Deleterious effect of nano, biological and chemical fungicides on mycorrhizal symbiosis with maize (*Zea mays L.)* roots

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Abstract: The injurious impacts of fungicides on mycorrhiza colonization efficiency in plant's growth are notoriously variable. With regard to the crucial roles of Arbuscular Mycorrhizal (AM) symbiosis on nutrient uptake, improvements of grain quality and enhancements of root traits, an experiment was executed in field to investigate the effects of different nano, biological and chemical fungicides (Benomyl, Nano-silver, *Bacillus subtilis*; respectively) on these key functions with four *Glomus* species. Fungicide applications had significant impacts on protein and grain yield and thousand- kernel weight ($P \leq 0.05$) and on root dry weight and phosphorous content ($P \leq 0.01$) whilst the effects of AM inoculation had significant impacts on root colonization ($P \leq 0.01$) and on root dry weight and grain phosphorous content ($P \leq 0.05$) as well. The interaction of fungicide application and AM inoculation revealed significant effects on grain yield and protein and phosphorous content and thousand-kernel weight ($P \leq 0.05$) and on root dry weight ($P \leq 0.01$). Results imparted that applications of bio-fungicide without mycorrhiza inoculation increased grain yield. Applications of nano-silver fungicide without inoculations of mycorrhiza altered grain phosphorous content and grain protein content . Also applications of Benomyl fungicide and inoculation with *G.intraradices* and *G.etunicatum* increased root colonization. Nano silver fungicide had a favourable impact on the phosphorous and protein content of the grain but the benefit of this application was declined when accompanied with Mycorrhizal inoculations.

Keywords Nano fungicide, Biological fungicide, Chemical fungicide, Mycorrhizal symbiosis,

Zea mays.

1. Introduction

Vesicular–arbuscular Mycorrhiza (VAM) fungi has been well documented to be a bio-fertilizers that have a symbiotic relationship with many crops and by increasing the uptake of nutrients mainly phosphorus, and enhances the water absorption and resistance to pathogens, improves growth and yield of host plants in sustainable agricultural systems (Sainz et al. 1998; Sajedi et al. 2010; Sarajuoghi et al. 2011). Application of fungicides is a common practice in intensive agriculture, some of these fungicides affect different stages of the development and function of Arbuscular Mycorrhizas adversely, but some of fungicides has not demonstrated such harmful impacts on these fungi (Schweiger et al. 2001).The researchers have found that the effects of foliar-applied systemic fungicides on different *Glomus* species ranged from inhibitory, through less inhibitory to neutral or slightly stimulatory (Dodd and Jeffries 1989; Kough et al. 1987). In the meanwhile it's been expressed that interactions of AM fungi and fungicides were highly variable and biological responses depend on fungus-fungicide combination and environmental conditions (Schreiner and Bethlenfalvay 1997). According to Benomyl and Benlate, a propriety form of the fungicide, found to have deleterious effects on vesicular-arbuscular (VA) mycorrhizas; delaying, diminishing or preventing the formation of symbioses between fungi and roots and disrupting their ability of phosphate translocation (Carr and Hinkley 1985). Results emerged from a case study of applications of PCNB on oats (*Avena sativa*) imparted that high levels of PCBN led to decline of VAM symbiosis and root growth (Gnekow and Marschner 1989). On a field-based experiment it has been illustrated that monthly applications of Benomyl has reduced root colonization by AM fungi throughout a 2 year experiment. Benomyl had shown to have no effective impact on the availability of soil phosphorous, but reduced P concentration in all parts of the plant and correspondingly measuring grain phosphorous content tends to be a powerful tool for defining efficiency because it indicates the pathways that transfer phosphorous in soil as in presence of mycorrhiza and fungi hyphae (Merry-Weather and Fitter 1996). According to the results of some experiments, applications of a chemical fungicide known as Vitavax had the most unfavorable effect on the growth of Maize, grain yield and grain phosphorous content (Samarbakhsh et al. 2009). The arising results from an experiment conducted on a plant genus manifested that implementation of 11 mg of silver nano-particle caused 22% of reduction in microbial population over non-implemented treatments. Although in previous studies under lab condition, no harmful effects found when treatments with 1.250 mg of Nano-silver screened (Colman 2000). It is reported that Nano-silver has decontaminated the *invitro* media of some explants while it has no poisonous effect on human and environment, despite the evidences that testified submerging explants in high concentrations of nano-silver caused grand damages

to explants (Rostami and Shahsavar 2009). Mycorrhiza fungi interact with a large number of other soil microorganisms and in some cases these interactions have resulted in reduced mycorrhiza phosphorous uptake (Schweiger et al. 2001). *Bacillus* can have a regulating impact on interactive relation between plant and other rhizosphere microorganisms e.g. mycorrhiza, through alternation in rate and variable root exudates, hence change plant metabolism and leave a significant impact on growth and production (Schonwitz and Ziegler 1988). In an experiment, the influence of *Bacillus subtilis* JA on arbuscular mycorrhiza fungi was evaluated in pot culture and results indicated that inoculation could significantly decrease the frequency of maize colonization by indigenous Arbuscular Mycorrhiza fungi (Xiao et al. 2008).

The aim of the present study was to determine the best fungicide with least unfavorable impacts on colonization efficiency with the intention of improvement and maintenance in maize grain yield and quality.

2. Materials and methods

2.1. Experimental procedure: The experiment was a factorial on the basis of Randomized Complete Block Design including 2 factors and 4 replicates and was conducted in spring of 2010, at the research field of Islamic Azad University , Karaj Branch, Iran. Four fungicide treatments including control (F0), Benomyl (F1), Nano-silver (F2), Bacillus (F3) and four AM treatments including control (M0), *Glomus mosseae* (M1), *G.etunicatum* (M2), *G.intraradices* (M3) were screened. Seeds were treated with the fungicides concentrations of 1 in 1000 in the form of wettable for benomyl, 2 in 1000 and 3% per liter for Biological fungicide containing *Bacillus subtilis* of commercial trademark Biosubtyl, silver nano-particles with concentration of 60 mg per kilogram in the form of colloid L2000 for Nano-silver fungicide. Hence, 16 experimental treatments were tested in each replicate making the total of 64 plots. Mycorrhizal fungi species used in the study isolated from dry lands of wheat in Iran and after initial recognition by Soil and Water Research Institute of Iran, then the inoculum used to inoculate sorghum plants as a host plant to produce mycorrhizal inoculum in a four-month period under greenhouse conditions using sterilized sand in Soil and Water Research Institute of Iran. Seeds were sowed in the rows with 60 cm distance between rows and 20 cm between plants and inoculated with 4 g of AM inoculums (a mixture of plant roots and sand and spore and hyphae) with 150-200 active propagules. Before field preparations, soil samples were taken in depth of 0-30 cm in order to determine physical and chemical characteristics of soil. Based on the results of soil analysis, soil texture was loam-sand with pH=7.85. The soil was fertilized according to soil analysis at 120 kg per hectare urea at two different stages and 100 kg per hectare of phosphate from Triple Super Phosphate (half of the recommended amount) and 250 kg per hectare potassium phosphate. Harvest performed in October from 2 lines in the middle of each plot with 50 cm removal from margins of the planting area of 6 Square meters to measure the grain yield and yield components (Ear and stem diameter, plant height, number of kernels per cob, kernel 1000 weight and ear length).

Grain protein percentage was measured with NMR (Nuclear magnetic resonance) method using Inframatic apparatus then grain protein yield was calculated per kilogram per hectare. Grain phosphorous content was measured via molybdenum Yellow color calorimetric method. Maize Samples were taken in order to estimate root colonization percentage at flowering stage. Roots stained (Philips and Hayman 1970) and then root colonization percentage was calculated according to Gridline Intersect Method (Giovannetti and Mosse 1980) . In order to estimate root dry weight three plants were chosen randomly then roots were separated, washed and dried in 70 degree for 48 hour in oven. Data were subjected to analysis of variance using SAS software. Means were separated using Duncan's multiple range tests.

3. Results

Main effects of Mycorrhizal inoculations and fungicide applications and their interactive effects were significant on root dry weight, grain phosphorous content and fully developed kernel number per cob while no significances observed in plants height, stem and ear diameter and ear length. The main effect of Mycorrihzal inoculations

revealed the only significant impact on root colonization (Table 1). The effects of fungicide applications and interactive effects of Mycorrhizal inoculations and fungicide applications found to be significant on yield, protein content and thousand-kernel weight. Results regarding the treatments inoculated with mycorrhiza with no fungicide applications imparted no significant effects on protein yield and content as well as on the kernel number per cob (Fig. 1, 2 and 8). Inoculations with *G.intraradices* marked the highest value for thousand-kernel weight (Fig. 7) while the most inferior value for phosphorous content resulted in treatments inoculated with *G.etunicatum* (Fig. 4). Mycorrhizal inoculations with different species exhibited a decreasing trend of root dry weight in comparison with (F_0M_0) and this reduction by *G.intraradices* (Fig. 6) measured as (76.01%). Different species of Mycorrhiza demonstrated distinct susceptibilities to fungicide applications. Inoculations with different species of mycorrhiza with the applications of Benomyl exhibited improvements in grain yield and the superior value obtained in plants inoculated by *G.mosseae*. Protein and phosphorous content of grains were decreased in treatments inoculated with *G.etunicatum* in comparison with (F1M0) as (4.69%) and (37.93%) respectively (Fig. 2 and 4) in which, results for reductions in protein content are in agreement with results by Samarbakhsh et al, 2009. Mycorrhizal inoculations with *G.etunicatum* and *G.intraradices* and applications of Benomyl led to enhancements of root dry weight as (35.89%) and (105.12%) in comparison with (F1M0) respectively (Fig. 6). Treatments inoculated with *G.mosseae* and *G.intraradices* demonstrated reductions in number of kernel per cop (13.02%) and (21.89%) respectively while it acted to increase the thousand-kernel weight(Fig. 8 and 7) in comparison with (F1M0).

The highest reduction (Fig. 1) in grain yield (37.60%), the highest enhancement of protein content (Fig. 2) and the highest enhancement of grain phosphorous content (Fig. 4), all (F0M0) were designated in treatments with nano-silver fungicide applications without Mycorrhizal inoculations. But inoculations with different Mycorrhiza species led to reductions in protein content (Fig. 2), grain phosphorous content (Fig. 4), number of kernels in cop (Fig. 8) and thousand-kernel weight (Fig. 7). The highest reduction in phosphorous content, protein content and thousand kernels weight observed in *G.intraradices* and *G.etunicatum* inoculated plants over (F2M0) whilst the highest reduction in the number of kernels per cop was recorded in plants inoculated with *G.mosseae* in relation to (F2M0). In treatments inoculated with *G.intraradices* when the nano-silver fungicide applied, the highest enhancement of root dry weight (63.93%) observed vis-à-vis (F0M0). As a biological fungicide, *B.subtilis* was applied and in treatments without Mycorrhizal inoculations, the highest reduction (125.92%) in root dry weight (Fig. 6) and the highest enhancement (48.12%) of grain yield (Fig. 1) were obtained over control (F0M0). Meanwhile, in relation to (F0M0) it showed descents (8.03%) in grain protein content (Fig. 2) and ascents (11.23%) in number of kernels per cob (Fig. 8). In treatments, separately inoculated with different Mycorrhiza species and applications of *B.subtilis* fungicide, *G.mosseae* and *G.etunicatum* acted to increase the grain protein content (9% and 10.35%) and decreased grain yield (45.66% and 51.04) in comparison with (F3M0) respectively (Fig. 1). In the meanwhile, inoculations with *G.intraradices* and applications of *B.subtilis* resulted in the highest increase in number of kernels per cop (Figure8) and the highest reductions in thousand-kernels weight (Figure7) and grain protein content (Fig. 2) in relation to (F0M0). In comparison with (F3M0) separate inoculations with *G.mosseae*, *G.etunicatum* and *G.intraradices* led to an extensive enhancement (96.29%, 122.22% and 103.7) of root dry weight (Fig. 6). Applications of different fungicides unaccompanied with Mycorrhizal inoculations, in regard of grain protein yield, demonstrated increases with Benomyl and *B.subtilis* applications (7.17% and 12.29%) and exhibited reduction (16.1%) when nano-silver fungicide was applied (Fig. 3). Therefore it can be expressed that the reductive impact of nano-silver fungicide on grain protein yield might be due to its deleterious effects on grain yield. Inoculations with *G.etunicatum* and *G.intraradices* caused the expecting enhancement of root colonization percentage (5.59% and 8.98%) while inoculations with *G.mosseae* surprisingly decreased (22.43%) the parameter in question in comparison with non-Mycorrhizal treatments (Fig. 5).

4. Discussion

The promising role of fungicides as a pledge of germination and the Mycorrhizal symbiotic relations act to enhance the yield under such circumstances. The formation and function of Mycorrhizal relationships are affected by edaphic conditions such as soil composition, moisture, temperature, pH, cation exchange capacity, and also by anthropogenic stressors including soil compaction, metals and pesticides (Entry et al 2002). Results from this experiment implied that fungicides caused different impacts on different mycorrhiza species. The variable reports regarding the effects of fungicides has been alleged to be due to the different environmental conditions of experiments (Schreiner and Bethlenfalvay 1997). The effects of fungicide applications would be influenced by different sites and modes of action, edaphic characteristics, rainfall and the time of application (Dodd and Jeffries 1989). Enhancements of grain yield in treatments with Benomyl fungicide and inoculations of *G.mosseae* are in accordance with results of

(Samarbakhsh et al. 2009). It seems that as a community, AM fungi modified and alleviated fungicide stress, resulting in high levels of plant performance and soil aggregation (Schreiner and Bethlenfalvay 1997). Based on the results derived from this experiment, seed inoculations with mycorrhiza decreased the unfavorable effects of Benomyl and nano silver on maize yield significantly. pH and Cation Exchange Capacity may be used as indicators clarifying the rate of deactivated Benomyl through absorption on the soil's surface (Carr and Hinkley 1985). In our experiment, noting the pH of soil, Cation Exchange capacity and applications of Benomyl with the concentration of 1 in thousand, may be the causes of promoting the Mycorrhization especially in *G.mosseae* while no negative effects observed on the symbiosis of Mycorrhiza with maize which might be the reason for improving the grain yield because of the increased thousand kernel weight. Fungicides affected the nutrient quality of grains via influencing the symbiotic relation of mycorrhiza with maize. External hyphae could be expected to be more sensitive to the direct effects of fungicides than internal hyphae as the root surface may protect the latter (Kgoller and Rosendahl 2000). Polyphosphates- containing vesicles appear to be the main vehicles for phosphorous transport from external mycelium of VA Mycorrhizal fungus to the host plant. Interference with vesicle transport due to application of Benomyl might be the reason for the damages concerning the phosphorous flow from soil to the root of host plant (Hale and Sanders 1982). The break down products of Benomyl, methyl-2-benzimidazole carbamate (MBC) and butyl isothiocyanate (BIC) have different modes of action. The latter inhibits growth by inhibiting respiration but its effects seem to be transient. MBC is known to disrupt mitosis by interfering with spindle formation and is more lethal in its effects, causing gross distortion of germ tubes, hyphae and nuclei and such effects may act to decrease the root dry weight in comparison with control which these findings are in accordance with the results of (Dodd and Jeffries 1989; Carr and Hinkley 1985; Kgoller and Rosendahl 2000; Menge 1982; Ramakrishna 2001). Results imparted the negative impact of nano silver fungicide on grain yield and this impact caused a harmful effect on the protein yield of grains but by inoculations with different Mycorrhiza species no significant effects observed in grain yield compared to treatments inoculated with Mycorrhiza without the application of nano silver fungicide. Microorganisms such as fungi, algae and bacteria are known to absorb silver ions and highly bioactive silver ions bind with proteins inside and outside bacterial cell membranes, thus inhibiting cell respiration and reproduction. But this destructive effect was not observed in our experiment. Probably VAM colonized the host's root and by producing a mycelium mass which can act most effectively as a protective barrier against the destructive effect. Mycorrhizal fungi interact with a large number of other soil microorganisms and in some cases these interactions have resulted in reduced Mycorrhizal phosphorous uptake (Hetrick 1988). Results of our experiment revealed that inoculations with *G.intraradices* and applications of *B.sublitis* led to the highest kernel number per cob. One of the possible reasons that have been expressed is that phyto-hormones may participate in interactions between roots and bacteria (Schonwitz and Ziegler 1988). *B.subtilis* had been identified to produce Hormone-like substances (Vivas et al. 2003). Results derived from an experiment indicated that organs of Mycorrhizal plants are capable of producing high levels of hormones hence, substantially affecting the reproduction physiology of the host plants (Daft and Okusanya 1973). It's been declared that lipopeptides or unknown volatile produced by *B.sublitis* might act as one of the early inhibitory factors and may damage the fungal hyphae destined to absorb nutrients in general and particularly phosphorous (Xiao et al. 2008). In the other hand, *B.sublitis* produces compounds with antibiotic activities and even at low levels this might cause damages to roots and can result in the leakage of materials. In this experiment, regarding the alterations of plant metabolism induced by *B.sublitis* and root exudates, modifications might have occurred in the microflora of rhizosphere. This might be the reason for alleviated effect of *B.sublitis* when interacted with Mycorrhiza as it's been well documented that in grain filling stage of cereals the flow of carbohydrates towards roots ought to be reduced (Hapkins and Huner 2004). It should borne in mind that the positive effect of bacteria on Mycorrhiza leads to additional carbon costs for plants and as a result root dry weight was increased while thousand kernel weight was decreased. The fungus might increase surface area indirectly by stimulating root growth and increase the surface area through hyphal growth (Safir et al. 1971). It has also been reported that Mycorrhizae species even isolates from a specific species are highly variable regarding the rate of infecting, symbiotic extent and degrees of impaction on host plant and this differences are functions of the relationships between host plant and fungus and also soil characteristics (Dodd and Jeffries 1989).In concern of lower levels of infections by *G.mosseae* it can be argued that the barriers to infection are intrinsic and that outside

the roots physical factors such as root hair density or structure of the epidermis, perhaps, may be more inhibitory than chemical factors such as root exudates or seed-coat components (Ocampo 1980).

5. Conclusion

Along with progressing toward sustainable agriculture, applications of biological and nano fungicides to avert plants from pathogens has now gained the conspicuous importance which lies in the fact that they can substitute chemical fungicides and alleviate the harmful impacts on humans and environment. Responses of the different studied Mycorrhizal inoculations to the applications of distinctive fungicides, indicates the distinguishable patterns of behaviors and implies the possibility to determine and obtain the favorable environmentally efficient combinations of inoculations and fungicides. Deleterious impacts of nano fungicide observed on quantified yield of maize. While, applications of *B.subtillis* without Mycorrhizal inoculations revealed significant effects on growth enhancement and quantified yield through adjustments in plants metabolism, although it caused negative impacts on root dry weight. In the meanwhile, applications of *B.subtillis* and *G.intraradices* at the same time imparted more negative effects on grain nutrient content in comparison with the other two fungicides. Nano silver fungicide had a favourable impact on the phosphorous and protein content of the grain but the benefit of this application was declined when accompanied with Mycorrhizal inoculations.

Table 1 Analysis of variance for mycorrhiza fungi and fungicides on traits

MS

SOV	df	Grain yield	Grain protein yield	Grain protein	Grain phosphorus	Root colonization	Root dry weight	Kernel 1000 weight	Number of grain per ear
block	3	ns	ns	**	ns	ns	**	**	**
Fungicide(F)	3	*	*	ns	**	ns	**	*	**
Mycorrhiza (M)	3	ns	ns	ns	*	**	*	ns	*
×Fungicide Mycorrhiza(M×F)	9	*	ns	*	*	ns	**	*	*
Error	45	1830415.9	10237.50	0.11	0.00	36.79	563.56	270.86	3942.94
(CV)	-	23.66	22.66	4.33	18.34	19.99	34.72	5.30	10.32

ns not significant ; ** ≤ 5% ; ** ≤ 1%

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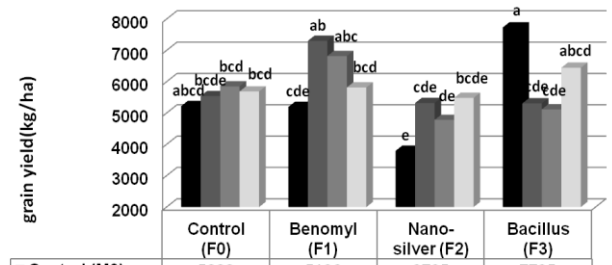
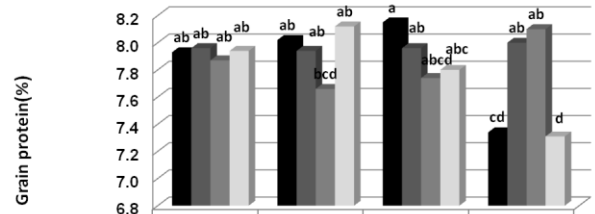


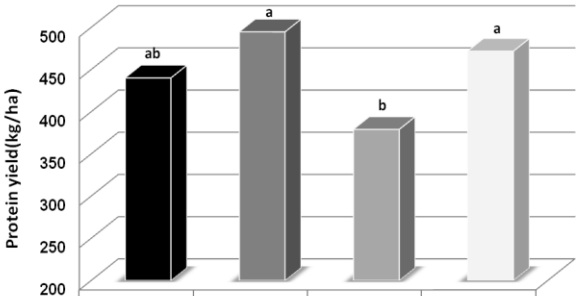
	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
Control (M0)	5222	5196	3795	7735
Mossea (M1)	5544	7306	5322	5310
Etunicatum (M2)	5854	6821	4783	5121
Intraradices (M3)	5692	5819	5486	6448

Fig 1. Mean comparison of the interactive effects fungicide and mycorrhizae on grain yield



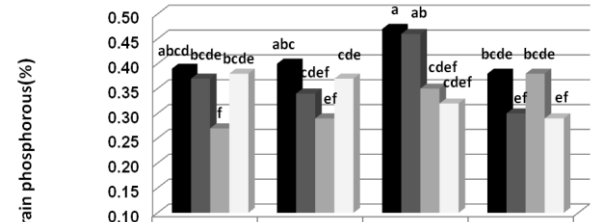
	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
	7.93	8.02	8.15	7.34
Mossea (M1)	7.96	7.94	7.96	8.00
Etunicatum (M2)	7.87	7.66	7.74	8.10
Intraradices (M3)	7.94	8.12	7.80	7.31

Fig 2. Mean comparison of the interactive effects fungicide and mycorrhizae on grain protein



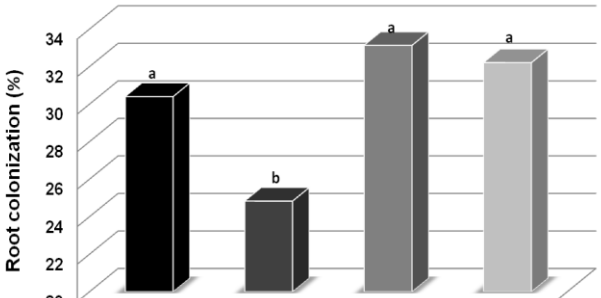
	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
Mean	440.22	494.78	379.16	471.81

Fig 3. Mean comparisons of the main effect of fungicide on protein yield



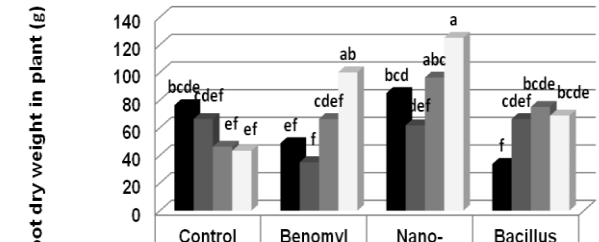
	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
Control (M0)	0.39	0.40	0.47	0.38
Mossea (M1)	0.37	0.34	0.46	0.30
Etunicatum (M2)	0.27	0.29	0.35	0.38
Intraradices (M3)	0.38	0.37	0.32	0.29

Fig 4. Mean comparison of the interactive effects of fungicide and mycorrhizae on grain phosphorous



	Control (M0)	Mossea (M1)	Etunicatum (M2)	Intraradices (M3)
Mean	30.40	24.83	33.13	32.21

Fig 5. Mean comparisons of the main effect of mycorrhizae on root colonization



	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
Control (M0)	76.25	48.75	85.00	33.75
Mossea (M1)	66.25	35.00	61.66	66.25
Etunicatum (M2)	46.25	66.25	96.25	75.00
Intraradices (M3)	43.32	100.00	125.00	68.75

Fig 6. Mean comparison of the interactive effects of fungicide and mycorrhizae on root dry weight in plant

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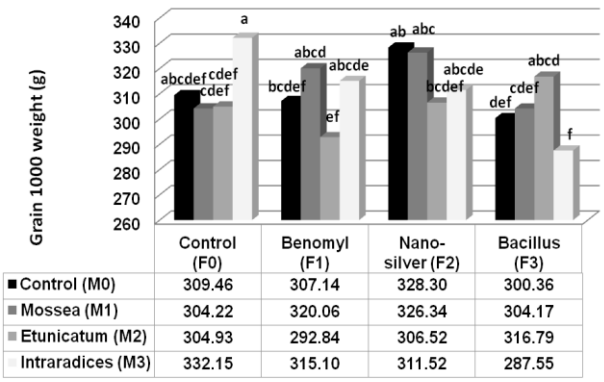
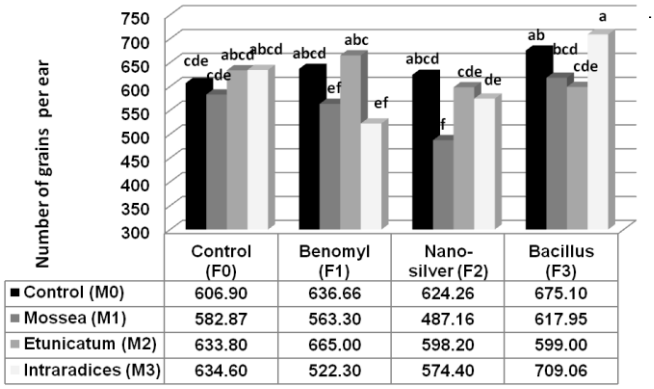


	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
Control (M0)	309.46	307.14	328.30	300.36
Mossea (M1)	304.22	320.06	326.34	304.17
Etunicatum (M2)	304.93	292.84	306.52	316.79
Intraradices (M3)	332.15	315.10	311.52	287.55

Fig 7. Mean comparison of interactive effects of fungicide and mycorrhizae on grain 1000 weight



	Control (F0)	Benomyl (F1)	Nano-silver (F2)	Bacillus (F3)
Control (M0)	606.90	636.66	624.26	675.10
Mossea (M1)	582.87	563.30	487.16	617.95
Etunicatum (M2)	633.80	665.00	598.20	599.00
Intraradices (M3)	634.60	522.30	574.40	709.06

Fig 8. Mean comparison of interactive effects of fungicide and mycorrhizae on number of grains per ear

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