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INVESTIGATING IRON OXIDE NANOPARTICLES AND *Piriformospora indica* ROLES IN MITIGATING THE HARMFUL EFFECTS OF DROUGHT STRESS IN SOYBEAN: ANTIOXIDANT ENZYMES AND OIL CONTENT

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ABSTRACT

Drought stress is one of the most important environmental stresses that affects the quantity and quality of soybean production. This study was conducted to explore the role of iron oxide nanoparticles (NPs) application and *Piriformospora indica* inoculation in alleviating the adversities of drought stress on fatty acid composition and oil production in soybean. The results showed that seed oil synthesis was considerably reduced by drought stress, whereas the membrane lipid peroxidation was accelerated under drought stress condition. The applied NPs (especially 0.75g L^{-1}) and *P. indica* enhanced the activities of enzymatic antioxidants (SOD, CAT, PPO and APX) with simultaneous decrease in malondialdehyde. Under drought stress, an inverse relationship between protein and seed oil was observed, as by increasing protein content, seed oil decreased linearly. In addition, the main oil constituents, oleic and stearic acids increased, while linoleic, linolenic and palmitic acids reached to the lowest level in severe drought stress (FC 20%). Interestingly, the beneficial effects of NPs and *P. indica* led to improvement in grain weight, fatty acid composition and oil content under drought stress. During drought stress, oleic, linoleic, linolenic and palmitic acids increased and stearic acid decreased considerably in *P. indica* and NPs treatments. However, combined NPs and *P. indica* was generally more effective in alleviation of drought stress deleterious effects than individual treatments.

Keywords: iron oxide nanoparticles, *Piriformospora indica*, drought stress, oil content, fatty acid composition.

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most economically valuable crops, and a source of oil (23%) and high-quality protein (42%), which its demand has grown dramatically in recent decades. It is widely used as food and pharmaceutical industries, raw material in animal feed industry, biodiesel production, as well as the production of biodegradable materials as an alternative to plastics (Song et al., 2011). Soybean has unsaturated fatty acid like oleic acid which plays an important role in antifungal, antimicrobial and cancer prevention activities as well as cholesterol lowering activity. It is composed of five fatty acids: approximately 16% saturated fatty acids (palmitic and stearic), 24% monounsaturated fatty acids (oleic), and 60% polyunsaturated fatty acids (linoleic and linolenic) (Primomo et al., 2002). Nowadays, the sustainability of soybean yields is negatively affected by climate change in many parts of the world (Bilal et al., 2020). Drought stress is one of the most devastating environmental stresses causes physiological and biochemical alterations which lead to the reduction of oil biosynthesis and crop productivity worldwide. The use of rhizosphere microorganisms is an important environmental strategy to mitigate damages to physiological processes in plants under drought stress. *Piriformospora indica* is one of the most important microorganisms that form a symbiosis with the roots of many plant species and provide beneficial effects for plants under various environmental stresses. It is used as a growth and yield promoter, biofertilizer, bioregulator as well as a bioprotector. *P. indica* inoculation increased growth and yield in soybean under normal and stressful growth conditions by improving the nodulation and stimulating root growth

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(Mansotra et al., 2015). It also significantly increased human health-promoting compounds in various plants by improving the concentrations of various compounds such as menthol, menthone and 1, 8-cineole in peppermint (Khalvandi et al., 2019). Calcareous soils of arid and semi-arid regions decrease the solubility of micronutrients, which leads to a decrease in micronutrients absorption by plants. Application of novel technologies such as nano-fertilizers can be an effective step towards achieving sustainable and environmentally friendly agriculture (Kalia and Kaur, 2019). Synthetic iron oxide nanoparticles are currently utilized to raise the obtainability of Fe in plants and to maintain its necessary amount (Janmohammadi et al., 2018). Iron plays important roles in many physiological processes including lignin production, metabolism of sulfur substances, nitrogen fixation, oxidation and reduction in energy exchange cycles, respiration, chlorophyll biosynthesis, and activation of enzymes which are involved in photosynthetic and mitochondrial electron transfer (Qureshi et al., 2010). It has been reported that the nano-Fe has a positive effect on components of safflower (Janmohammadi et al., 2018) and protein content in *Catharanthus Roseus* (Askary et al., 2017).

There is a little information about the application of iron oxide nanoparticles besides the *P. indica* fungi symbiosis on the performance of soybean plants, especially under drought stress condition. We comprehensively investigated the alleviative effects of iron oxide nanoparticles and *P. indica* on soybean plants and its seed oil compounds under drought stress.

MATERIAL AND METHODS

A field experiment was conducted as a factorial based on a randomized complete block design with three replications in 2017 and 2018. Treatments included inoculated fungi and non-inoculated control, three irrigation regimes (irrigation after 20%, 50% and 70% soil moisture depletion) and three levels of iron oxide Nano particles (0, 0.5 and 0.75 g L^{-1}). Soybean seeds were inoculated by dipping in fungal suspension for 3 hours. Then the seeds were sown in plots by dimensions of 5×3.5 m^2. NPs foliar spraying was performed on the six-leaf-stage of the plants, and drought stress was applied before flowering and continued until physiological maturity. NPs were obtained from Pishgaman Mashhad Company. The mixture of materials and distilled water was placed in an ultrasonic device for half an hour (ultrasonic disperses the nanomaterials). Then, in order to dissolve the nanoparticles and to prevent the nanomaterials from sticking together, a magnet was inserted into the shaker for 4 hours. The leaves samples were homogenized according the method of (Coban and Göktürk Baydar, 2016). The superoxide dismutase (SOD) activity was measured according to the method described by Khalvandi et al. (2019). Absorbance was recorded at 560 nm using a spectrophotometer. Catalase (CAT) activity was measured by the method of Alici and Arabaci (2016), and APX activity was defined as the consumption of ascorbate per minute, according to the method described by Nakano and Asada (1981). Polyphenol oxidase (PPO) was measured according to Chance and Maehly, 1955. Grain from each replicate were analyzed for oil, fatty acids, and protein, the oil from soybean seeds (about 10 g) was extracted using petroleum ether at a solvent (v/w plant material) for 6 h according to AOCS Official Method. The fatty acid composition of the oil samples was determined by gas chromatography (Agilent Technologies, United States) equipped with a split injector (split ratio 1:40) (nitrogen was used as the carrier gas) and a flame ionization detector (FID) (Ghassemi-Golezani and Farhangi-Abriz, 2018). Lipid peroxidation was estimated by measuring leaf malondialdehyde (MDA) content as described by Heath and Packer (1968). Data were analyzed using SAS (9.2) statistical program and means were compared using an LSD (Least Significant Difference) test ($P<0.05$). Person’s correlation analysis was used to evaluate correlation coefficients among the measured traits, also principal component analysis (PCA) were analyzed by XLSTAT software.

RESULTS AND DISCUSSION

In microscopic inspection, the chlamydospores and hyphae of *P. indica* were observed in the root cortex, as an extensive network of hyphae was formed around the roots (Figure 1).

Figure 2 showed the interaction between drought stress × NPs × *P. indica.* Our results demonstrated that antioxidant enzymes activity significantly affected by drought stress, NPs and *P. indica*. Drought stress significantly increased SOD, CAT, PPO, and APX activity in soybean plants compared to the control. In the severe drought stress treatment (FC 20%), an increase was observed in SOD, APX, CAT and PPO activity, respectively, compared to the control (Figure 2 a, b, c and Figure 3). As expected, under drought stress condition, the activity of ROS scavenger enzymes in soybean significantly increased by *P. indica* inoculation. The enhancement was much higher at the severe drought stress (FC 20%) (61.39, 45.21, 60.8 and 65.55%, respectively, higher than the control). The same increasing trend was observed in NPs treatments for SOD and CAT and PPO. Under drought stress condition (50% and 20% Fc), the greatest effect of NPs on SOD, CAT and PPO activity was observed at 0.75 g L^{-1} of NPs. Also, Figure 2c shows that application of NPs, especially 0.75 g L^{-1}, increased PPO compared to the control. In addition, the positive effect of fungi on SOD, CAT and PPO activity was greater when these plants were sprayed with different concentrations of NPs. Also, the results indicated that drought stress significantly increased the accumulation of MDA (Figure 2d). The highest amount of MDA was observed in severe drought stress treatment (non-inoculated * non-use of NPs). In normal condition, no significant differences were observed between the control and treatment application of NPs × *P. indica*. Fungi-inoculated and NPs treatments significantly ameliorated the adverse effects of drought stress condition on membrane lipids; however, under moderate and severe drought stresses there was no significant difference between two NPs concentrations (0.5 and 0.75 g L^{-1}). The positive effect of fungus on reducing MDA was much greater when it used in combination with iron oxide nanoparticles. Figure 4 indicated the interaction between year × NPs × drought stress. Seed protein increased when plants were exposed to drought stress (Figure 4a). The increase was even greater when severe drought stress was applied. However, only CAT activity showed a significant difference between 2017 and 2018 (Figure 4b). The main difference was observed in the application of 0.75 g L^{-1} NPs, which resulted in an increase in the amount of catalase enzyme in 2017. The increase of seed protein was not significant compared with the control in 2017, whereas in 2018 application of NPs and *P. indica* increased seed protein content. In both years, no significant difference was observed between 0.5 and 0.75 g L^{-1} of NPs. The application of 0.5 g L^{-1} NPs (Figure 4c) significantly increased MDA in 2018. The findings of this study showed that drought stress significantly decreased oil content (Figure 5), and it reached to the lowest level in severe drought stress in both years, especially 2017. In addition, fungal coexistence remarkably increased oil content in control treatments as well as in drought stress condition, however, in inoculated plants the decrease in oil content was observed only at severe drought stress. In both NPs treated and non-treated plants, oil content was higher in 2017. The highest value was recorded in 0.5 g L^{-1} of NPs. However, there was no significant difference in oil content between fungal treatments and treatments in which inoculated plants were exposed to 0.5 g L^{-1} of NPs. It can be seen that, the positive effect of *P. indica* on oil content in fungal inoculation treatments was greater when inoculated plants were sprayed with 0.75 g L^{-1} of NPs.

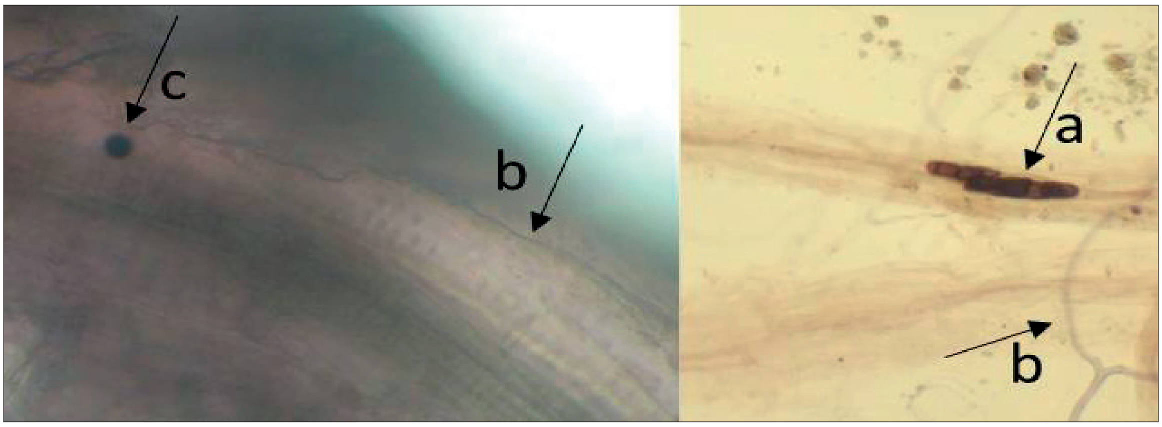

Figure 1. In microscopic analysis, development of *P. indica* [intracellular chlamydospores (a), hyphal growth (b) and spores (c)] was observed in root cells and around the root of soybean

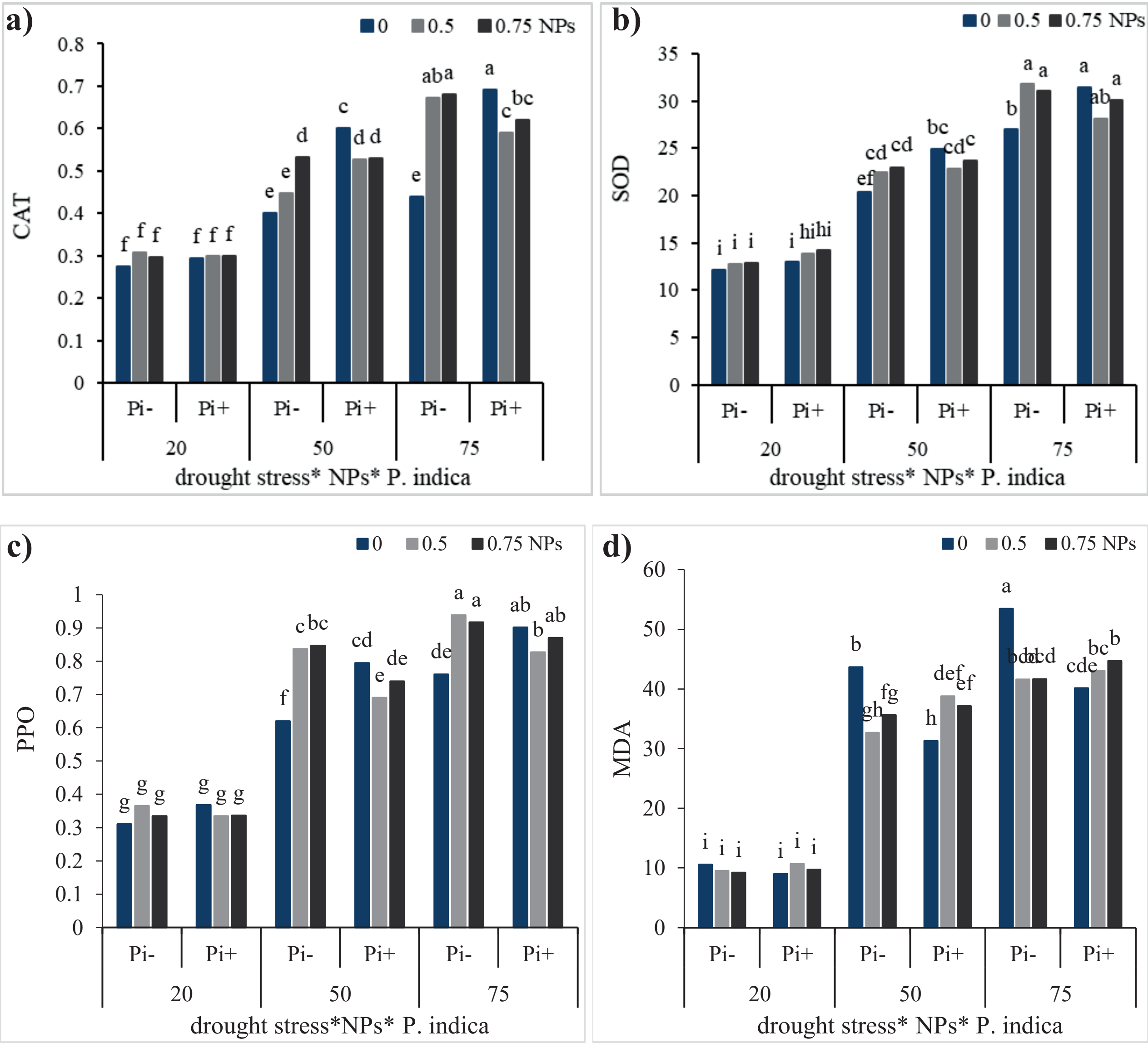

Figure 2. Interaction between drought stress × NPs × *P. indica* on CAT (a), SOD (b), PPO (c) and MDA (d) in soybean. In each figure, means with the same letter are significantly different according to LSD test at P<0.05. Means±S.D from the three experiments.

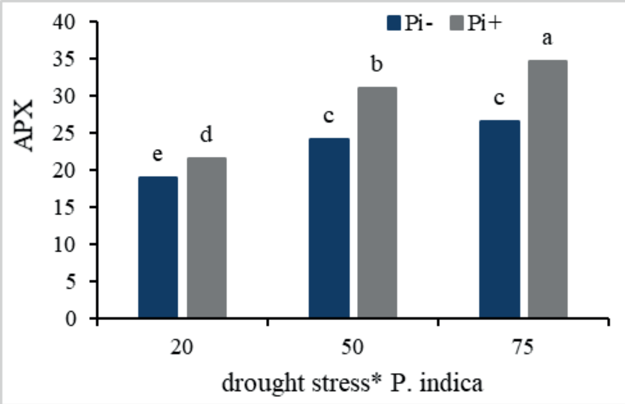

Figure 3. Interaction between drought stress × *P. indica* on APX in soybean. In each figure, means with the same letter are significantly different according to LSD test at P<0.05. Means±S.D from the three experiments.

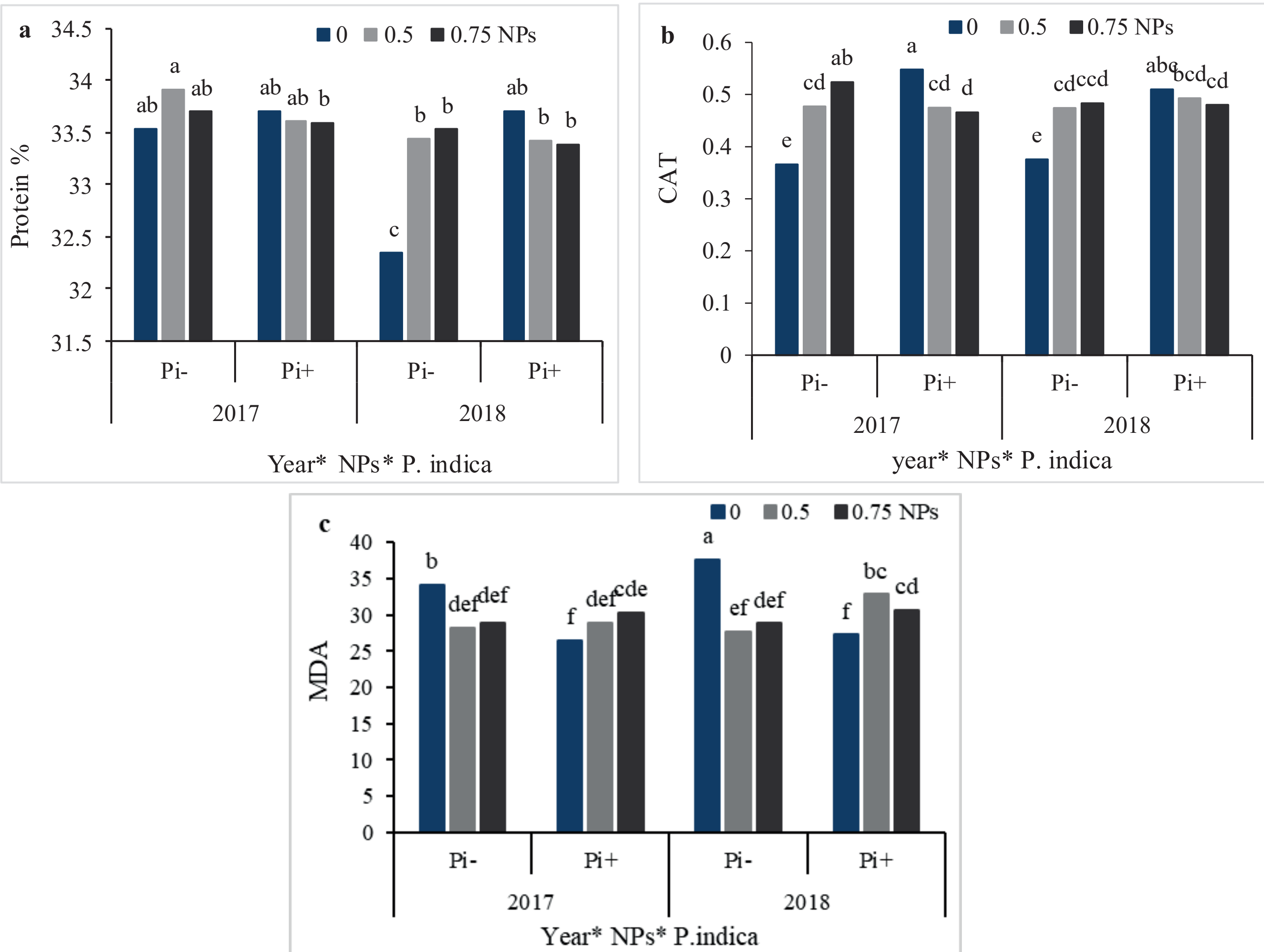

Figure 4. Interaction between year × NPs × drought stress on protein (a), CAT (b) and MDA (c) in soybean. In each figure, means with the same letter are significantly different according to LSD test at P<0.05. Means±S.D from the three experiments.

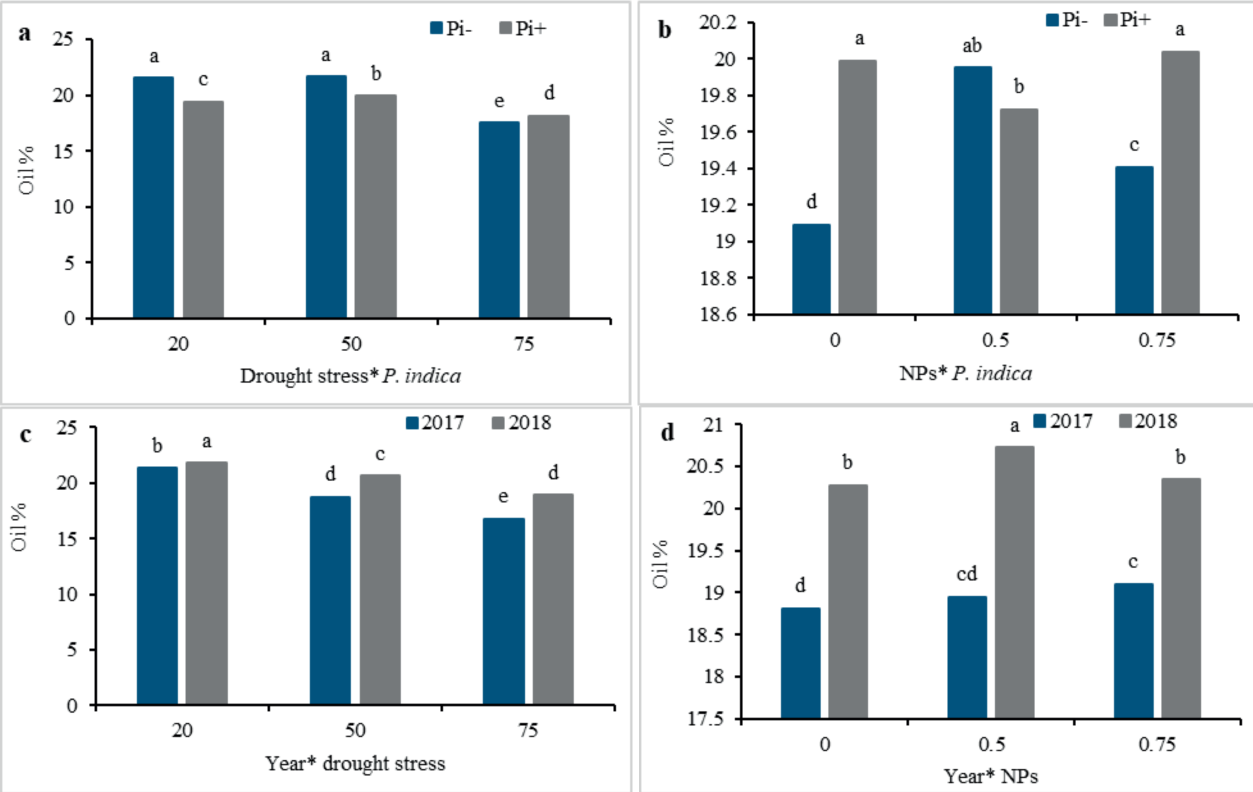

Figure 5. Interaction between *P. indica* × drought stress (a), NPs × *P. indica* (b), Year × drought stress (c), year × NPs (d) on oil content in soybean. In each figure, means with the same letter are significantly different according to LSD test at P<0.05. Means ±S.D from the three experiments.

As shown in Table 1, severe drought stress (FC 20%) caused significant changes in soybean fatty acid composition. Based on the results, in drought stress treatments, plants exhibited a significant increase in oleic and stearic acids, while some fatty acids such as linoleic, linolenic and palmitic acids were decreased. In this regard, our findings illustrated that application of NPs and root fungal symbiosis increased soybean oil quality by increasing the amount of oleic, linoleic, linolenic and palmitic acids and decreasing stearic acid (Table 1). The highest amount of oleic acid was observed in severe drought stress when inoculated plants were exposed to 0.75 g L^{-1} NPs. Under drought stress conditions, moderate stress increased the amount of linoleic acid and linolenic acid, whereas the level of linolenic and linolenic acid reached the lowest level in severe drought stress. The highest amount of linoleic acid and linolenic acid was observed in *P. indica* * 0.5 g L^{-1} NPs treatment in moderate drought conditions.

Drought stress significantly reduced seed yield (35.51% and 51.15%, respectively, compared with control plants) by exposure to medium and severe drought stress. The results show that foliar application of NPs significantly enhanced seed yield. The highest and lowest seed yield was recorded in 0.75g L^{-1} of NPs and control plant, respectively. As shown in Figure 6, seed yield significantly increased by *P. indica* inoculation (44.95% higher, respectively, compared with the control).

Table 1. Effect of *P. indica*, NPs and drought stress on seed fatty acid composition in soybean plants

Drought stress (after depleting %FC)		Oleic	Linoleic	Linolenic	Palmitic	Stearic
20	control	16.44 (±0.132)	50.01 (±0.955)	7.58 (±0.096)	12.05 (±0.019)	4.42 (±0.057)
	0.5	16.57 (±0.277)	50.5 (±0.966)	7.84 (±0.034)	12.09 (±0.053)	4.37 (±0.057)
	0.75	17.30 (±0.646)	49.81 (±0.579)	7.89 (±0.09)	12.12 (±0.043)	4.40 (±0.046)
	Pi	16.90 (±0.063)	50.24 (±0.709)	7.92 (±0.086)	12.02 (±0.054)	4.35 (±0.028)
	Pi + 0.5 g L^{-1} NPs	17.46 (±0.277)	50.29 (±0.710)	7.77 (±0.048)	12.06 (±0.047)	4.31 (±0.031)
	Pi + 0.75 g L^{-1} NPs	17.30 (±0.427)	50.39 (±0.836)	7.95 (±0.069)	12.06 (±0.042)	4.30 (±0.023)
50	control	17.76 (±0.254)	49.98 (±0.579)	7.77 (±0.075)	12.01 (±0.043)	4.80 (±0.024)
	0.5	19.54 (±0.381)	50.42 (±0.835)	7.87 (±0.063)	12.03 (±0.036)	4.75 (±0.084)
	0.75	20.19 (±0.363)	49.85 (±0.580)	7.96 (±0.094)	12.01 (±0.023)	4.76 (±0.02)
	Pi	18.76 (±0.525)	50.26 (±0.710)	8.03 (±0.088)	11.89 (±0.029)	4.61 (±0.142)
	Pi + 0.5 g L^{-1} NPs	19.40 (±0.629)	50.29 (±0.705)	7.61 (±0.142)	12.00 (±0.062)	4.77 (±0.03)
	Pi + 0.75 g L^{-1} NPs	19.49 (±0.178)	50.51 (±0.841)	8.05 (±0.017)	11.91 (±0.024)	4.81 (±0.051)
75	control	21.10 (±0.600)	48.91 (±0.579)	6.96 (±0.073)	11.61 (±0.039)	5.01 (±0.038)
	0.5	25.08 (±0.282)	49.49 (±0.837)	7.10 (±0.091)	11.81 (±0.02)	4.88 (±0.053)
	0.75	24.97 (±0.271)	48.75 (±0.588)	7.08 (±0.179)	11.90 (±0.034)	4.92 (±0.033)
	Pi	25.26 (±0.161)	49.86 (±0.713)	7.47 (±0.189)	11.66 (±0.079)	4.86 (±0.076)
	Pi + 0.5 g L^{-1} NPs	25.86 (±0.554)	49.38 (±0.708)	7.39 (±0.172)	11.95 (±0.012)	4.85 (±0.056)
	Pi + 0.75 g L^{-1} NPs	25.81 (±0.092)	49.62 (±0.866)	7.91 (±0.093)	11.69 (±0.19)	4.92 (±0.047)

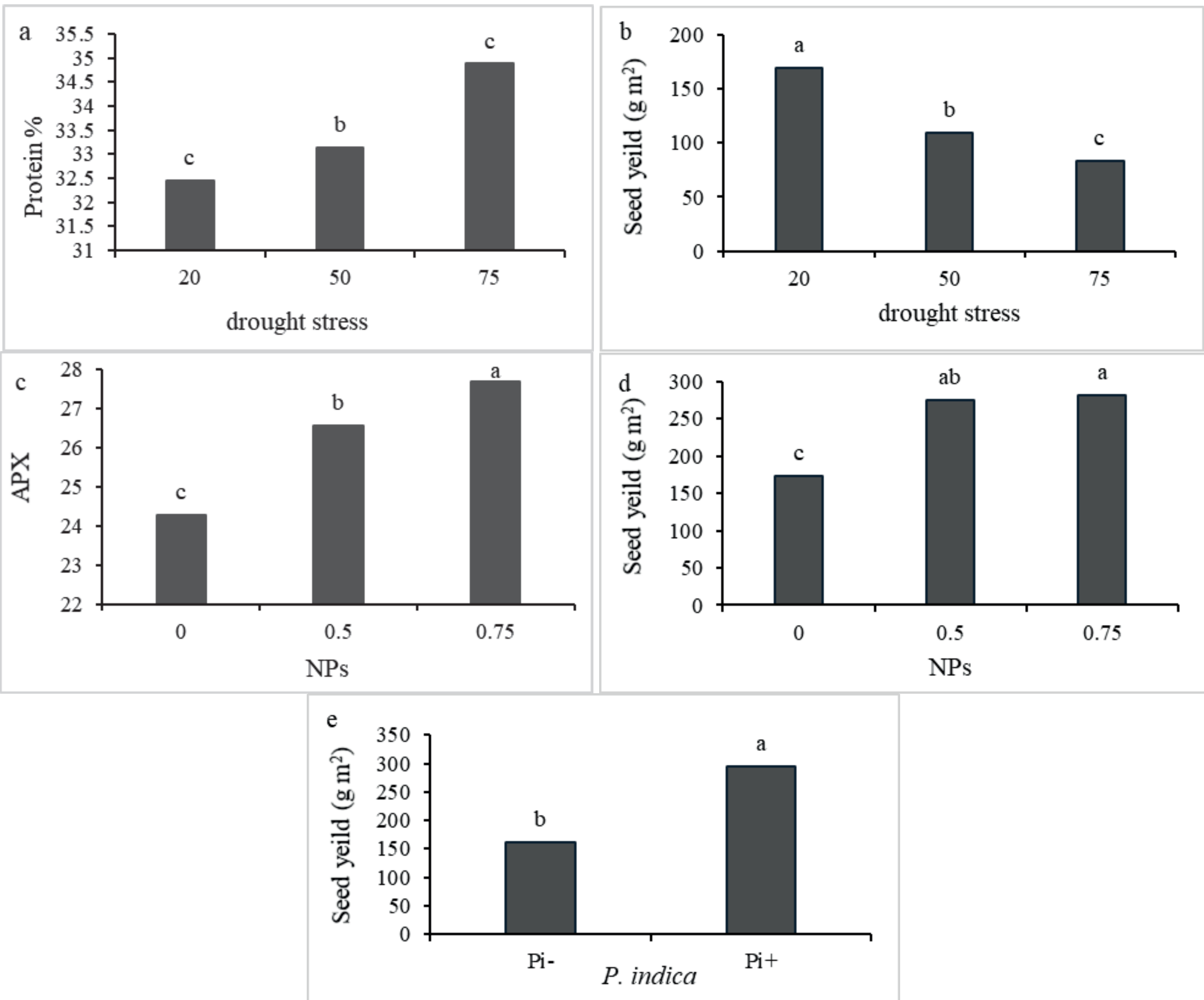

Figure 6. Effects of drought stress on seed protein (a), seed yield (b) and NPs on APX (c), seed yield (d) and *P. indica* on seed yield (e) in soybean. In each figure, means with the same letter are significantly different according to LSD test at P<0.05. Means ±S.D from the three experiments.

The Principle Component Analysis (PCA) results of data obtained from fatty acids composition, seed oil and protein content, antioxidant enzyme, MDA and seed yield of soybean leaf exposed to irrigation regimes are shown in Figure 7. It shows that the first component with 76.63% relative variance and the second component with 8.48% had the highest variation among the obtained components; and the two main components together accounted for 85.11% of the total variation in traits in this study (Figure 7). In the first and second components of the biplot, it was shown that the treatments were divided in three distinct groups. Based on the results of the biplot, the control treatments were placed into one group. On the other hand, moderate and severe stress treatments were included in two set. The biplot results showed that the treatments were divided in two distinct groups (Figure 7). Based on the results of the biplot, linoleic, linolenic and palmitic acids, protein, oil and seed yield traits had a strong positive correlation with non-stress treatments, while, oleic and stearic acids, antioxidant enzyme and MDA had a strong positive correlation with severe drought stress treatments.

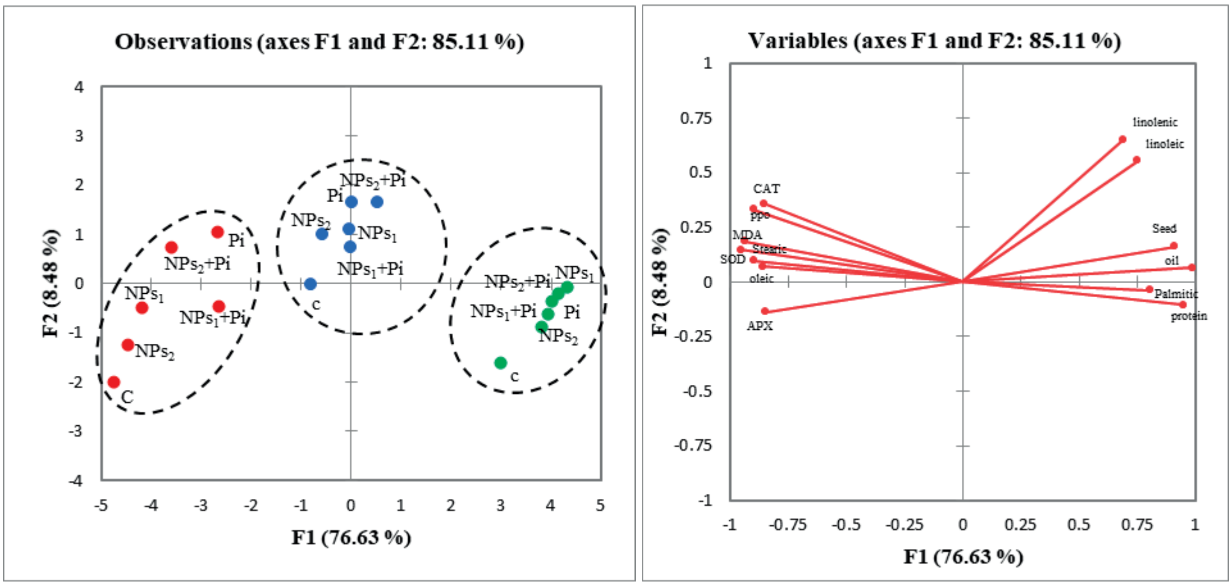

Figure 7. Biplot of principle component analysis for the first two principle components of all parameters, drought stress, *P. indica* and NPs. PCA biplot for traits studied under drought stress and *P. indica* and NPs treatment (a) and parameters (b). Abbreviations: C (control), NPs (iron oxide nanoparticles), Pi (*Piriformospora indica*).

Drought stress results in overproduction of reactive oxygen species (ROS), which can cause peroxidation of cell membrane lipids and intracellular organelles. Our results showed that application of NPs and endophytic fungi contribute to the integrity and structure of soybean membrane. NPs and fungal symbiosis treatments reduced MDA, which could be attributed to activation of defense mechanisms in the cytosol, which increased drought tolerance as a result of ROS scavenging and improved plant cell membrane integrity. High activity of antioxidant enzymes in NPs treatment may be due to the role of iron in stimulating the expression of antioxidant enzymes genes (Vansuyt et al., 1997). Our results indicated that, there is a direct relationship between inhibition of MDA production and free radical scavenging (such as CAT, PPO and SOD activity) in inoculated plants. It can be concluded that endophytic fungi can enhance ROS scavenging activity which lead to alleviation of negative effects of drought stress on the membrane system. Some studies have shown that mycorrhizal fungi are involved in the detoxification of free radicals through the accumulation of H_2O_2 in the fungal cytosol, hyphae walls, and arbuscules, resulting in a decrease in membrane lipid oxidation (Wu et al., 2009). The results showed that seed oil content had a direct relationship with grain dry weight, so the low grain yield in soybean plants could be an explanation for the low oil content in plants under severe drought stress. It is mainly associated with: reducing the length of grain filling period under stress conditions reduces the ability to convert carbonate to oil, in addition, lipid accumulation is highly sensitive to drought stress, and the oxidation of some unsaturated fatty acids can be another reason for the decrease in seed oil (Wijewardana et al., 2019). In our experiment, the reason of high percentage of seed oil in plants inoculated with fungi can be related to the increasing of mineral uptake, especially phosphorus. Inorganic phosphorus content in the plant plays an important role in the production of ATP for the biosynthesis of proteins, oils and fats and the formation of structures such as phospholipids (Malavolta et al., 1997). In addition, it was shown that spraying of iron oxide nanoparticles can dramatically increase grain oil content, which is consistent with reports by Mutlag et al. (2019) who reported that application of NPs fertilizer significantly increased oil and protein content in rapeseed seeds compared to control. They showed that this may be due to the increase in leaf area and surface area of the capsules involved in the carbon delivery process, and it accumulates dry matter, which has a positive effect on the oil content of the seeds. There was also an inverse relationship between protein and seed oil under drought stress conditions, which is not necessarily due to stimulation of protein synthesis. It is probably for this reason that reduction in hydrocarbon production is greater than the amount of protein stored in the grain, so, the percentage of protein in the grain increases (Rotundo et al., 2009). Also noteworthy is that the protein content in *P. indica* plants increased. The reason can be related to the ability of this fungus to increase the activity of nitrate reductase and phosphate transporters and consequently better absorption of N and P (Khalid et al., 2018). The increase in grain protein in plants exposed to NPs may also be due to the role of iron in nitrogen metabolism and the reactions associated with nitrogen fixation. In addition, iron also participates in the structure of nitrate and nitrite reductase enzymes, which has a direct effect on nitrogen fixation and nitrogen reduction in the plant, which may explain the increase in protein content in the presence of NPs (Marschner, 1995). The quality and nutritional properties of soybeans are highly dependent on its chemical elements, especially the fatty acid composition. Changes in seed composition under drought stress can be attributed to changes in nutrient accumulation and allocation in soybean seeds (Wijewardana et al., 2019). Most unsaturated fatty acids, up to 90% in non-photosynthetic tissues of plants, are synthesized through the (18:1) desaturase in the endoplasmic reticulum. The endoplasmic reticulum - dependent desaturase [FAD2 (1-acyl-2-ololel-sn-glycero-3-fosphocholine 12-desaturase)] is the key enzyme responsible for the production of linoleic acid in plants. In addition, the change in grain fatty acid composition under stress conditions is related to the change in desaturase fatty acid activity (Zhang et al., 2014). The increase in oleic acid under drought conditions indicates its possible role in environmental or chemical stresses. Drought stress enhances oleic acid biosynthesis by affecting the activity of D-9 desaturase [responsible for accumulation of oleic acid (18:1)] by desalting stearic acid (18:0) and D-12 desaturase enzymes (catalyst for spontaneous saturation of oleic acid in linoleic acid), resulting in oleic acid accumulation (Petcu et al., 2001). It is noteworthy that *P. indica* consistently improved oil content and quality of oil. It seems that endophytic microorganisms reduced the negative effects of drought stress on grain fatty acid quality through induction of enzyme activity, gene expression and mineral nutrients uptake especially phosphorus (Baltruschat et al., 2008). The important point is that phosphorus absorbed by the fungus is stored as orthophosphate (a form that is easily used for the synthesis of phospholipids and DNA) (Wu et al., 2009). Increased grain fatty acids quality was also observed in NPs application. Consistent with these results, positive effects of NPs on lipid content in canola (Mutlag et al., 2019) and increased linoleic and oleic acid content in soybean (Sheykhbaglou et al., 2018).

CONCLUSIONS

In conclusion, utilization of NPs and *P. indica* individually or in combination ameliorate the detrimental effects of drought stress by improving antioxidant defense system and altering seed components. Effective use of latent ecological relationships in plant rhizosphere and potential of nano fertilizers can be an effective way to increase plant resistance to environmental stresses and to achieve maximum yield of soybean oil, especially under drought stress conditions.

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