernatant was mixed with lithium chloride. After overnight incubation, samples were centrifuged again to precipitate RNA pellets. Which was then washed with 2 M lithium chloride, mixed in 1/10 volume of 2 M potassium acetate and incubated on ice to precipitate other unwanted contaminants. DNA-free total RNA (4 µg) was used to synthesize cDNA. For that, a kit of SuperScript® III Reverse Transcriptase (Life Technologies, Inc) was used. For qRT-PCR, cDNA was further diluted, and 200 ng of cDNA was mixed with Maxima Sybr green qPCR master mix (Thermo Scientific, Inc.) + 250 nM of forward and reverse primers for each gene (Table 2). Amplification and detection of the product assays were carried out in iQ5 cycler (Bio-Rad, Inc.). Melting curve analysis was also performed to check the specificity of primers[55].

Protein Profiling By Sds-page

Protein samples were run on 10% SDS-PAGE gels [(separating gel: 1.5 M Tris pH 8.8, 10% SDS (w/v), 30% (v/v) acrylamide, 10% (w/v) $(NH_4)_2S_2O_8$, 0.05% (v/v) TEMED; stacking gel: 1 M Tris pH 6.8, 10% (w/v) SDS, 30% acrylamide, 10% $(NH_4)_2S_2O_8$, 0.01% (v/v) TEMED)]. About 2 µL of this protein was mixed with 8 µL 1X running buffer loading dye (60 mM Tris pH 6.8, 25% (v/v) glycerol, 5% (w/v) SDS, 1% (v/v) saturated bromophenol blue. After incubating for 30 minutes at room temperature, proteins were run in 1X SDS running buffer (250 mM Tris pH 8.3, 500 mM glycine, 1% [w/v) SDS] at 200 volts with a protein size marker, until the dye was 1–2 mm from the end of the gel. Gel was stained in a Coomassie Blue stain solution (0.1% (w/v) Coomassie brilliant blue, 45%

(v/v) methanol and 10% (v/v) acetic acid) for 20–30 minutes and then washed with PAGE-destain [10% (v/v) acetic acid, 45% (v/v) ethanol] for several times to visualize protein bands.

Growth, Yield And Cu Analysis

After 90 days of sowing, lengths of shoot and root were measured in cm, while weights were computed in grams. Yield assays like the number of pods/plant and the weight of pods were also taken at the time of harvest. The dry mass of shoots and roots was recorded after keeping them in a scientific oven at 60°C for 48 h.

The soil was analyzed for texture, organic matter, and macronutrients (nitrogen, potassium, and phosphorus) before and after mixing with 2% FYM according to methods explained by International Soil Reference and Information Centre[56]. For determination of total Cu concentration in the treatments provided with Cu, the dried soil, root, stem, leaf, and seeds samples of the plants were powdered, and digested separately using 2 mL 70% v/v nitric acid at 100 ºC for 2 h exposures and analysed through Atomic absorption spectroscopy (Thermo scientific ICE 3000 SERIES). The translocation factor was calculated by the following equation (Cshoot and Croot are metal concentrations in the shoot and root of the plant, respectively. TF > 1 represents that the translocation of metals effectively was made to the shoot from the root)[57]. The bioconcentration factor was also calculated (SMC: shoot metal concentration, SDW: shoot dry weight; RMC: root metal concentration, RDW: roots dry weight)[58]

$$\mathrm{BCF}\,(\backslash\%) = \mathrm{SMC} \times \mathrm{SDW} + \mathrm{RMC} \times \mathrm{RDW}\,(\mathrm{A}) = \mathrm{SMC}{*}\mathrm{RMC}/\mathrm{A}$$

Statistical Analyses

Data were subjected to the LSD test ($p \leq 0.05$), and Pearson correlation was used to analyze the correlation between metal accumulation in the plants and morpho yield-related attributes. Moreover, all the statistical analyses were done by using the computer software Statistics 8.1. Principal components analysis was performed to summarize the variability of the treatments and to determine the association among the measured traits

Conclusions

It was concluded that mash bean plants were more drastically affected when exposed to individual stress of *M. phaseolina* or Cu than their combined stress. Cu was found to exhibit a generally positive effect on the defense response of the mash beans against the fungal infection by a hormesis-like phenomenon. Therefore, morph-growth attributes were not affected as severely under the combined stress of Cu + MP as was observed due to separate effects of either stress. Besides, soil amendment with 2% FYM mitigated the individual and simultaneous stress factors by inducing resistance in mash bean plants and improving various biological attributes. This data suggests that the application of soil application with 2% FYM to crops might be a valid strategy to overcome charcoal rot disease in mash bean in Cu contaminated soils.

Declarations

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Author contribution

A. S. Designed and, supervised the experiments, prepared figures and tables, and drafted the manuscript; **S.A.** Performed experiments and collected data; **I.J.** PCA, Gene Expression, SDS-PAGE; **U. Q.** Gene Expression; **R. T**: SDS-PAGE. All authors have substantial contributions to the final manuscript and approved this submission.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures in this experiment were carried out in accordance with relevant guidelines of the university field of the University of the Punjab, Lahore, Pakistan.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Tables

Table 1: Treatments designed for the current experiment.

Experiment treatments	
T_1	-ve Control
T_2	+ve Control (*Macrophomina phaseolina*, MP)
T_3	Cu (50 mg/kg)
T_4	Cu (100 mg/kg)
T_5	Cu (50 mg/kg) + MP
T_6	Cu (100 mg/kg)+ MP
T_7	2% FYM
T_8	2% FYM + MP
T_9	2% FYM + Cu (50 mg/kg)
T_{10}	2% FYM + Cu (100 mg/kg)
T_{11}	2% FYM + Cu (50 mg/kg)+ MP
T_{12}	2% FYM + Cu (100 mg/kg)+ MP

Table 2: Sequences of primers used in qRT-PCR

No.	Primer name	Sequence (5' – 3')
1	ActinTF	ATTGAGCATGGATTGTGAG
2	ActinTR	GGCGACATACATAGCAGGAG
3	CatalaseTF	GCAAAGGGTTTCTTTGAGGT
4	CatlaseTR	GAAGACGGGAAGGTTGTTTC
5	CYR1 TF	AAAAGGTGCTTTGCTTATTGTG
6	CYR1 TR	TGCCAAGTCATTCAAAAGGT
7	Ascorbate PeroxidaseTF	TTCGGAACCATCAAGCACC
8	Ascorbate PeroxidaseTR	CTCAACTGCGACAACTCCAG

Table 3: Effect of soil amendment with Farmyard manure (FYM) on disease and metal toxicity in mash bean due to *Macrophomina phaseolina* (MP) under Cu toxicity.

Treatments	Disease severity index (%)	Plant mortality (%)	Yellow patches on leaves due to Cu toxicity (%)
T_1: -ve Control	0.0^{f}	0.0^{e}	0.0^{f}
T_2: + ve Control (*Macrophomina phaseolina*, MP)	100^{a}	87.0^{a}	-
T_3: Cu (50 mg/kg)	-	0.0^{e}	33.0^{c}
T_4: Cu (100 mg/kg)	-	0.0^{e}	60.0^{a}
T_5: Cu (50 mg/kg) + MP	61.0^{b}	41.0^{b}	12.0^{c}
T_6: Cu (100 mg/kg) + MP	49.0^{c}	38.0^{b}	38.0^{b}
T_7: 2% FYM	-	0.0^{e}	-
T_8: 2% FYM + MP	25.0^{d}	19.0^{c}	-
T_9: 2% FYM + Cu (50 mg/kg)	-	0.0^{e}	3.0^{f}
T_{10}: 2% FYM + Cu (100 mg/kg)	-	0.0^{e}	17.0^{e}
T_{11}: 2% FYM + Cu (50 mg/kg)+ MP	2.0^{f}	0.0^{e}	0.0^{f}
T_{12}: 2% FYM + Cu (100 mg/kg)+ MP	15.0^{e}	15.0^{d}	13.0^{c}

Different letters (superscript) in the column depict significant differences ($p \leq 0.05$) as determined by LSD Test.

Table 4: Translocation factor and bioconcentration factor of mash bean leaf due to the effect of soil amendment with 2% FYM under *Macrophomina phaseolina* and Cu stress.

Treatments	TF	BF
T_1: -ve Control	**0.70**	**0.15**
T_2: + ve Control (*Macrophomina phaseolina*, MP)	**0.70**	**0.18**
T_3: Cu (50 mg/kg)	**0.88**	**3.56**
T_4: Cu (100 mg/kg)	**1.18**	**5.32**
T_5: Cu (50 mg/kg) + MP	**0.79**	**1.48**
T_6: Cu (100 mg/kg) + MP	**1.11**	**2.36**
T_7: 2% FYM	**0.20**	**0.01**
T_8: 2% FYM + MP	**0.43**	**0.07**
T_9: 2% FYM + Cu (50 mg/kg)	**0.75**	**1.10**
T_{10}: 2% FYM + Cu (100 mg/kg)	**1.02**	**2.13**
T_{11}: 2% FYM + Cu (50 mg/kg)+ MP	**0.98**	**0.54**
T_{12}: 2% FYM + Cu (100 mg/kg)+ MP	**0.87**	**0.88**

Figures

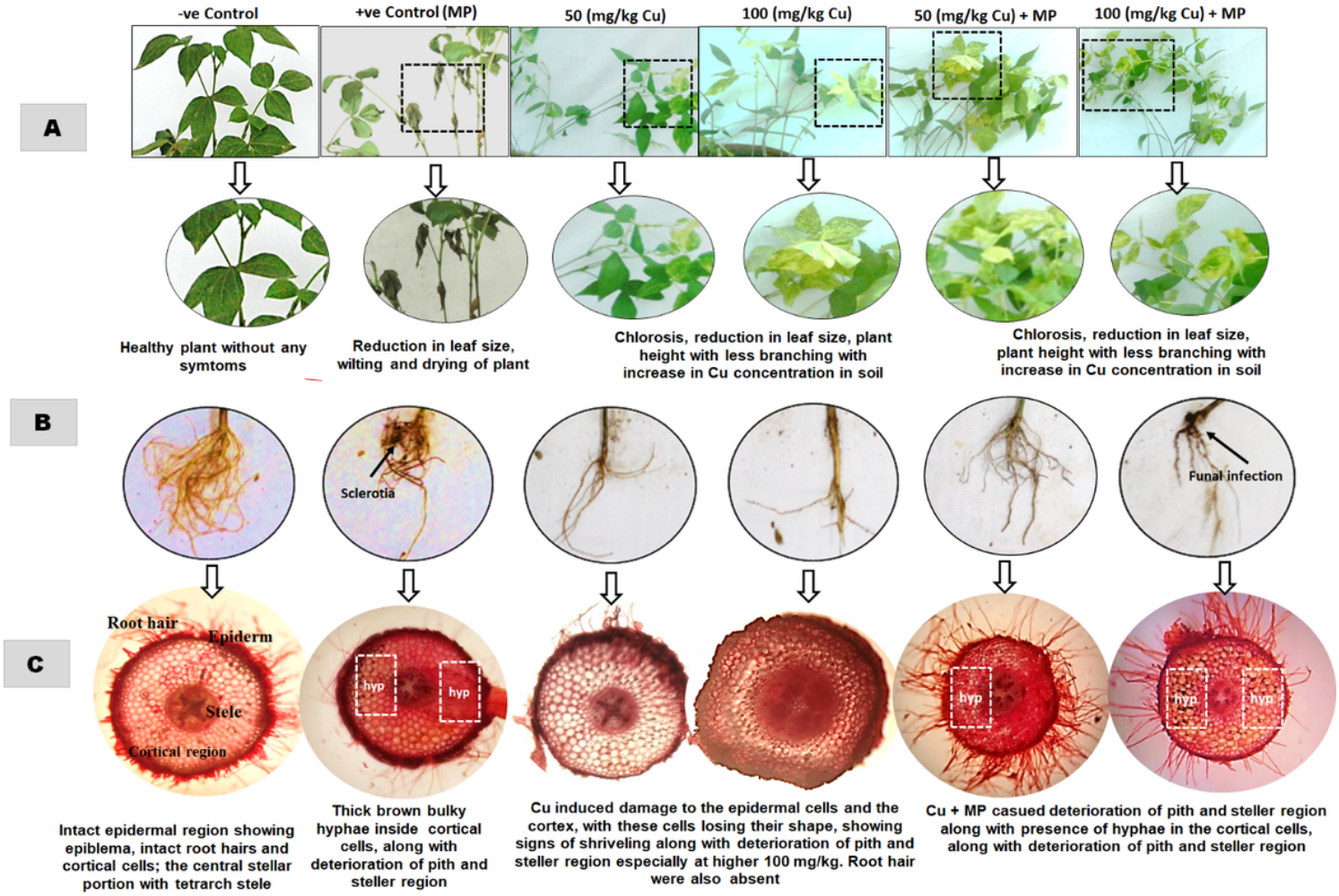

Figure 1

(A-C): Morphological and anatomical alterations in mash bean plant due to effect of *Macrophomina phaseolina* (MP) and Cu at 90th days of sowing. Symptoms of leaves (A); roots (B); and cross-section of the root (C).

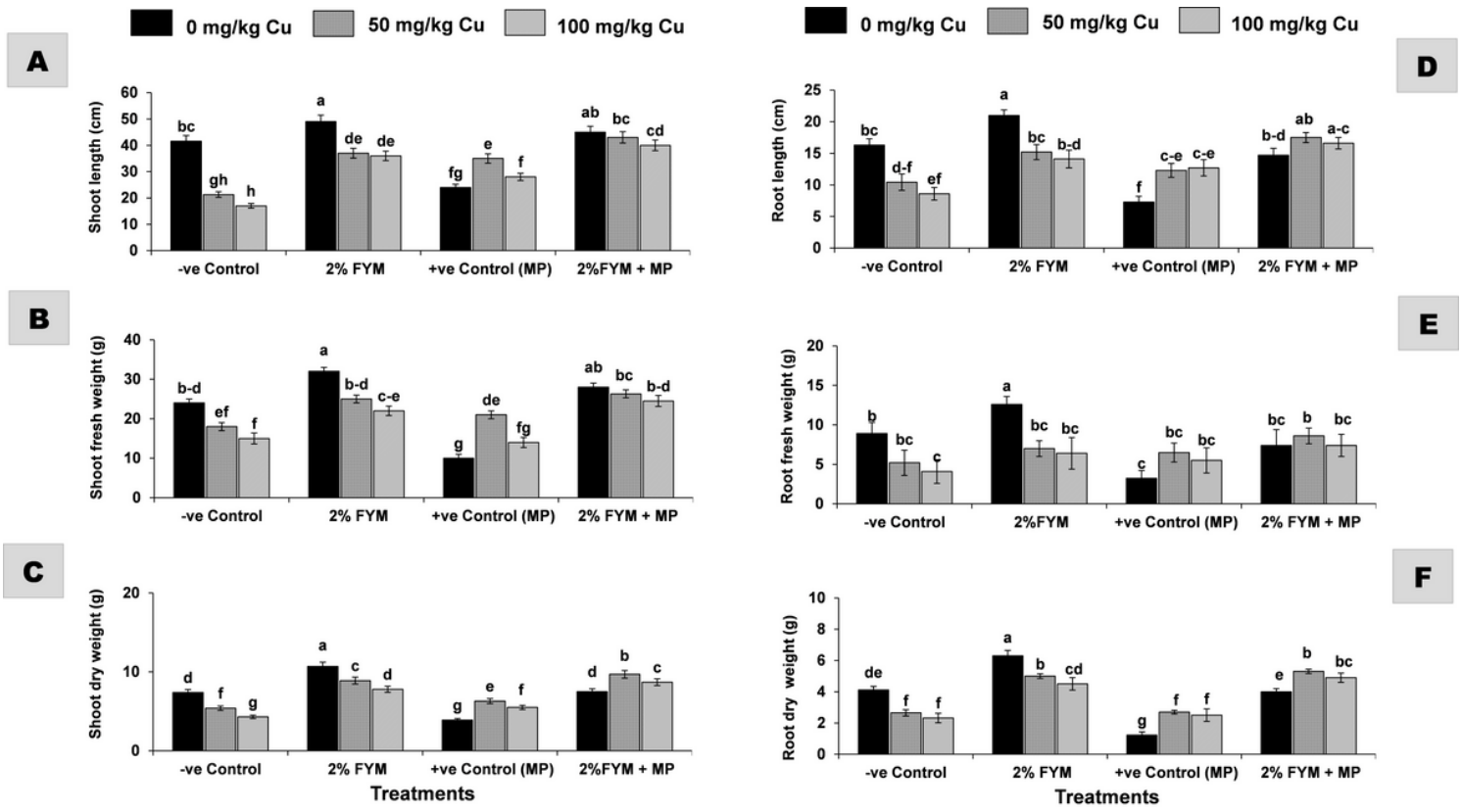

Figure 2

(A-F): Effect of 2% FYM on vegetative growth-related attributes of mash bean under separate and simultaneous stress of *Macrophomina phaseolina* (MP) and excess copper (Cu) at 90th days of sowing. Vertical bars show standard errors of means of six replicates. Values with different letters at their top show significant difference ($p \leq 0.05$) as determined by LSD-test.

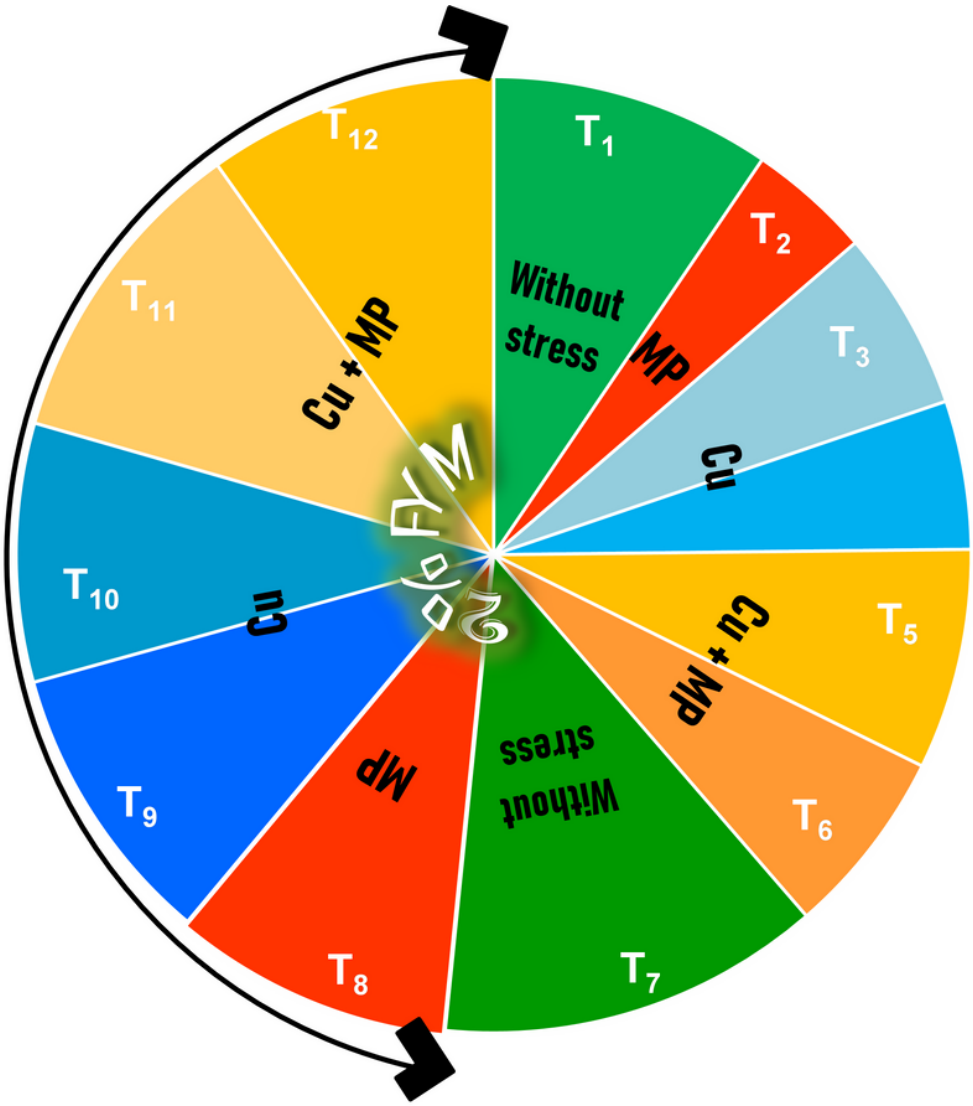

Figure 3

Cumulative growth tolerance indices in mash bean including vegetative growth attributes of mash bean plants exposed to separate and simultaneous stress of *Macrophomina phaseolina* (MP) and excess copper (Cu) at 90th days of sowing.

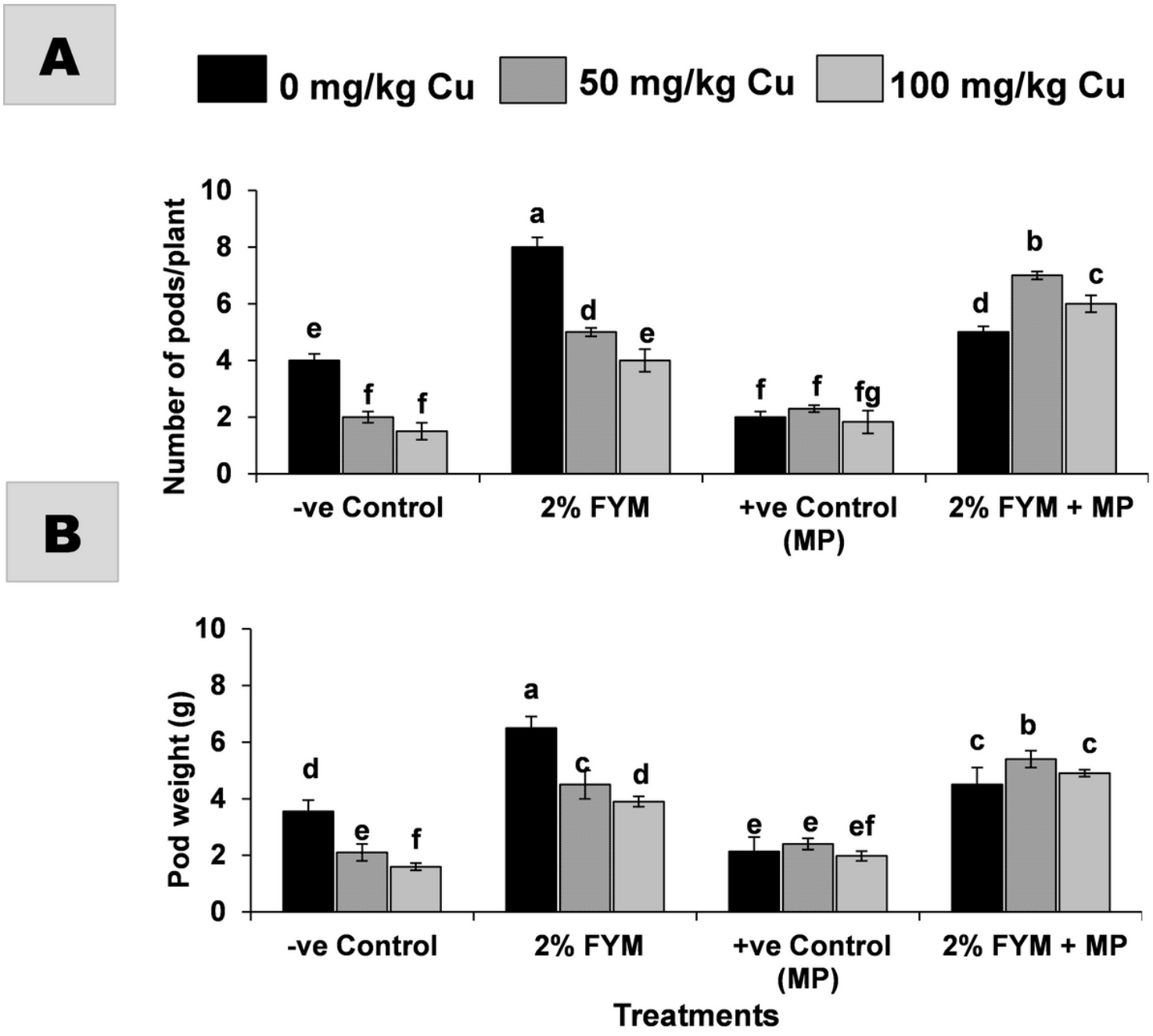

Figure 4

(A and B): Effect of 2% FYM on yield-related attributes of mash bean under separate and simultaneous stress of *Macrophomina phaseolina* (MP) and excess copper (Cu) at 90th days of sowing. Vertical bars show standard errors of means of six replicates. Values with different letters at their top show a significant difference ($p \leq 0.05$) as determined by LSD-test.

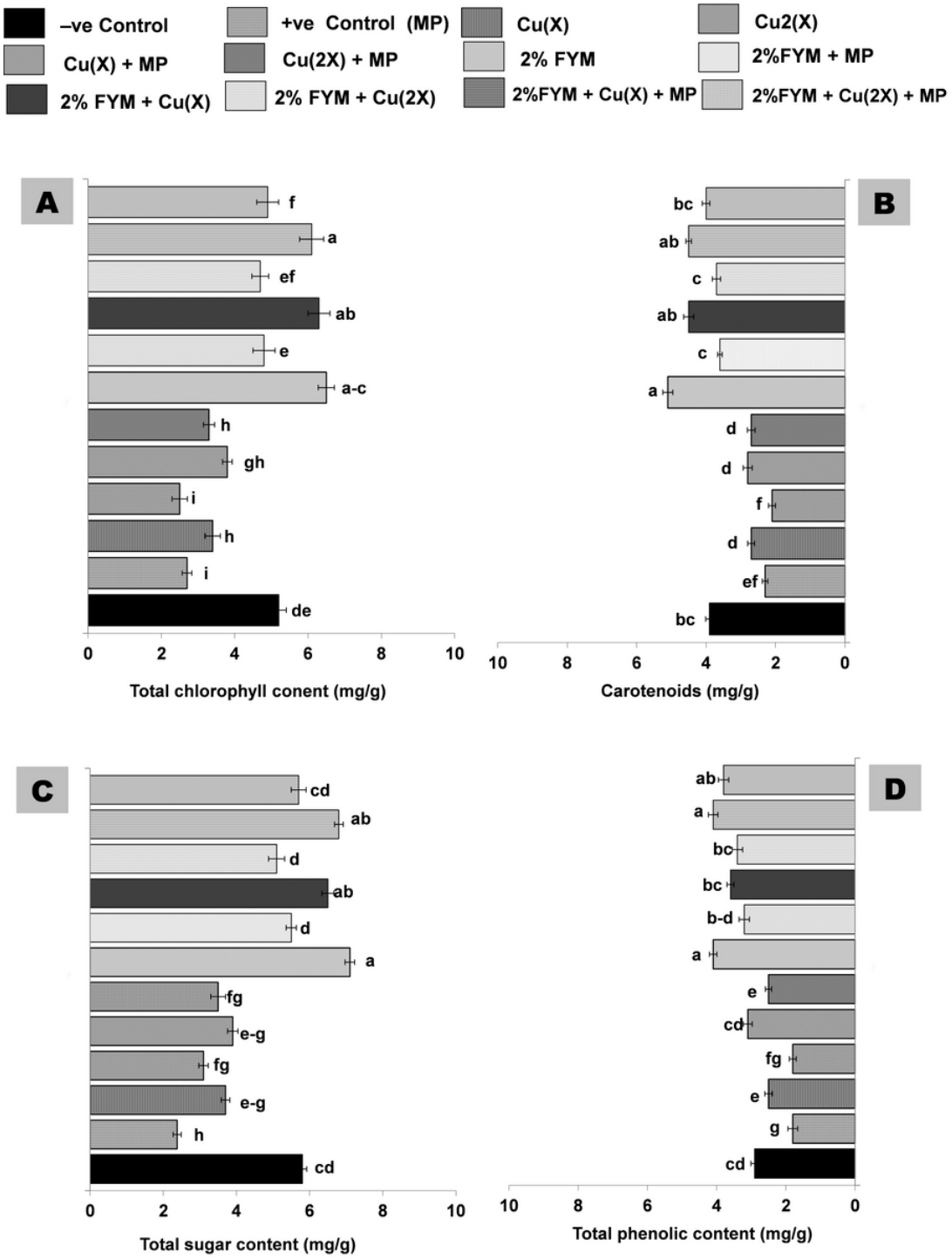

Figure 5

(A-D): Effect of 2% FYM on physiological attributes of mash bean leaf under separate and simultaneous stress of *Macrophomina phaseolina* (MP) and excess copper (Cu) at 45th days of sowing. Vertical bars show standard errors of means of six replicates. Values with different letters at their top show a significant difference ($p \leq 0.05$) as determined by LSD-test.

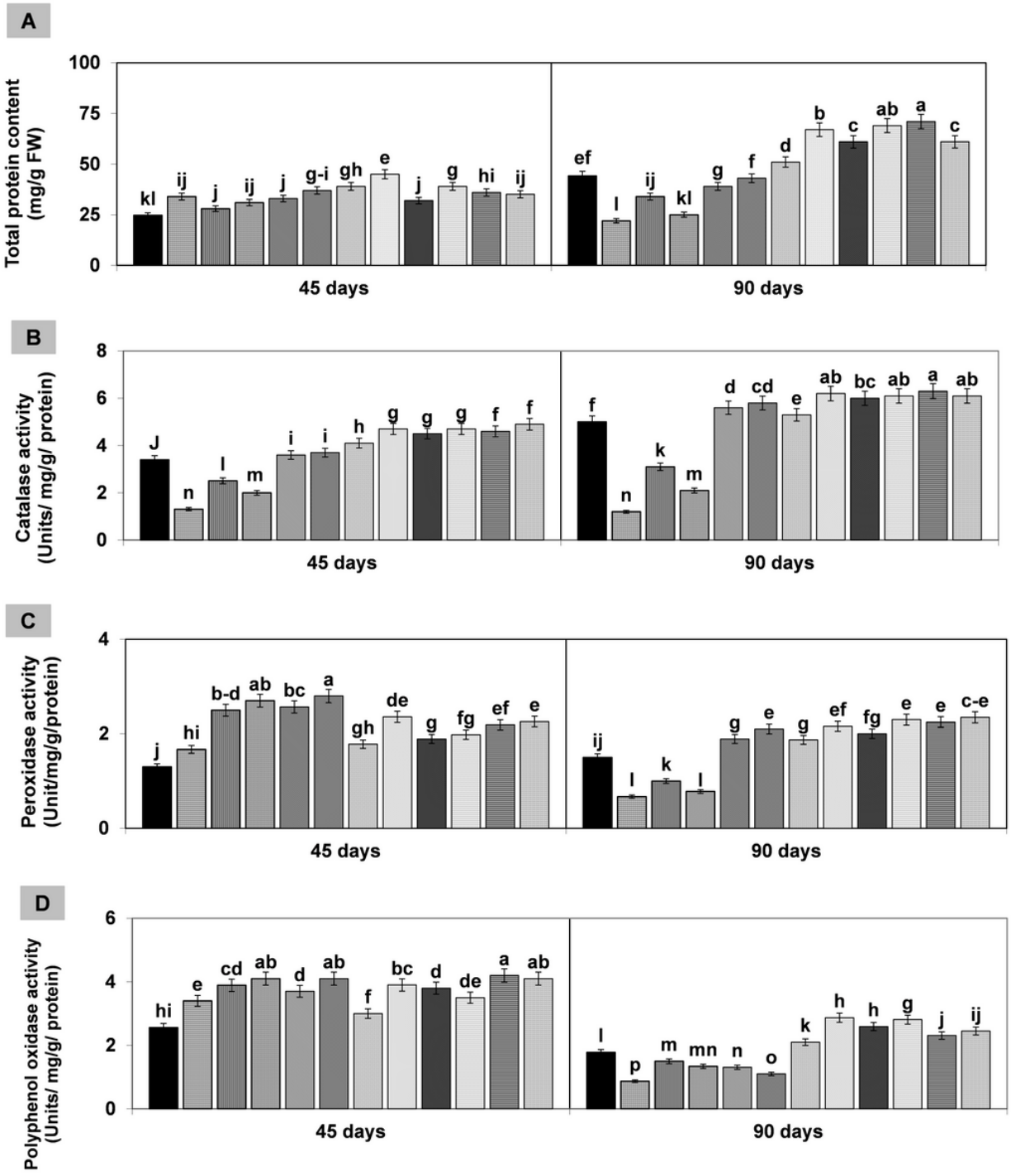

Figure 6

(A-D): Effect of 2% FYM on biochemical attributes of mash bean leaf under separate and simultaneous stress of *Macrophomina phaseolina* (MP) and excess copper (Cu) at 45th days of sowing. Vertical bars show standard errors of means of six replicates. Values with different letters at their top show a significant difference ($p \leq 0.05$) as determined by LSD-test.

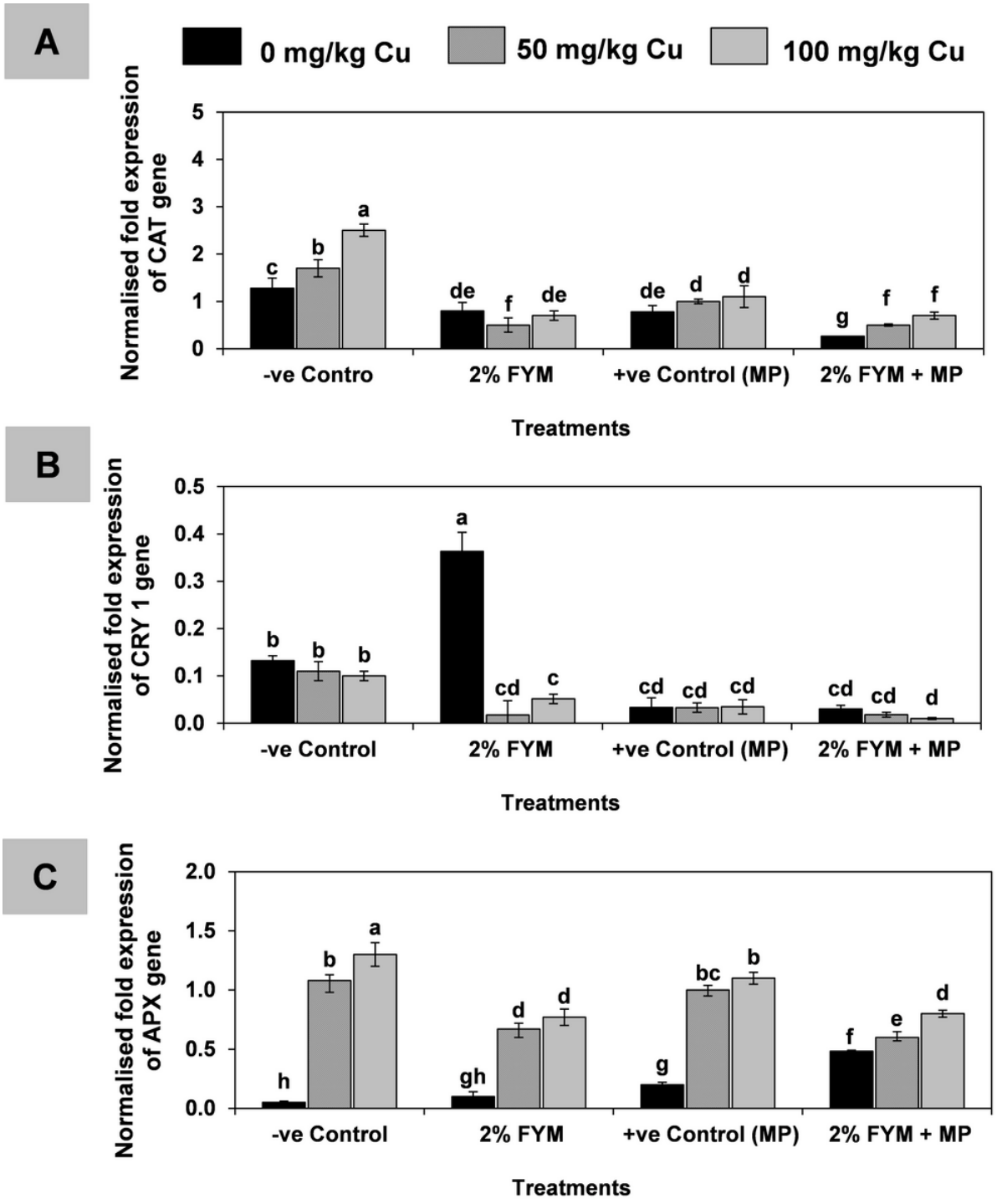

Figure 7

(A-C): Quantitative analysis of catalase (CAT), cytokinin-resistant genes (CYR1) and ascorbate peroxidase (APX) expression levels in mash bean leaf upon treatment with 2% FYM, *Macrophomina phaseolina* (MP) and excess copper (Cu) at 45th days of sowing. Vertical bars show standard errors of means of six replicates. Values with different letters at their top show a significant difference ($p \leq 0.05$) as determined by LSD-test.

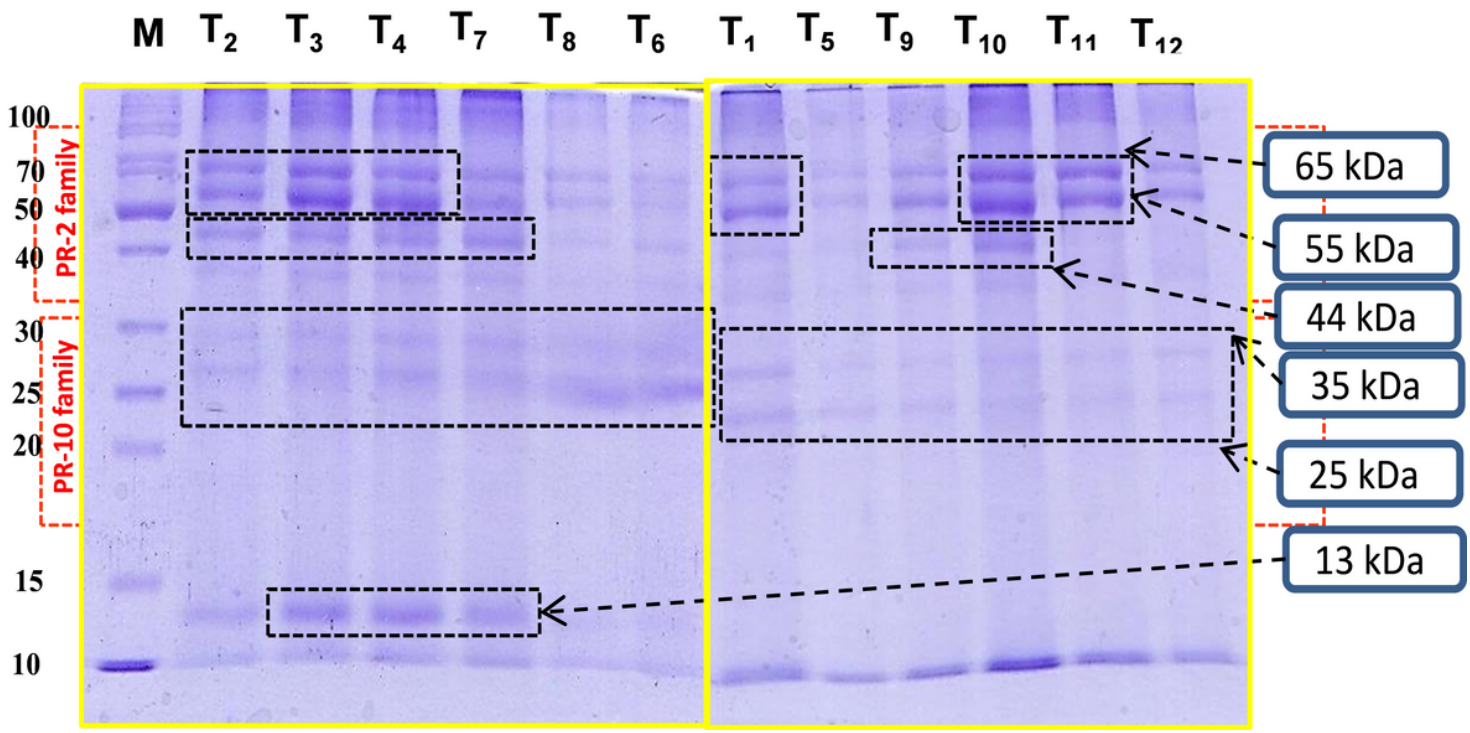

Figure 8

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 45-days old mash bean leaf due to the effect of soil amendment with 2% FYM on charcoal rot disease caused by *Macrophomina phaseolina* (MP) and excess copper (Cu) at 45th days of sowing. T_1: -ve Control; T_2: + ve Control (MP); T_3: Cu (50 mg/kg); T_4: Cu (100 mg/kg); T_5: Cu (50 mg/kg) + MP; T_6: Cu (100 mg/kg) + MP; T_7: 2% FYM; T_8: 2% FYM + MP; T_9: 2% FYM + Cu (50 mg/kg); T_{10}: 2% FYM + Cu (100 mg/kg); T_{11}: 2% FYM + Cu (50 mg/kg)+ MP and T_{12}: 2% FYM + Cu (100 mg/kg) + MP. Yellow boxes indicate the grouping of gels cropped from different parts of the same gel.

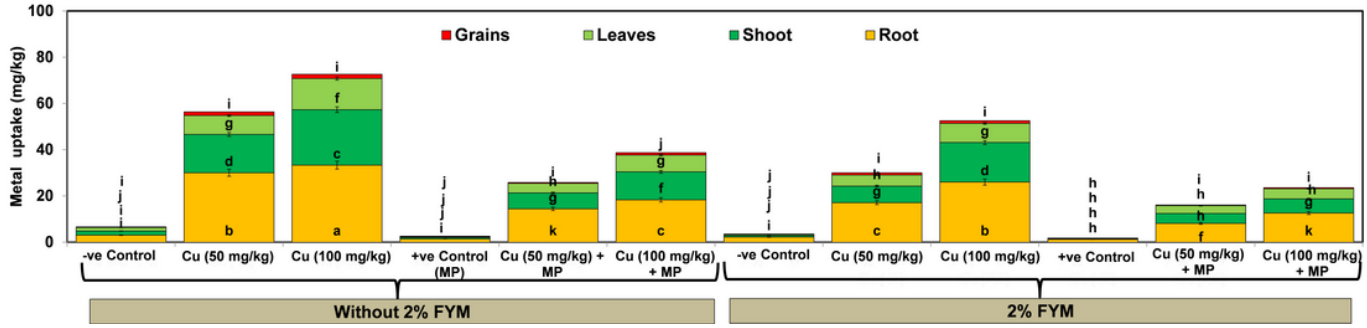

Figure 9

Copper (Cu) uptake by different parts of mash bean due to the effect of soil amendment with 2% FYM, *Macrophomina phaseolina* (MP), and excess Cu at 90th days of sowing. Vertical bars show standard errors of means of six replicates. Values with different letters at their top show a significant difference ($p \leq 0.05$) as determined by LSD test.

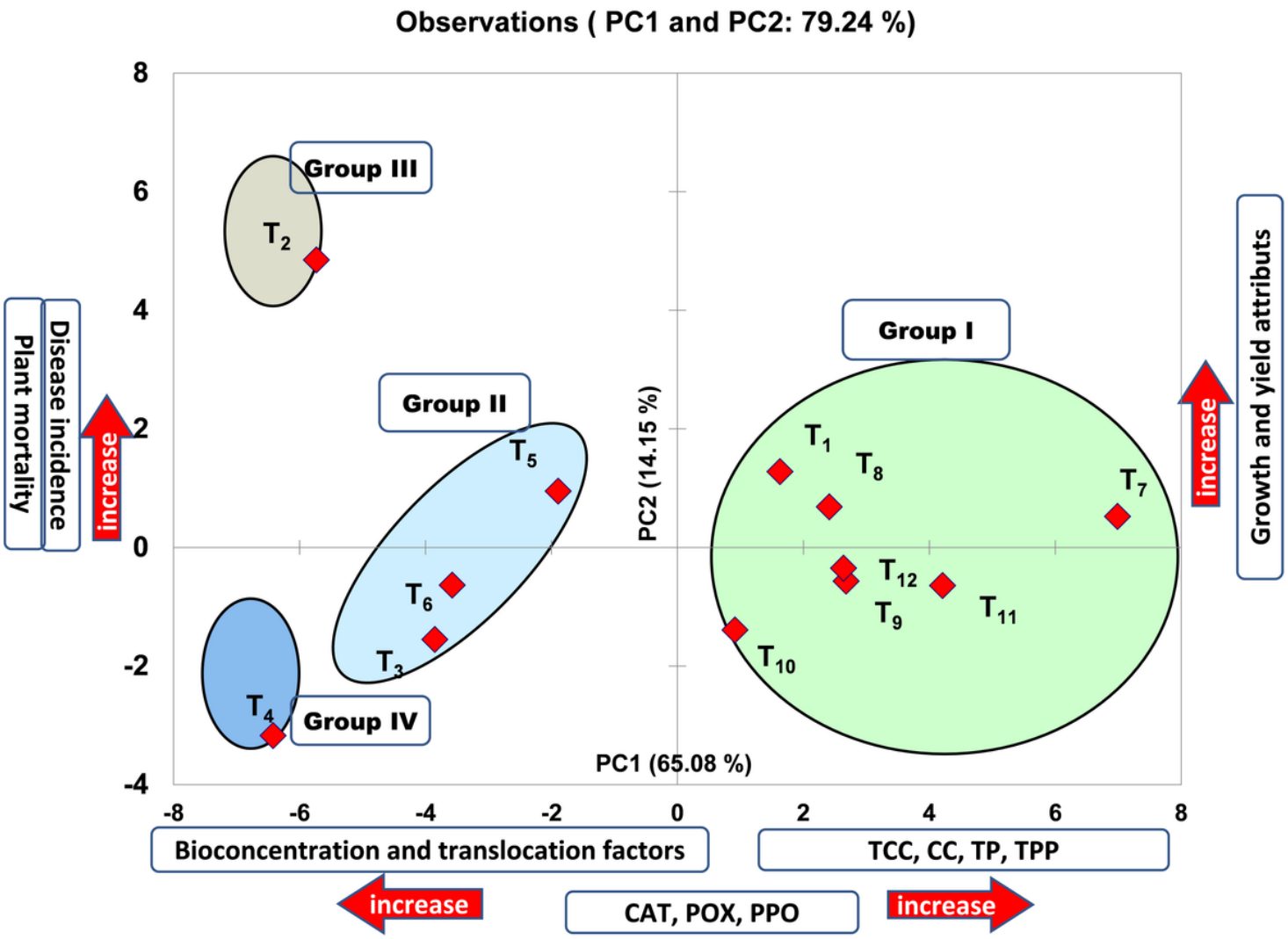

Figure 10

Principal component analysis of biophysical, biochemical, molecular and metal accumulation attributes in mash bean plants due to the effect of soil amendment with 2% FYM, *Macrophomina phaseolina* (MP), and excess copper (Cu). TCC: total chlorophyll content, CC: carotenoids; TRS: total reducing sugar; TP: total phenolic; TPP: total protein content; CAT: catalase; POX: peroxidase; PPO: polyphenol oxidase. T_1: -ve Control; T_2: + ve Control (MP); T_3: Cu (50 mg/kg); T_4: Cu (100 mg/kg); T_5: Cu (50 mg/kg) + MP; T_6: Cu (100 mg/kg) + MP; T_7: 2% FYM; T_8: 2% FYM + MP; T_9: 2% FYM + Cu (50 mg/kg); T_{10}: 2% FYM + Cu (100 mg/kg); T_{11}: 2% FYM + Cu (50 mg/kg)+ MP and T_{12}: 2% FYM + Cu (100 mg/kg)+ MP.

Supplementary Files

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