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SEPARATE AND COMBINED EFFECTS OF *MACROPHOMINA PHASEOLINA* AND COPPER ON GROWTH, PHYSIOLOGY AND ANTIOXIDATIVE ENZYMES IN *VIGNA MUNGO* L

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ABSTRACT

Food poisoning due to toxicity of heavy metals and pathogenic microorganisms is a major food safety issue that impacts on agriculture production. In the current research work, polygonal interaction of plant-pathogen-metal was investigated *in vitro* in terms of seed germination, seedling growth, physiology and antioxidant enzymes activity in *Vigna mungo* L. An experiment was conducted in growth chamber in glass Petri plates contained mash bean seeds on sterilized filter papers provided with spore suspension of *M. phaseolina* and four different concentrations of copper [Cu(II)] (25, 50, 75 and 100 ppm) either alone and in combination. Germination and seedling growth were significantly decreased by 40-98% and 30-98% due to separate effect of *M. phaseolina* and Cu, respectively. Germination and seedling growth were either insignificantly affected or stimulated at lower concentration (25 ppm) of Cu, while declined by 10-62% with increase in metal concentration from 50-100 ppm given in combination with pathogen. Total chlorophyll, sugar and carotenoid contents were reduced, while proline and total phenolic contents were increased due to given stresses. However, both proline and total phenolic contents were gradually decreased with increasing amount of Cu treatment (25-100 ppm). Antioxidant enzymes activities (peroxidase, polyphenol oxidase, catalase and superoxide dismutase) were increased to variable extents in all treatments. It was concluded that detrimental effect of Cu was increased with increase in its concentration. The simultaneous occurrence of *M. phaseolina* and excess Cu decreased harmful effect of individual stress on the plant.

Keywords: Copper; charcoal rot; *M. phaseolina*; toxicity; legumes; black gram.

INTRODUCTION

Mash bean (*Vigna mungo* L.) belongs to popular family Papillionaceae, is one of the commonly grown, nutritionally rich and nitrogen fixing summer pulse crop worldwide particularly in Asia having short span of life cycle (3-4 months). In Pakistan, due to insufficiency of research on mash bean, its cultivation and production area have gradually declined (Achakzai *et al.*, 2011). It occupies an area of over 27600 hectares with 13600 tones production, and Punjab is the major mash bean producing province (Anonymous, 2010). However, its yield is comparatively low in Pakistan as compared to its potential yield obtained in many other countries (Iqbal *et al.*, 2011). Diseases are the main contributors of pulse crops losses and among 26 diseases of mash bean, charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid is notorious for causing charcoal rot in pulses. Almost all growth stages of the plant are infected by this pathogen through formation of dark lesions on the epicotyls and hypocotyls. When pathogen progress, it hinder normal physiological and biochemical activities in plant through impeding xylem vessels followed by reduction in growth and yield. Successful infection finally results in seedling blight, stem and pod rot and seedling death. Moreover, occurrence of the pathogen in seed poses alarming threat to some overseas sprouting seed markets as disease may consequences with 100% yield losses (Iqbal *et al.*, 2011).

Within the context of plant-pathogen interactions the availability and toxicity of heavy metals can have a substantial impact on disease development. Metals are essential for defensive generation of reactive oxygen species (ROS) and can be used directly to limit pathogen growth (Fones and Preston, 2012). Heavy metal copper (Cu) is oldest known, 25^{th} most prevalent element in the ecosphere, and its contamination in lithosphere and hydrosphere from natural and anthropogenic activities such as agriculture, mining, and metallurgical industries is well-known (Mamat *et al.*, 2015). The highest acceptable content of Cu in soil is 100 µg kg^{-1} and in plant the permissible limit is 10 mg kg^{-1} (Hassan *et al.*, 2012). Within permissible limits vital role of Cu in key biochemical reactions of plants is very important and excessive concentration consequence in morphological, growth and physiological disorders (Nenovaa and Bogoeva, 2014). Enhanced production of ROS is common stress response of plant. The function of ROS may be damaging, protective, or signalling factors (De Gara *et al.*, 2010). Greater accumulation of ROS results in activation of stress related antioxidant enzymes like catalase, peroxidase, superoxide dismutase and polyphenol oxidase to combat with ROS (Sharma *et al*., 2012). These enzymes may be manipulated, overexpressed or down regulated depending on the strength of stress, duration, enzyme type and plant species etc. (Jouili *et al*., 2011)

Exceeding level of Cu not only alters plant growth but might leave deleterious or favourable effects on soil flora including pathogenic microorganisms (Puig and Thiele, 2007). The toxic level of Cu considered as noxious for plant might not be toxic for pathogen, so far, plant may suffer combined action of biotic and abiotic stress simultaneously through modifying effect of single stress (Nenovaa and Bogoeva, 2014). Interaction between the heavy metals and pathogenic fungi is conditioned on degree of tolerance of the fungi and their possibility of absorption of the metal. In this regard, Golubović-Ćurguz *et al.* (2010) reported that mycelium of *Fusarium oxysporum* and *Pythium debaryanum* grew slowly under stress Zn, Cu, Pb and Cd. *F. oxysporum* f. sp. *lycopersici* exhibited potential to degrade humic substances with subsequent increase in metal solubilization and bioaccessibility to tomato plant (Escobosa *et al*., 2010). Increase in disease severity caused by *F. oxysporum* f. sp. *radicis lycopersici* has been reported with increased in lead uptake by tomato plant (Maneva *et al*., 2013). Likewise, Nenova and Bogoeva (2014) suggested more severe disease development under excessive concentration of Cu. Shoaib *et al*. (2015) found that *Alternaria alternata* can grow up to 90 ppm of copper sulphate, therefore they suggested considering fungus-metal tolerance range before formulation of Cu-based fungicides.

Up till now mostly scientists focused their work either on the interaction between plant and pathogen or relations of plant with metal but few reports are available on simultaneous impact of pathogen and heavy metal on plant growth. There is a need to explore hidden fact behind combined influence of heavy metal and pathogenic fungi on the growth of plants. Therefore, the current study was conducted *in vitro* to compare separate and combined effect of *M. phaseolina* and Cu on seedling growth, physiology and antioxidative enzymes of mash bean seedling.

MATERIALS AND METHODS

Preparation of metal solution: About 3.93 g of copper sulphate ($CuSO_4.5H_2O$) (MERCK) was added in 1000 mL of distilled sterilized water to make of 1000 ppm stock solution. Required concentrations of 25, 50, 75 and 100 ppm were made by diluting stock solution with double distilled water.

Procurement of fungal pathogen: Pure culture of *M. phaseolina* (FCBP # 0751) was provided by First Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. Synthetic growth medium (2% Malt extract agar) was used to sub-culture and maintain the fungus culture.

Experiment: Seeds of mash bean var. Maikhaldia 6066 were surface sterilized with 0.1% sodium hypochlorite solution and were placed in pre-sterilized glass Petri plates (90 mm diameter) on double layered filter papers (Whatman No. 1). Experiment was conducted in four sets of treatments, and each set contained 100 seeds of mash bean. First set of treatments comprised of spore suspension (2.0 x 10^5 spores mL^{-1}) of *M. phaseolina*. In second set of treatment each of four concentrations (25, 50, 75 and 100 ppm) of Cu were applied. However, in third set, a mixture contained 3 mL of each concentration of metal solutions and spore suspension (2.0 x 10^5 spores mL^{-1}) of the fungus was applied. Control treatments were provided with 3 mL of distilled water only. There were four replicates of each treatment. Petri plates were kept in a completely randomized designed under controlled conditions in growth chambers (at 25 ± 2°C with 10 hours light period daily).

Assessment of germination assays: Germination rate (GR) and relative injury rate (RIR) of mash been seedlings in each treatment were calculated in each treatment by formulae described by Bekheet *et al*. (2006).

Assessment of growth parameters: The root and shoot lengths (cm) and fresh and dry weight (g) of germinated seeds were evaluated after 8 days of germination. Tolerance index (T_{index}) was calculated for all treatments by the following formula (Mahmood *et al.*, 2007).

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

Where: G_i = normalized growth parameter; G_x = individual growth parameter; G_{max} = maximum value; T_{index} = Tolerance index.

Assessment of physiological parameters: For total chlorophyll, carotenoid and sugar contents, the plant extraction was prepared in 80% acetone. The absorbance for chlorophyll a (645 nm), chlorophyll b (663 nm) and carotenoid (270 nm) were quantitatively analysed according to the formula of Lichtenthaler and Buschmann (2001). Sugar content was quantified according to the method of Nelson (1944) and proline content was estimated by method of Bates *et al.* (1973). Modified method of Bray *et al*. (1954) was used to assess total phenolic content. The plant material was homogenized with 80% ethanol, centrifuged at 10,000 rpm for 10 minutes to get supernatant 1, and in residue 10 mL of ethanol (80%) was added followed by re-centrifugation to get supernatant 2. To the mixture of supernatant 1 and 2, 1 mL of 20% Na_2CO_3 along with 0.5 mL of Folin-Ciocalteatou's reagent was added. This mixture was made up to 20 mL with distilled water and its absorbance was measured at 660 nm for total phenol content.

Assessment of antioxidative enzymes: Activities of antioxidant enzymes of mash bean seedling were analyzed at 48, 72 and 96 hours after germination. Protocols of Kumar and Khan (1982) and Mayer *et al.* (1965) were used for estimation of peroxidase (POX) and polyphenol oxidase (PPO), respectively. Beers and Sizer (1952) procedure was used for analysis of catalase (CAT) activity. Superoxide dismutase (SOD) activity was assayed with slight modification in the methods proposed by Maral *et al*. (1977). Two test tubes each containing 2.0 mL of 1.0 mM sodium cyanide, 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, and 2.0 µM riboflavin were as a substrate. One tube was used as sample containing reaction mixture + 5.0 µL enzyme extract, placed in light for 15 minutes. The other tube containing reaction mixture without enzyme extract was covered with black cloth at the same time. The absorbance of samples was compared to non-illuminated mixture at 560 nm.

Statistical analysis: All the data from laboratory bioassays were subjected to analysis of variance followed by LSD test to differentiate the treatment means at probability 5% through Statistics 8.1.

RESULTS AND DISCUSSION

When mash bean seedling was exposed to separate as well as combined stress of charcoal rot pathogen (*M. phaseolina*) and excess Cu, overall changes in growth, physiology and activities of antioxidant enzymes were more pronounced due to effect of individual stress.

Germination and growth assays: When seeds of mash bean were inoculated with spore suspension of the *M. phaseolina*, the GR and RIR were significantly decreased by 40% as compared to control (Fig. I A and B). Shoot length and biomass was significantly inhibited by 70-98%, while these parameters for root were declined by 70-80% over control (Fig. I C-H). The inhibitory effect of spore suspension of *M. phaseolina* on overall growth and biomass of mash bean seedling revealed sensitivity of plant to the fungus. Production of extra cellular enzymes and toxins by the fungus likely to break main polymeric components of the cell wall and the cell membrane of mash bean seedling (Seregin and Ivanov, 2001) with net negative effect of growth and biomass. Typical symptoms of charcoal rot disease were visible as black sclerotial head on the shoot and fungus produced ashy gray lesions on basal end of the shoot with root rotting (Zhang *et al*., 2011).

Seeds provided with different concentrations of Cu only, exhibited a significant and progressive reduction of 13-35% in GR and RIR with improving Cu concentrations from 25-100 ppm over control (Fig. I A and B). Seedling length and dry biomass were considerably declined by 20-50% and 30-100%, respectively due to effect of 25-100 ppm of metal over control (Fig. I C-H). Significant reductions in seedling biomass and growth are due to either direct or indirect Cu phytotoxicity. Direct phytotoxicity could occur through oxidative damage or by interaction with protein, while in indirect mode, Cu may disturb absorption, transportation and functioning of other nutrient elements in mash bean seedlings (Yruela, 2009).

When spore suspension of *M. phaseolina* was given along with different concentrations of Cu, GR and RIR were significantly decreased by 10-30% over control (Fig. I A and B). It was also observed all four concentrations (25-100 ppm) of Cu significantly increased shoot length up to 60%, however increase in shoot length was comparatively less pronounced with increased in Cu concentrations. Besides, 25 ppm exhibited stimulatory effect on all growth parameters. However, rest of three Cu concentrations (50-100 ppm) either declined or insignificantly influenced on all growth parameters with respect to control. Fresh as well as dry weights of shoot were decreased by 30-60% due to effect of 50-100 ppm of the metal along with the pathogen. Root length was decreased by 10-35% and dry weight by 20-50% at 50-100 ppm, respectively combined with the pathogen (Fig. I A-F). Different stress factors occurring in combination may cause change in their individual effects (Nenovaa and Bogoeva, 2014). Antifungal activity of Cu against many phytopathogenic fungi including *M. phaseolina* is well-known. Hence, inhibition in spore germination and mycelial growth by antimycotic action of Cu (Niinemets, 2010) is likely to decrease the infection level in plants. It has been documented that higher concentration of Cu could induce disruption of membrane integrity that inevitably leads to loss of cell viability in the pathogenic fungi (Kumbhar, 1991). Therefore, more harmful effect of Cu rather fungus could be anticipated on mash bean seedling under combined stress of Cu and pathogen. It was further evident from the absence of fungal spores and typical disease symptoms on mash bean seedling.

T_{index} was significantly reduced up to 30% due to infection of *M. phaseolina* only, from 90-30% and 100-50% with increase in concentrations from 25-100 ppm only and combined with the pathogen, respectively (Data not shown). The low T_{index} might have been due to the changes in physiological mechanisms of mash bean seedlings during growth stages after exposure to stress (Khan *et al.*, 2006)

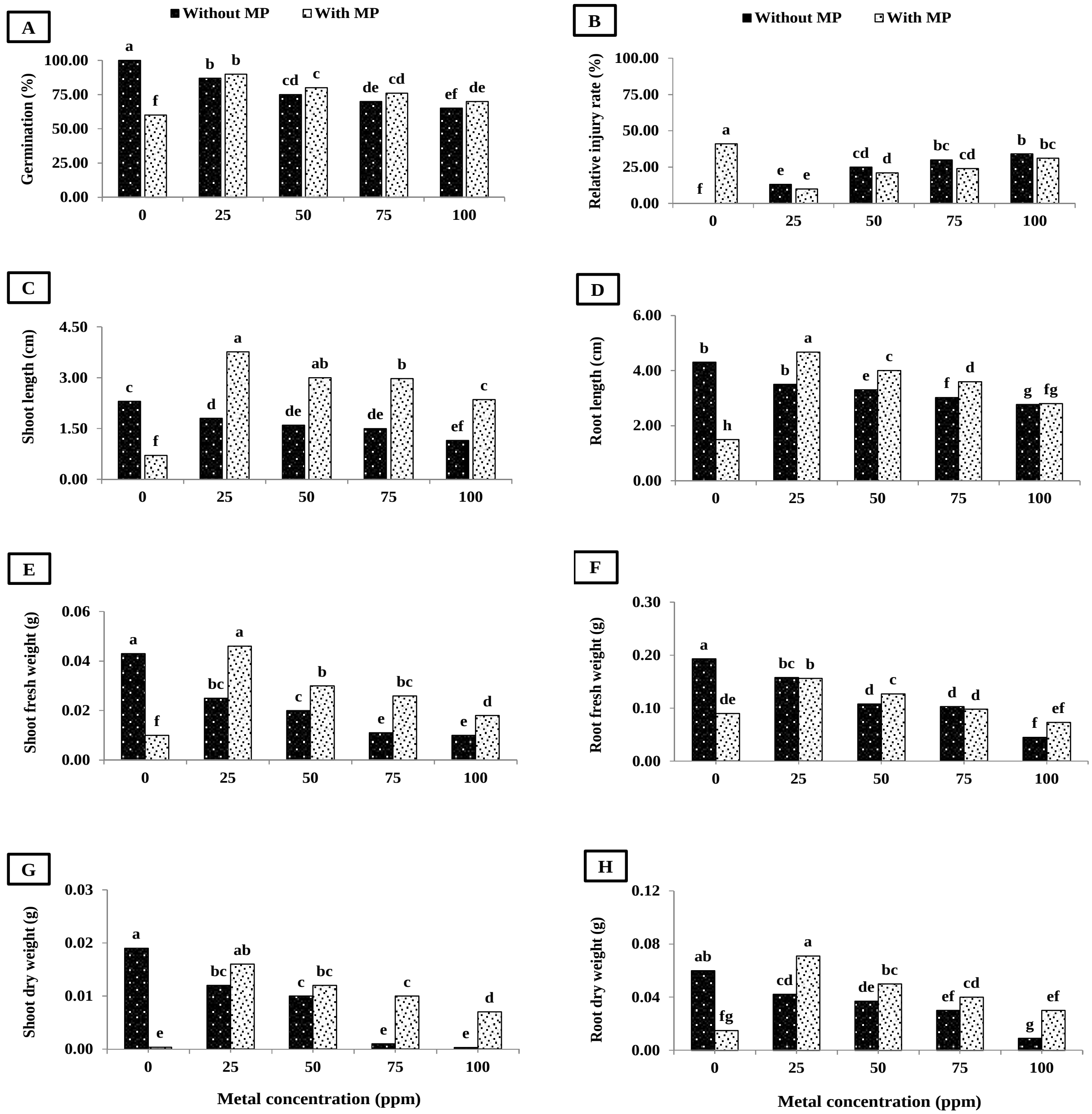

Fig. I A-F: Separate and combined effect of *Macrophomina phaseolina* (MP) and copper on germination, growth and biomass of *V. mungo*

Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test.

Physiological assays: Total chlorophyll, carotenoid, sugar, total phenolic and proline contents in control treatments were 0.36, 0.22, 0.30, 0.07 and 0.14 mg g^{-1} fresh weight, respectively (Table I). The investigated parameters were more considerably affected due to separate effect of *M. phaseolina* or Cu than under their simultaneous stress. Therefore, inoculation with *M. phaseolina* resulted in significant reduction of 72, 51 and 77% in the contents of total chlorophyll, carotenoid and sugar, respectively, while considerable increase of up to 2 and 3 folds in the contents of proline and total phenolic, respectively as compared to respective control. The total chlorophyll, carotenoid and sugar contents were significantly dropped off by 10-80% and

10-50% with increase in metal concentration from 25-100 ppm either alone or combined with *M. phaseolina*, respectively over control. So far, proline and total phenolic contents were significantly increased as compared to respective control, but were decreased from 70-30% with increase in metal concentration (25-100 ppm) applied separately or simultaneously with pathogen, respectively (Table I). Reduced synthesis or enhanced oxidative degradation of the chloroplast pigment imposed by oxidative stress could be negative out of the given stresses (Chaneva *et al.*, 2010). Role of carotenoids was documented against ROS and photochemical damage, and presently reduction in carotenoids could provide their supportive role against oxidative stress (Kachout *et al*., 2015). Decrease in sugar content due to pathogen or metal could be attributed to diversion of metabolites to other synthesis processes or due to destruction of chlorophyll molecules (Khurshid *et al*., 2016). Decreased level of proline and total phenolic contents with increasing amount of Cu treatment in mash bean seedling indicated varied level of defences in plant under stress. Increased in proline content could be due to loss of water from plant, while proline function has been suggested as stabilizer of plasma membrane and some macromolecules and free radical scavenger (Azooz *et al*., 2013). Increase in total phenolic content might be attributed to increase in resistance in mash bean seedlings to protect itself under biotic stress by hypertensive response and abiotic stress by chelating the metals as well as inhibit ROS generation (Mamat *et al*., 2015).

Table 1. Separate and combined effect of *Macrophomina phaseolina* (MP) and copper (Cu) on the physiology of mash bean seedlings after 8 days of germination

Physiological parameters (mg^{-1} g FW)	Cu concentration (ppm)									
	Without MP					With MP				
	0	25	50	75	100	0	25	50	75	100
Total chlorophyll content	0.36^{a}	0.33^{a}	0.24^{cd}	0.21^{e}	0.15^{f}	0.10^{f}	0.34^{a}	0.27^{bc}	0.29^{b}	0.22^{f}
Carotenoid content	0.22^{a}	0.19^{a-c}	0.16^{b-d}	0.13^{de}	0.09^{e}	0.11^{de}	0.20^{ab}	0.19^{a-c}	0.16^{a-d}	0.14^{c-e}
Sugar content	0.30^{a}	0.25^{bc}	0.19^{d}	0.15^{ef}	0.09^{g}	0.07^{g}	0.28^{b}	0.22^{c}	0.18^{de}	0.14^{f}
Proline content	0.14^{g}	0.38^{ab}	0.35^{bc}	0.29^{cd}	0.24^{de}	0.41^{a}	0.35^{bc}	0.29^{de}	0.21^{ef}	0.17^{fg}
Total phenolic content	0.09^{f}	0.29^{a}	0.25^{b}	0.22^{c}	0.17^{d}	0.28^{a}	0.24^{bc}	0.19^{d}	0.14^{e}	0.10^{f}

In a row values in superscript with the different letters show significant difference ($P \leq 0.05$) as determined by LSD test.

Antioxidative enzymes assays: Activities of POX, PPO, CAT and SOD in control treatment were found to be within 0.40-0.43, 0.28-0.32, 0.43-0.48 and 0.48-0.51 Units mg^{-1} g^{-1} protein, respectively with advent of incubation period (48, 72 and 96 hrs). These all enzymes activities were not significantly increased in control, but pronouncedly increased in rest of the treatments with increased in time and percentage of increase was reached at the highest level after 96 hours. Enzymes activities were significantly improved with increased in Cu concentration and the separate effect of pathogen or metal was more substantial as compared to their combined effect. There was 3-4, 2-3, 2 and 2 folds enhancement in the activities of PPO, POX, CAT and SOD, respectively during 48-96 hours in the mash bean seedling provided with spore suspension of *M. phaseolina* over respective control. There was 10-40% and 0-30% increase in the activities of all four enzymes due to 25 ppm of Cu only and in combination with pathogen, respectively at 24-96 hrs. With increase in metal concentration from 50-100 ppm, either alone or with pathogen, the highest increase was observed in activity of PPO, it was increased by 60-150% at three time intervals over respective control. While, the activities of POX, CAT and SOD were significantly increased by 10-70%, 30-50% and 30-70% over corresponding control (Table II). The increase in activities of antioxidant enzymes indicated activation of plant defense system against production of reactive oxygen species (ROS) (Morsy *et al*., 2012). Free radicals and ROS could be dismutated to H_2O_2 and O_2 by SOD, and H_2O_2 could be eliminated by CAT and POX (Scndalions, 1993). The most obvious change was observed in activity of PPO. The function of PPO is to catalyse the final step in biosynthesis pathway of lignin and other oxidative phenol (Raghavendra *et al*., 2013). Therefore, up-regulation of PPO might indicate role of antioxidative polyphenols after pathogen or metal stress. It could be speculated that when mash bean seedling was under biotic or abiotic stress, overall changes in plant growth, physiology and antioxidant enzymes could be due to destabilization of membrane function that may consequences in loss of plant intrinsic balance. As there is threshold of enzyme activity, and it seems that increase in activity of stress related enzyme was not sufficient to protect plant from oxidative stress both due to pathogen and at higher concentration of Cu (Azooz *et al*., 2012).

Table 2. Separate and combined effect of *Macrophomina phaseolina* (MP) and copper (Cu) on antioxidative enzymes of mash bean seedlings at different time intervals (48, 76 and 92 hrs) after germination

Antioxidative enzyme activity (Units mg^{-1} g^{-1} protein)	Time (hrs)	Cu concentration (ppm)									
		Without MP					With MP				
		0	25	50	75	100	0	25	50	75	100
Catalase activity (CAT)	48	0.43^{lm}	0.46^{kl}	0.54^{g-i}	0.58^{f-h}	0.63^{d-f}	0.71^{c}	0.36^{n}	0.5^{i-k}	0.54^{g-i}	0.57^{f-h}
	76	0.45^{j-l}	0.49^{i-l}	0.59^{e-g}	0.64^{de}	0.69^{cd}	0.83^{b}	0.39^{mn}	0.54^{g-i}	0.58^{f-h}	0.61^{ef}
	92	0.46^{h-j}	0.53^{g-i}	0.61^{ef}	0.69cd	0.71^{c}	0.99^{a}	0.44^{k-m}	0.57^{f-h}	0.62^{ef}	0.65^{c-e}
Peroxidase activity (POX)	48	0.40^{m}	0.42^{lm}	0.44^{k-m}	0.47^{i-l}	0.58^{g-j}	0.93^{b}	0.40^{m}	0.42^{lm}	0.45^{j-m}	0.49^{h-i}
	76	0.42^{lm}	0.48^{n-l}	0.52^{f-j}	0.57^{e-g}	0.64^{d}	0.99^{b}	0.43^{k-m}	0.53^{f-i}	0.54^{f-h}	0.58^{d-f}
	92	0.43^{k-m}	0.53^{e-h}	0.58^{d-f}	0.63^{de}	0.71^{c}	1.2^{a}	0.48^{h-l}	0.57^{e-g}	0.61^{de}	0.64^{d}
Polyphenol oxidase activity (PPO)	48	0.28^{r}	0.33^{p}	0.42^{o}	0.47^{k-m}	0.54^{gh}	0.87^{c}	0.32^{q}	0.37^{p}	0.45^{l-o}	0.52^{g-i}
	76	0.31^{r}	0.42^{o}	0.49^{i-k}	0.51^{h-j}	0.71^{e}	0.95^{b}	0.38^{o}	0.44^{no}	0.48^{j-l}	0.68^{e}
	92	0.32^{qr}	0.46^{k-n}	0.53^{gh}	0.58^{f}	0.80^{c}	1.43^{a}	0.43^{no}	0.48^{j-l}	0.55^{fg}	0.75^{d}
Superoxide dismutase activity (SOD)	48	0.48^{s}	0.52^{pq}	0.64^{kl}	0.69^{hi}	0.73^{fg}	0.89^{c}	0.49^{rs}	0.56^{o}	0.61^{mn}	0.68^{ij}
	76	0.49^{rs}	0.58^{o}	0.68^{jk}	0.74^{f}	0.78^{e}	0.95^{b}	0.54^{p}	0.59^{no}	0.66^{jk}	0.74^{f}
	92	0.51^{qr}	0.63^{lm}	0.72^{fg}	0.79^{e}	0.85^{d}	1.23^{a}	0.58^{o}	0.63^{lm}	0.71^{gh}	0.79^{e}

Different letters (superscript) in rows depict significant difference ($P \leq 0.05$) in the treatments of individual parameters as determined by LSD Test.

Conclusions: Excess Cu and *M. phaseolina* infection changed plant growth, physiology (chlorophyll, sugar, carotenoid, proline and total phenolic contents) and antioxidative enzymes activities (POX, PPO, CAT, and SOD). The simultaneous occurrence of both stress factors modified their separate effects on plant. However, it was found that mash bean seedling has low potential to survive under Cu contamination present alone or in combination with *M. phaseolina* within range of 50-100 ppm, therefore plant should not exposed to Cu toxicity.

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