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COMPARATIVE ASSESSMENT OF POTENTIAL OF MICRONUTRIENTS, BIOFERTILIZERS AND CHEMICAL FUNGICIDES AGAINST CHARCOAL ROT IN SUNFLOWER

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Abstract

Charcoal rot incited by *Macrophomina phaseolina* is yield limiting disease of sunflower (*Helianthus annuus* L.). Micronutrients and biofertilizers are safer and sustainable alternatives to fending off disease over chemical fungicides. The present investigation aimed to investigate and compare the potential of zinc (Zn), boron (B) and commercial biofertilizers with chemical fungicides on disease management and seedling growth attributes in sunflower. According to principal component (PCA) analysis, the treatments were classified into 4 groups. Highly infected seedlings displaying 81% disease incidence and 50-90% reduction in growth in positive control (provided with *M. phaseolina* only) occupied group I (left side) as compared to negative control (un-inoculated) located opposite side in group III. Higher concentrations (1000 and 2000 ppm) of both fungicides (carbendazim and thiophanate-methyl) highly significantly managed 80% of the disease also occupied cluster III, while lower (150-350 ppm) and medium (500 ppm) concentrations reduced 20-60% disease belong to group II. Zn (2.5-3.5 ppm) and B (0.4-0.7 ppm) reduced 38-62% and 34-50% disease, respectively in cluster VI, hence these treatments were significantly and closely associated with treatments in cluster III. Biofertilizers and lower doses of micronutrient in cluster I were less effective in managing disease. Effective doses of fungicides, Zn and B were significantly and positively associated with growth variables. However, Zn (2.5 ppm) exhibited comparable effect on disease and growth attributes as was recorded with higher doses of fungicides. Hence, Zn (2.5 ppm) could be used as encouraging control means of charcoal rot disease in sunflower.

Key words: *Macrophomina phaseolina*, PCA, sunflower, zinc

Introduction

Charcoal rot incited by fungus *Macrophomina phaseolina* (Tassi) Goid (synonym: *Rhizoctonia bataticola* (Taub.) Butler) is a serious and economically important disease of sunflower, which has hampered sunflower production in the warmer areas around the world and can cause massive yield losses up to 90% under epidemic conditions (Darwesh and Elshahawy, 2023). The severe intensity of the disease has also been reported in sunflower growing regions of Pakistan, and the disease has been regarded as threat for sunflower growers (Siddique *et al*., 2021).

M. phaseolina is a killer pathogen, which through bulk of black microsclerotia turns coal like appearance of infected plant tissues (Hemmati *et al*., 2018). The microsclerotia thrive over three years or more in soil and are stimulated by root exudates to germinate and infect host plant material (Akhtar and Shoaib, 2020).

After infecting root and stem, the pathogen clog vascular tissues in the tap root, and also reduced germination that leads to seedling rots (Sun *et al*., 2015). Deeper penetration of pathogen leads to structural damages and death of infected plant particularly when conductive conditions (water stress and high heat and temperatures) are available for the pathogen (Coser *et al*., 2017).

Disease management strategies viz. regulatory, cultural, biological, physical, and chemical have provided limited control (Mengistu *et al*., 2015). Micronutrients and biofertilizers are among the suitable and eco-friendly approaches use to reduce pathogens damage either by inducing resistant and/or by stimulating antagonist microbial population in the rhizosphere. Micronutrients like zinc (Zn) and boron (B) are integral for several metabolic pathways and constituents of the defense response in plants when available at optimum level (Pérez *et al*., 2020). The effect of B and Zn to reduce incidence and/or severity of charcoal rot diseases has been poorly studied. Zn deficiency has been documented to reduce yield of rice and other crops in Asian countries (Rehman *et al*., 2013; Khan *et al*., 2018; Awan *et al*., 2019). It has been shown that application of Zn either in soil or on seeds has improved grain yield and grain Zn of bread wheat (Rehman *et al*., 2018). Haider *et al*. (2020) findings revealed that Zn-treated mung bean seeds have displayed better stand establishment, seedling growth, and other agronomical attributes due to contribution of Zn in physiological processes. It has been reported that seed priming with Zn induces disease resistance by improving photosynthesis, cell division, protein synthesis, carbohydrate and nitrogen metabolism (Farooq *et al*., 2012). Zn can directly arrest fungal growth, secondary metabolism and effect host-pathogen relationship (Li *et al*., 2016). Silva *et al*. (2016) observed a 45-56% reduction of eucalyptus powdery mildew after applying 2 ml L^{-1} of Zn compounds (phosphite and sulphate) to soil or leaves and root. Machado *et al*. (2018) described Zn as a major player in plant immune helped to reduce disease symptoms. Zn finger (Znf) proteins are reported to be involved in plant growth, development, and response to biotic stress (Noman *et al*., 2019). Awan *et al*. (2019) reported 2.5 and 5.0 ppm of $ZnSO_4$ mitigated early blight stress in tomatoes and improved growth by acting against reactive oxygen species.

Boron (B) is reported to be involved in cell division, calcium utilization, flowering and fruiting, metabolism of carbohydrates, protein, phenol, nucleic acid and indole acetic acid, synthesis of cell wall, integrity and function of membrane as well as disease resistance (Tanaka and Fujiwar, 2008; Farooq *et al*., 2012). B is known to activate defense mechanisms by assisting release of calcium cations from the cell wall, the calcium ions in turn participate in interaction with salicylic acid to induce systemic resistance in plant against pathogen (Fu and Dong, 2013). Jiang *et al*. (2016) experimentation results showed that 0.05, 0.50 and 2.50 ppm of B exhibited 95.2%, 72.6% and 63.4% disease severity of bacterial wilt in tomato. Dong *et al*. (2016) also found that 0.05, 0.2 and 2.0 ppm of B in combination with iron (Fe) helped to reduce *Fusarium oxysporum* infection in banana by maintaining the integrity of the membrane and cell wall. It was also revealed that plants deficit in B may show fragmented cell wall with fewer antifungal compounds at the infection site (Thompson and Raizada, 2018).

Biofertilizer (biological fertilizer) is a modernized form of organic fertilizer, which not only boost plant productivity but also help in managing diseases (Itelima *et al*., 2018). These are enriched with microbial isolates and has shifted fortunes in agriculture by acting as key players in sustainable agriculture. Biofertilizers effectively manage disease through lysis, antibiosis, hyper-parasitism and antagonism for space and nutrients. Additionally, through siderophore production (Olanrewaju *et al*., 2017) and detoxifying phytopathogen virulence factor (Ramette *et al*., 2006), biological fertilizer can help to hamper pathogen growth. Many diseases including Fusarium wilt (Xiong *et al*., 2017), bottom rot disease in lettuce (*Rhizoctonia solani*) (Windisch *et al*., 2017) and charcoal rot diseases of sunflower (Nafady *et al*., 2019) have been successfully managed using biofertilizers. Hence, biofertilizers and micronutrients may play beneficial roles in eco-friendly agricultural systems in comparison with the conventional chemical treatments to manage charcoal rot of sunflower. The present study was designed to evaluate and compare the effect of micronutrients (Zn and B) and commercially available biofertilizers with chemical fungicides on germination, seedling growth and charcoal rot disease in sunflower.

Materials and Methods

Previously identified isolate of *M. phaseolina* (MPSS-01) isolated from sunflower stem infected with charcoal rot disease, submitted to the GenBank (MH999811) was used and refreshed aseptically on PDA (potato dextrose agar) media. Sclerotial suspension of 2×10^5 sclerotia mL^{-1} was prepared from seven days old culture of *M. phaseolina* (Siddique *et al*., 2021).

Seeds of sunflower hybrid (FH-331) procured from Ayub Agriculture Research Institute, Faisalabad, Pakistan were surface sterilized in 1% NaOCl solution for 3-4 minutes. Three layers of sterilized blotter were placed in pre-sterilized Petri plates and seeds were placed in them (7 seeds $plate^{-1}$). Different concentrations of micronutrient (Zn and B) and fungicides (carbendazim and thiophanate-methyl) were applied (1.5 mL) separately in respective plates (Table 1). For biofertilizers application, seeds were presoaked in 10% sugar solution to ensure the attachment of beneficial bacteria with seed surface and then 1.5 mL of respective biofertilizer (100,000ppm) was applied. After 24 hours the sclerotial suspension (2×10^6 sclerotia mL^{-1}) of *M. phaseolina* was applied on seeds in each plate. Negative control treatment received distilled water only, while positive control, seeds were provided with sclerotial suspension only. Plates were placed undisturbed at room temperature 28 ± 2 °C. Experiment was run in completely randomized block design with three replicates for each treatment.

Data concerning germination and growth parameters (shoot length, root length, seedling biomass and vigor index) was recorded after 10 days of germination. Following formulas were used to determine seed germination percentage, disease incidence percentage and vigor index (Jadhav *et al*., 2019).

$$\textbf{Germination}\ \% = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

$$\textbf{Disease incidence}\ \% = \frac{\text{Number of seeds infected}}{\text{Total number of seeds sown}} \times 100$$

$$\textbf{Vigor index} = (\text{Shoot length} + \text{root length}) \times \text{Germination percentage}$$

Data were analyzed using one-way analysis of variance (ANOVA) followed by LSD test to monitor significance among treatments. The types of treatment and growth variables were subjected to ordination by a principal component analysis (PCA), which allowed to simplify the data set and identify positive and negative correlations among variables.

Table 1: Treatments used *in-vitro* to manage the infection of *M. phaseolina* on sunflower seeds.

Sr #	Trade name	Active ingredient and concentration (%)	Concentrations used (ppm)	Recommended doses per acre
1	Zinc sulphate	Zinc 33%	1, 1.5, 2, 2.5, 3, 3.5, 4	5 kg
2	Borax	Boron 11.3%	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8	3 kg
3	Shincar	Carbendazim 50% SC	150, 250, 350, 500, 1000, 2000	100 mL
4	Topsin® M	Thiophanate-methyl 70% WP	150, 250, 350, 500, 1000, 2000	250 mL
5	Biopower	Microbial preparations containing *Rhizobium, Azorhizobium, Pseudomonas, Azospirillum, Bradyrhizobium* etc.	100,000	100 g Kg^{-1} of seeds
6	Feng shou	Microbial preparations containing *Azospirillum* and *Azotobacter* along with other plant growth hormone producing bacteria	100,000	100 mL Kg^{-1} of seeds

Results

Effect on disease

There was no disease in negative control, while disease incidence was 81% in positive control. Disease incidence was significantly decreased from 47 to 19% and from 52 to 23% with increase in concentrations (150-2000 ppm) of carbendazim and thiophanate-methyl, respectively. Micronutrients (Zn and B) also found effective in reducing disease incidence. Significantly lowest disease incidence (38%) was noticed with 2.5 ppm of Zn, followed by 46, 52, 62 and 67% with 3.5, 4.0, 2.0 and 1.5 ppm, respectively. Likewise, application of 0.4 and 0.5 ppm of B exhibited the significantly lowest disease incidence of 50% followed by 67% at remaining concentrations of 0.2, 0.3, 0.6 and 0.7 ppm. Seeds treated with biofertilizers (BF1 or BF2) exhibited 60% disease incidence, which was statistically at par with each other, but significantly less as compared to positive control (Figure 1).

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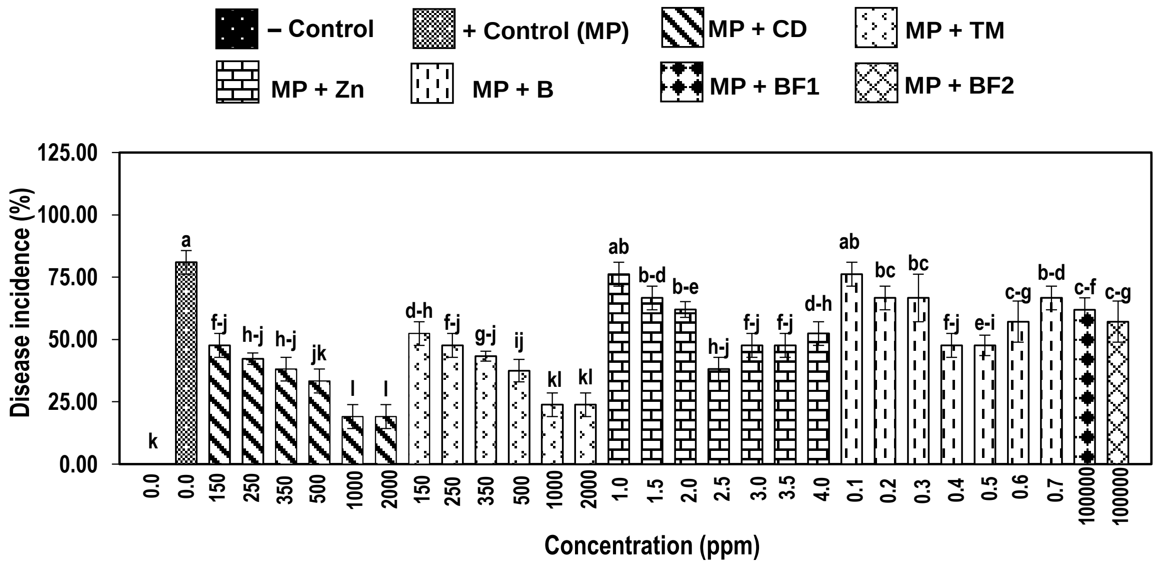

Figure 1: Effect of carbendazim (CD), thiophanate-methyl (TM), zinc (Zn), boron (B), biofertilizers (BF) on disease incidence in sunflower seedling under *Macrophomina phaseolina* (MP) stress. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test.
BF 1: Biopower; **BF 2:** Feng Shou

Effect on germination (%)

Germination was 100% in negative control, while it was declined significantly by 52% in positive control under pathogen stress. Application of fungicides, micronutrients, and biofertilizers significantly improved germination (%) as compared to positive control (Figure 2a). Both fungicides consistently and significantly improved germination by 70-95% and 60-90% with increase in concentration (150-2000 ppm) of carbendazim and thiophanate-methyl, respectively. Application of either micronutrient did not significantly improve germination at low concentrations, while medium concentrations gave the maximum germination, and the highest concentrations exhibited less pronounced effect with respect to positive control. Accordingly, the said attribute was significantly enhanced to 90, 86, 85 and 70% with 2.5, 3.0, 3.5 and 4.0 ppm of Zn, respectively. Application of 0.4 ppm of B showed the highest germination (86%) followed by 81, 70 and 66% at 0.5, 0.6 and 0.7 ppm, respectively. BF1 and BF2 significantly improved germination to 67% and 61%, respectively (Figure 2a).

Effect on seedling growth

Generally, shoot and root lengths were significantly improved at all applied concentrations of fungicides, micronutrients, and biofertilizers with respect to positive control. However, among all treatments, Zn (2.5 ppm) proved highly effective for improving the said indices, and the improvement was at par with negative control as well (Figure 2 b and c).

Sunflower seedlings provided with sclerotial suspension of *M. phaseolina* in positive control were drastically affected, therefore lengths of shoot and root were reduced significantly by 65% (1.5 cm) and 80% (0.6 cm) as compared to negative control (4.3 and 3.1, cm, respectively). Different concentrations (150-2000 ppm) of carbendazim showed significant improvement of 70-135% and 177-367% in length of shoot and root, respectively over positive control. Likewise, with increase in concentration (150-2000 ppm) of thiophanate-methyl, the lengths of shoot and root were improved significantly by 50-127% and 167-333% over positive control. Moreover, the higher concentrations (1000 and 2000 ppm) of either fungicide statistically provided corresponding improvement in the studied attributes over positive control (Figure 2 b and c).

In case of micronutrients, the shoot and root lengths improved significantly by 100% & 250% at low concentrations (1.0-2.0 ppm), by 145% & 376% at medium concentrations (2.5 and 3.0 ppm), and by 115% & 325% at higher concentrations (3.5 and 4.0 ppm) of Zn, respectively as compared to positive control. Likewise, for B, the low concentrations (0.1-0.3 ppm) significantly improved shoot and root length by 70% & 200%, medium concentrations (0.4 and 0.5 ppm) enhanced attributes by 118% & 300% and higher ones (0.6 and 0.7 ppm) increased them by 85% and 250%, with respect to positive control, respectively (Figure 2 b and c).

Both biofertilizers (BF1 and BF2) statistically proved equally effective in improving shoot length by 55% and root length by 170% over positive control (Figure 2 b and c).

Effect on seedling biomass

Seedling dry biomass was drastically reduced by 84% in positive control as compared to negative control (3900 mg), while all treatments significantly improved the said attributes to variable extents over positive control. The dry biomass was significantly enhanced by 2-4 folds and 2-3 folds with increase in concentrations (150-2000 ppm) of carbendazim and thiophanate-methyl, respectively over positive control. Micronutrient like Zn improved the said attribute significantly up to 2 folds, 4 folds and 3.5 folds due to effect of low (1.0-2.0 ppm), medium (2.5 and 3.0 ppm) and higher concentrations (3.5 and 4.0 ppm). Low concentrations (0.1 and 0.2 ppm) of B did not improve the investigated attribute significantly, while rest of the concentrations (0.3-0.7 ppm) exhibited a significant improvement of 2 folds. BF1 and BF2 also enhanced the seedling dry weight up to 2 folds, as compared to positive control (Figure 2d).

Effect on vigor index

Lowest vigor index was calculated in positive control, which was 7-times less than negative control. Application of different concentrations of fungicides, micronutrients and biofertilizers variably and significantly improved vigor index by decreasing disease over positive control. Hence, various concentrations showed the lowest and highest peaks of 200-500%, 140-500%, 140-500% and 120-200% for increase in the vigor index due to effect of carbendazim, zinc, thiophanate-methyl and boron, respectively over positive control. BF1 as well as BF2 treated seeds also exhibited significantly greater improvement of 160% in vigor index with respect to positive control (Figure 3).

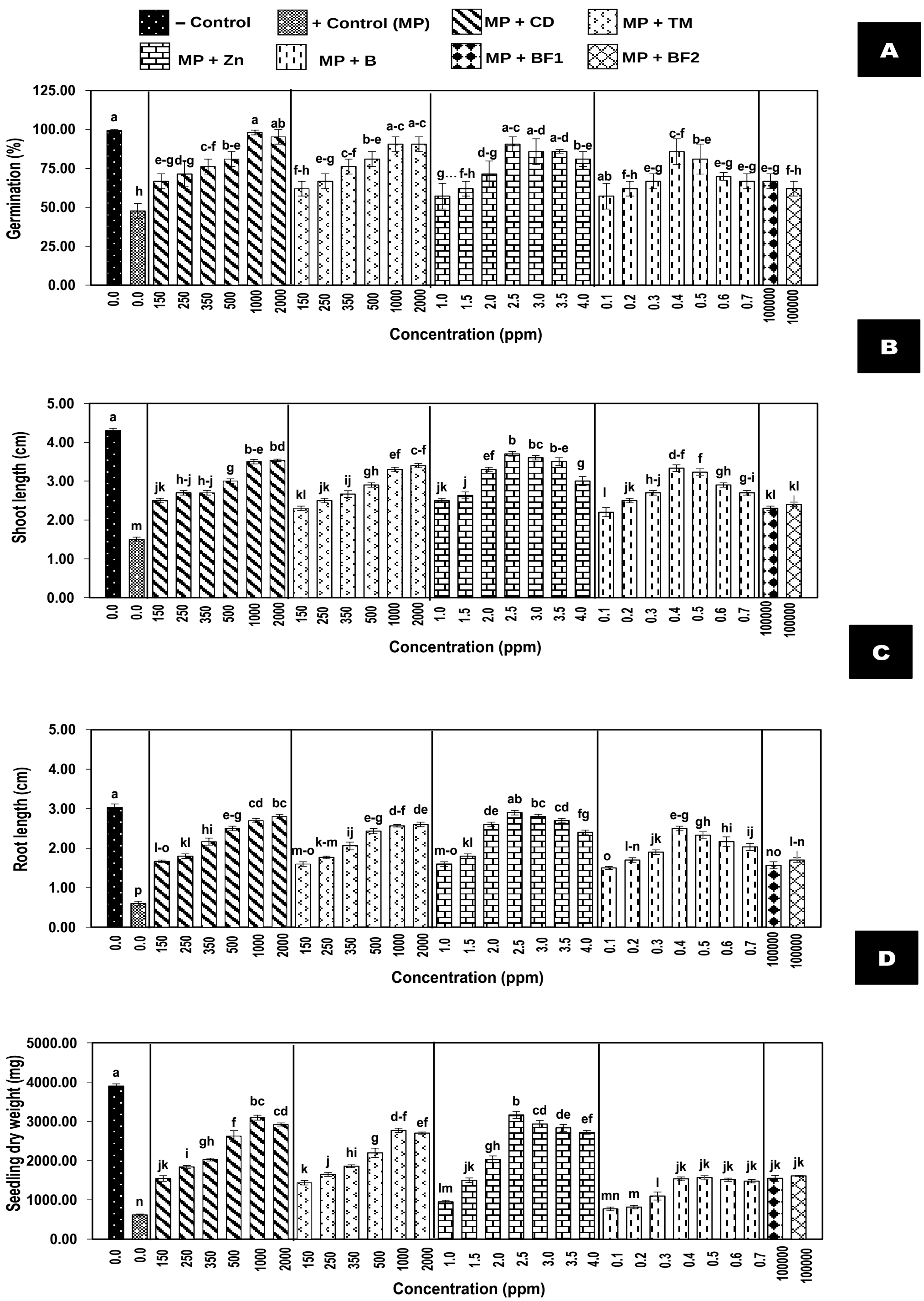

Figure 2 (A-D): Effect of carbendazim (CD), thiophanate-methyl (TM), zinc (Zn), boron (B), and biofertilizers (BF) on germination and seedling growth of sunflower under *Macrophomina phaseolina* (MP) stress. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P ≤ 0.05) as determined by LSD Test.
BF 1: Biopower; **BF 2:** Feng Shou

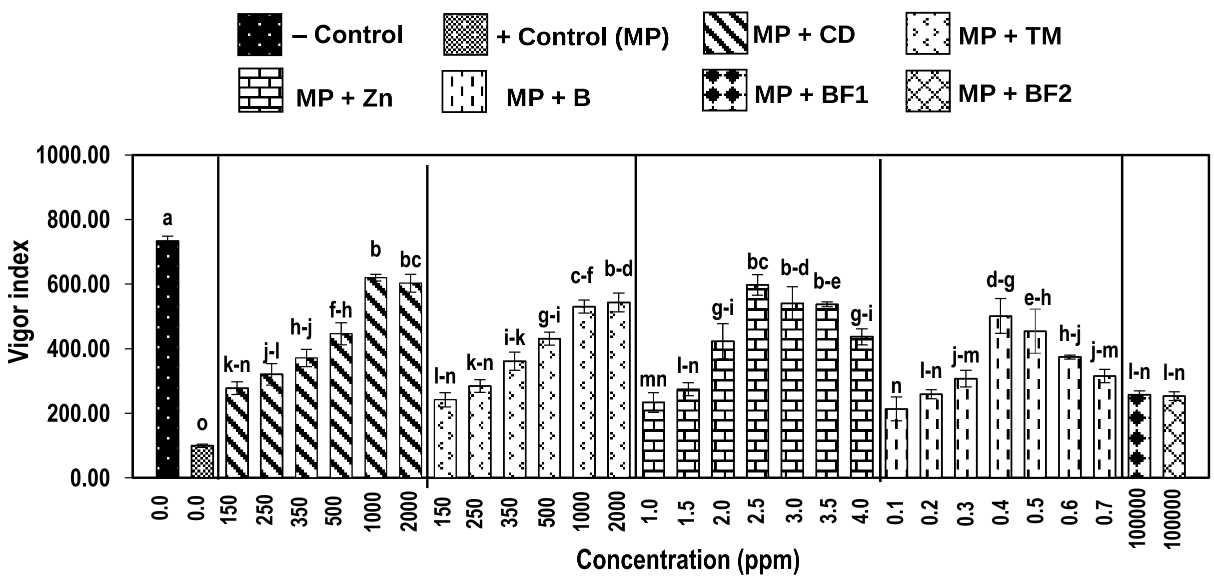

Figure 3: Effect of carbendazim (CD), thiophanate-methyl (TM), zinc (Zn), boron (B), and biofertilizers (BF) on vigor index of sunflower under *Macrophomina phaseolina* (MP) stress. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P ≤ 0.05) as determined by LSD Test.
BF 1: Biopower; **BF 2:** Feng Shou

PCA analysis

The analysis revealed that two components together accounted 96.65% of the variance and discriminated better the treatments according to their potential to decrease disease to improve germination and growth indices in sunflower seedling (Figure 4). All variables showing strong and positive correlation with each other (right side), while these variables were negatively associated with the disease incidence (left side). Furthermore, treatments and their levels on the right side indicated, these were highly effective in improving growth indices and managing disease than the other treatments on opposite side. Moreover, four distinct clusters were identified in biplot. Positive control occupied cluster1. Fungicides, lower doses in the cluster II and higher one in cluster III exhibited strong and positive correlations between two fungicides and their levels. Variables were also positively correlated with clusters III, and this cluster is very close to negative control. It also indicated that 1000 and 2000 ppm of the fungicides are optimum level for managing disease. The cluster VI specified medium (Zn_4: 2.5 ppm) to high (Zn_{5-7}: 3.0, 3.5 and 4 ppm) concentrations of Zn were closely and positively associated with each other and with growth variables. Likewise, in same cluster, B_4 (0.4 ppm) seems to work better in decreasing diseases and improving growth variables followed by B_5 (0.5 ppm), B_6 (0.6 ppm) and B_7 (0.7 ppm). The cluster I also includes low concentrations of Zn ($Zn_{1\&2}$: 1.0 and 1.5 ppm), B ($B_{1\&2}$: 0.1 and 0.2 ppm) and the biofertilizers, which were closely associated and were less effective in managing disease.

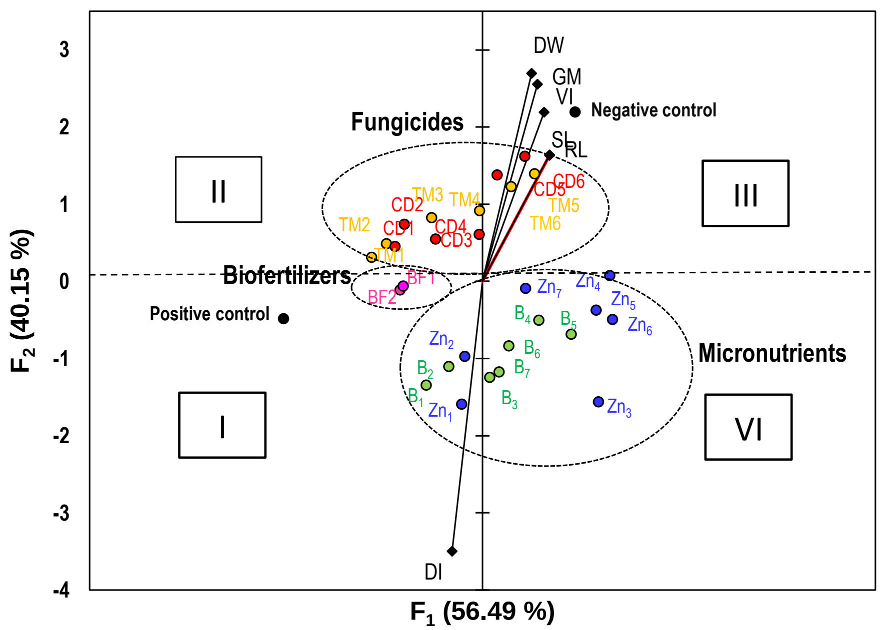

Figure 4: Principle Component Analysis (PCA) on correlation biplot indicating the correlations among studied variables and biplot scores on axis 1 and 2 are representing 98.56%
Variables: disease incidence (DI), germination (GM), shoot length (SL), root length (RL), seedling dry weight (DW) and vigor index (VI). **Treatments:** Carbendazim (CD_{1-6}: 150, 250, 350, 500, 1000 and 2000 ppm), thiophanate-methyl (TM_{1-6}: 150, 250, 350, 500, 1000 and 2000 ppm), zinc (Zn_{1-7}: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 ppm), boron (B_{1-7}: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ppm), and biofertilizers (BF1: Biopower; BF2: Feng Shou).

Discussion

Charcoal rot incited by *M. phaseolina* in sunflower causes huge yield losses, and registered fungicides and resistant varieties are still unavailable. Carbendazim (Shincar) and thiophanate-methyl (Topsin® M) belong to benzimidazole group of systemic fungicides, commonly used against *M. phaseolina* in Pakistan due to their broad-spectrum of antifungal activity with protective and curative action. However, chemical fungicides are a concern for sustainability of environment and global stability. Hence, effectiveness of micronutrients (Zn and B) and biofertilizers (Biopower and Feng shou) were tested and compared with above-mentioned chemical fungicides against the charcoal rot disease in sunflower. Results revealed incidence (81%) of charcoal rot disease significantly reduced (50-90%) germination and growth in sunflower seedling in positive control, while all treatments managed disease and improved growth attributes to variable extents. Moreover, a very clear separation of highly effective treatments was represented by PCA analysis as well.

Effect of both fungicides was highly significant and statistically similar against the disease, hence suppressed 50 to 79% disease with increase in concentration (150-2000 ppm). However, sunflower germination and growth attributes were more significantly improved by 2-5 folds with carbendazim than by 1.5-4.5 folds with thiophanate-methyl. Carbendazim and thiophanate-methyl being benzimidazole group of fungicides arrest fungal growth by inactivating tubulin assembly, and inhibiting nuclear division, which may result in hindrance in different regulatory cellular activities including mitosis, meiosis and cell shape maintenance etc. in fungi (Wang and Zhang, 2018). Benzimidazole fungicides have been used to control tobacco brown spot (*Alternaria alternata*) in China for more than a decade, later the mixture of carbendazim (or thiophanate-methyl) and diethofencarb have been recommended to manage this disease as well (Wang and Zhang, 2018). Reznikov *et al*. (2016) recommended application of carbendazim and thiophanate-methyl as seed treatment to enhance plant emergence and disease control caused by *M. phaseolina*. Iqbal and Mukhtar (2020) also reported reduction (79%) in growth of *M. phaseolina* and enhancement in germination and plant survival of green gram and black gram by application seed with carbendazim (150 ppm). They also found optimum results with carbendazim and benomyl as compared to mancozeb, copper oxychloride, captan, propineb and chlorothalonil.

Both nutrients (Zn and B) significantly managed disease at medium range of concentrations, but Zn had greatest effect. Different concentrations (1.0-4.0 ppm) of Zn improved germination and seedling growth (1.5-5.0 folds) by managing 20-62% of the disease. However, 2.5 ppm of Zn displayed potent antifungal activity particularly comparable to carbendazim (1000 ppm) by reducing 62% disease along with the 5 folds improvement in seedling attributes. In earlier investigations, 2.5 ppm $ZnSO_4$ was also recommended as ideal dose against mung bean charcoal rot disease (Khan *et al*., 2018; Haider *et al*., 2020) and tomato early blight disease (Awan *et al*., 2019). Zn may help to induce resistance in sunflower seedling through activation of defense signaling pathways (Haider *et al*., 2020). Wang *et al*. (2017) documented that Zn-binding protein (RAR1) might activate the salicylic acid mediated oxidative burst in wheat against stripe rust. Moreover, antifungal action of Zn through cell injury and deformed hyphal growth patterns has also been documented against fungal pathogen in coffee pink rot (Arciniegas-Grijalba *et al*., 2017). Over and above, a range of 2.5-4.0 ppm particularly 2.5 ppm of Zn may be beneficial dose for the plant, which might cause direct toxicity in *M. phaseolina* along inducing activation of defense signaling in sunflower (Martos *et al*., 2016). Gallego *et al*. (2017) related high Zn in *Noccaea caerulescens* with incompatible *Alternaria brassicicola* interaction, possibly in cooperation with high glucosinolate concentrations under high Zn (102 µM) supply.

Among seven concentrations of B (0.1-0.7 ppm), a significantly lowest disease incidence (50%), and higher germination rate along with 2-3 folds increase in seedling attributes were recorded at either 0.4 or 0.5 ppm. Remaining concentrations managed 33% disease, and improved seedling growth attributes up to 2 folds. Reduction in disease likely to improve germination and growth in sunflower seedling due to role of B in cell wall synthesis, structural integration, cell division and elongation, nitrogen and carbohydrate metabolism, sugar transport etc. (Shireen *et al*., 2018). Qin *et al*. (2010) also reported that B compounds (e.g. potassium tetraborate) managed gray mold in grapes caused by *Botrytis cinerea* by inducing disruptive effect on cell envelop, resulting in the breakdown of the cell membrane, loss of cytoplasmic materials from the fungal hyphae and cell death. Shi *et al*. (2012) found borate treatment of the plants controlled anthracnose in mango fruit possibly by stimulating ROS accumulation in *Cladospoium gloeosporioide*s spores, which may result in mitochondrial damage and inhibition in spore germination.

Seeds treated with biofertilizers (BF1 or BF2) showed less potential against charcoal rot disease as compared to other treatments, hence 40% of the disease was managed and 2 folds enhancement in growth-related parameters were recorded as compared to positive control. Currently used biofertilizers were in liquid formulations, since they are deprived of solid-carrier protection, so probably did not adhere to the seeds properly and may lose viability shortly after inoculation (Mącik *et al*., 2020), which resulted in their low disease managing potential.

Conclusion

Effect of both fungicides (carbendazim and thiophanate-methyl) was highly significant and statistically similar on the disease. Micronutrients exhibited more inhibitory effect on the disease and stimulatory effect on the growth indices at concentration range of 2.5-3.5 ppm (Zn) and 0.4-0.7 ppm (B). However, the effect of Zn (2.5 ppm) was statistically equal than either of the fungicides and better than B. Biofertilizers (100,000 ppm or 10 %) in general proved less effective in decreasing disease and improving growth attributes.

Conflict of Interest

Authors have no conflict of interest.

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