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Effect of AM *Rhizophagus irregularis* inoculation on growth and physiology of *Eleusine coracana* (L.) Gaertn. grown under Zn stress

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ABSTRACT

Heavy metals at more than threshold concentration interfere with usual physiological processes of plants. Arbuscular mycorrhizal (AM) fungi which form symbiotic association with plant root is reported to ameliorate abiotic and biotic stress. The present study was conducted to evaluate the effects of AM fungi *Rhizophagus irregularis* inoculation to *Eleusine coracana* growing under different concentration of Zinc in pot culture experiment. Plant growth parameters such as root length, shoot length and bio-mass, physiological parameters such as total chlorophyll, carbohydrate, protein, reducing sugar, free amino acid and proline content, antioxidative enzyme CAT and GPX activity were analyzed. Zn concentration of 100 ppm enhanced plant growth and physiology, where as high concentration of Zn (300 & 500 ppm) caused stress to the plant resulting in reduction in AM root colonization and spore density. However, the AM inoculation alleviated the Zn stress in all the treatments by enhancing anti-oxidation enzyme activities. The AM inoculation has, therefore, the potential to alleviate Zn stress in *E. coracana*.

1. Introduction

Zinc (Zn) is a heavy metal and essential micronutrient for plants (Reeves and Baker, 2000). It acts as cofactor for several enzymes such as RNA polymerases, anhydrases, dehydrogenases, oxidases, peroxidases (Cakmak, 2000) and regulates synthesis of protein, nucleic acid metabolism, Photosynthesis, carbohydrate metabolism and auxin synthesis (Palmer and Guerinot, 2009). The acute deficiency of Zn results in physiological stress with several visible symptoms like stunted growth, small leaves, chlorosis of leaves, necrotic leaf tips, sterility of spikelets etc. (Sharma *et al*., 2013). Besides, at high concentrations Zn is reported to be potentially toxic (Ozdener and Aydin, 2010). Anthropogenic releases of zinc and its compounds to the environment are from dust and fumes from mining, zinc production and processing facilities, brass works, coal and fuel combustion, refuse incineration, iron and steel production etc. (EPA 1980d; Raymond *et al*., 2011).

Mycorrhiza is the symbiotic relationship between a group of fungi and roots of higher plants (Smith and Read, 2008). Arbuscular Mycorrhiza (AM) is the most widespread mycorrhizal symbiosis in which the fungus develops hyphae, arbuscules and vesicles by entering the cortical cells of the plant roots. AM fungi provide a direct physical linkage between the soil and plant roots by their extrametrical mycelia. The AM association is reported to enhance plant tolerance to biotic and abiotic stresses (Augé, 2001; Beltrano *et al.,* 2008). The interaction between AM colonization and accumulation of toxic elements is an area of considerable interest relating to both production of safe food and bioremediation programs (Smith *et al.,* 2009).

Eleusine coracana (L.) Gaertn. commonly known as finger millet is one of the nutritious staple crops of India cultivated since ancient times. Its grains are rich source of calcium, magnesium, potassium, methionine and tryptophan, which is lacking in the diets of poor people living on starchy

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foods like cassava, polished rice and maize meal (Fernandez *et al*., 2003; Devi *et al*, 2014). Wheat and rice provide food security, but crops like finger millet promise nutritional security for the world (Singh and Raghuvanshi, 2012). It is a climate resilient crop and considered as a future crop in the context of climate change (Gupta *et al*., 2017). The present study was designed to assess the effect of Zn stress and AM association on growth and physiology of *E. coracana*.

2. Materials and methods

2.1 Seed collection and surface sterilization

Seeds of *Eleusine coracana* were collected from Regional Pulse Research Centre, Odisha University of Agriculture and Technology (OUAT), Berhampur, Odisha**.** Healthy seeds were selected and surface sterilized in 0.1% mercuric chloride for 5 minutes followed by washing with sterile distilled water several times to remove the traces mercuric chloride from seed. The surface sterilized seeds were then processed for seed germination.

2.2 AM inocula

Rhizophagus irregularis (Blaszk., Wuet, Renker & Buscot) C. Walker & A. Schüßler 2010 (Formerly *Glomus intraradices*) was procured from Ambika Biotech and Agro Services, Madhya Pradesh by the generic name Root Care containing 100000IP (IP: Infective Propagule) containing spores and hyphae per kg of the carrier material.

2.3 Zn treatment

Zinc Sulphate ($ZnSO_4$) was used as the source of Zn. To prepare 1000ppm stock solution, 4.39g of Zinc sulphate was added to 1000ml water. Appropriate dilutions were made to the stock solution to get different concentration of treatment solutions.

2.4 Seed germination study

For seed germination study 10 no. of surface sterilized seeds were placed in sterilized petriplates over different Zn treatment solution (0, 100, 200, 300, 400, 500 and 600ppm) saturated cotton pads with for germination. The seeds were allowed germinate at 25ÚC under darkness for 3 days.

2.5 Pot culture and experimental design

Pot culture experiment was carried out in polybags containing sterilized potting mix composed of dry soil and sand (2:1, v/v) sieved through 2mm diameter sieve with organic manure (3:1, v/v). Potting mixture was sterilized by autoclaving for 1hr at 121^{o}C and 15psi on alternate days 3 times to make the substrate free from any mycorrhizal contamination. The basic physico-chemical properties of the potting substrate were pH: 6.3, EC: 0.052 dSm^{-1}, organic carbon: 16g kg^{-1}, Avl. N: 148 mg kg^{-1}, Avl. P: 93 mg kg^{-1}, Avl. K: 298 mg kg^{-1}, Zn: 1.83 mg/kg. Each poly bag was filled with 2kg of substrate mixed with 20g of AM inocula containing approx. 2000IP and 12 no. of *E. coracana* seeds were sown. After 7 days of seed germination appropriate level of Zn dissolve in water was added. Experiment was randomized with 4x2 factorial designs consisting of four Zn addition level (0, 100, 300 and 500ppm) and two inoculation treatments (non-mycorrhizal and mycorrhizal). The treatments were (1) T0 (NM): Non-Mycorrhizal + 0ppm Zn, (2) T0(M): Mycorrhizal + 0ppm Zn, (3) T100 (NM): Non-mycorrhizal + 100ppm Zn, (4) T100(M): Mycorrhizal + 100ppm Zn, (5): T300 (NM): Non-mycorrhizal + 300ppm Zn, (6) T300(M): Mycorrhizal+300ppm Zn, (7) T500(NM): Non- mycorrhizal + 500ppm Zn, (8) T500(M): Mycorrhizal + 500ppm Zn stress. Each treatment had 3 replicates and different parameters were analyzed after 45days growth.

2.6 Growth and morphological parameters

For study of root and shoot length, the root and shoot were detached and individual length of root and shoot length were measured and expressed in centimeter. Fresh weight of the root and shoot were measured in electric balance. The plant materials were then kept in a hot air oven at 80ÚC for 3 hr and the dry weight was measured. Both fresh weight and dry weight expressed in g.

2.7 Biochemical parameters

Biochemical parameters such as chlorophyll content (Arnon *et al.,* 1949), total carbohydrate by the Anthrone reagent method (Hedge and Hofreiter, 1962), total protein content by Lowry Method (Lowry *et al.,* 1951), reducing sugar (Nelson, 1994), Free amino acid (Moore and Stein, 1963) and Proline content (Bates *et.al*., 1973) were estimated.

2.8 Anti-oxidative enzyme activity

Leaf tissues were ground to a fine powder in liquid N_2 and then homogenized in 2ml of 50 Mm/l potassium phosphate buffer (pH 7.0), 1mM EDTA, 2mM D-iso ascorbic acid, 2% (w/v) PVP & 0.05% (w/v) Triton X-100 using a chilled mortar and pestle. The homogenate was centrifuged at 10,000rpm for 10 min at 4^{0}C and the supernatant were collected and used for the enzyme assay. Catalase (CAT) activity was determined spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240nm (Aebi, 1984) and GPX activity was measured spectrophotometrically at 470 nm as increase in absorbance due to guaiacol oxidation

(Hemeda & Klein, 1990). The enzyme activity was expressed as U g^{-1} protein.

2.9 Estimation of mycorrhizal root colonization

The roots were washed thoroughly and were cut (1cm), cleared in 10%KOH, bleached in H_2O_2 for 5 min, acidified with 2% HCl and stained in trypan blue (0.05%) as per Phillips and Hayman (1970). The level of colonization in each root segment was measured by the method of Giovannetti & Mosse (1980) which involved gentle squashing of stained root segment on a microscope slide after covering with a cover slip. The percentage of mycorrhizal colonization was estimated by following formula:

$$\text{Mycorrhizal colonization (\%)} = \frac{\text{No. of root colonized with AM}}{\text{Total no. of roots inspected}} \text{ X } 100$$

$$\text{Occurance (\%) of a particular AM structure} = \frac{\text{No. of root with a particular AM structure}}{\text{Total no. of roots colonised with AM}} \text{ X } 100$$

2.10 Estimation of AM spore density

AM fungal spores were extracted from potting substrate by wet sieving and decanting method of Gerdemann & Nicholson (1963) followed by sucrose density gradient centrifugation technique as described by Daniel and Skipper (1982). The AM spores were washed into a filter paper using a stream from wash bottle and the filter paper containing spores were kept in a petriplates. Isolated spores were counted over a gridded filter paper under stereo zoom microscope at 40× magnification.

2.11 Statistical analysis

The significant difference between parameters by the level of Zn addition and AM inoculation is statistically analyzed by two way analysis of variance (ANOVA) at $P< 0.05$ using MS excel.

3. Results & discussion

3.1. Germination study

The percentage of seed germination of *E. coracana* in different conc. of Zn was presented in Figure 1. In control condition (0ppm) there was 100% seed germination and as conc. of Zn increased there was decrease in the rate of germination. At 600ppm of Zn there was 50% seed germination, hence it was considered as LC50. The findings indicated that higher than 200ppm Zn is showing toxicity and inhibiting the germination rate. Ionic toxicity and osmotic stress caused of drastic effects of heavy metal salts on seed germination (Shaukat *et al.,* 1999).

3.2 Mycorrhizal root colonization and AM spore density

The mycorrhizal root colonization was not detected in non-inoculated plants. Among the mycorrhiza inoculated treatments AM structures hyphae, arbuscules and vesicles were observed (Table 1). The AM colonization increased with increasing Zn conc. at 100ppm than control but decreased at 300 and 500ppm. The formation of vesicle is drastically reduced (32%) in response to 500ppm Zn than hyphae and arbuscules. The spore density was also highest at 100ppm and again decreased with increasing conc. of Zn.

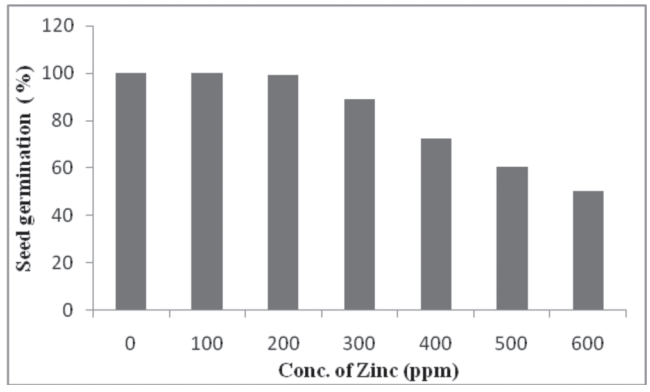

Figure 1. Seed germination under different concentration of Zn

Regarding the effects of heavy metal on mycorrhizal colonization positive, negative or neutral reports are available. AM root colonization of maize and mycorrhizal spore density in the heavy metal metal Cd, Zn, Pb, and Cu polluted field were higher than that in the uncontaminated field (Weissenhorn *et al*., 1995b) and higher mycorrhizal colonization in *Viola calaminari*a was observed in highly contaminated sites with Zn and Pb (Hildebrandt *et al*.,1999). Weissenhorn *et al*. (1995a) and Diaz *et al*. (1996) reported no correlation between AM association and the degree of heavy metal pollution in a field soil. Chao and Wang (1990) reported mycorrhizal infection rate of maize was reduced by

Table 1

Mycorrhizal root colonization (%) in *Eleusine coracana* under different treatments.

Zn Conc.	Total AM (%)	Arbuscules (%)	Vesicle (%)	Spore Density (No. of spore /100g soil)
0ppm	95	88	84	116
100ppm	100	89	68	138
300ppm	90	77	61	105
500ppm	86	73	52	94

the addition of heavy metals (Zn, Cu, Ni, Cr, Pb and Cd). Pb contamination inhibited mycohrrizal colonization of leguminous tress *Robinia pseudoacacia* (Yang *et al.,* 2015) and tomato plant (Chen *et al*., 2005). All these reports suggested impact of heavy metal on mycorrhizal colonization differ among host plant species. Thus, the observation of present study revealed that the *E. coracana* is sensitive to high level of Zn stress and reducing the AM colonization.

3.2 Growth parameters

Growth parameters like shoot length, root length, fresh weight and dry weight showed enhancement at 100ppm Zn, but significant reduction at 300 and 500ppm Zn addition in both mycorrhizal and non mycorrhizal plants (Table 2). However, all the growth parameters were higher in mycorrhizal plants than non mycorrhizal plants. Similar results were reported in tomato (López-Millán *et al*., 2009) and barley (Wu *et al*., 2008). Enhancement of growth at 100ppm of Zn indicated that it might have compensated the Zn deficiency in the soil. The reduction in plant growth under high levels of Zn was water potential, hampered nutrient uptake and secondary stress such as oxidative stress (John *et al.,* 2009). Shetty *et al.* 1994 reported that the plant growth inhibition in zinc contaminated sites was due to interference of zinc with phosphorous uptake by plants and the application of arbuscular mycorrhizae (AM formerly VAM) fungi increased plant biomass even at elevated levels of Zn in the soil.

3.3 Biochemical parameters

The total chlorophyll, total carbohydrate, reducing sugar and protein content (Table 3) in the leaves of *E. coracana* showed increase at 100ppm Zn, but there was significant decrease at higher conc. like 300 and 500ppm of Zn in both mycorrhizal and non mycorrhizal plants. Interesting to note that mentioned parameters were higher in mycorrhizal plants than non mycorrhizal plants in all the treatments. Further, total free amino acid and proline content (Figure 2 a & b) was recorded to increase in with Zn stress which was again reduced by AM association.

The reduction in chlorophyll content might be due to Zn induced oxidative stress (Gallego *et al.,* 1996). The heavy metal stress affect the synthesis of chlorophyll enzymes (Padmaja *et al.,* 1990), thereby reducing the photosynthesis of the plants and reduce the growth of plants under abiotic stress (Wu and Xia, 2006). Like other heavy metals, excess Zn reported to show marked alterations in electron transport, membrane permeability and uptake and transloca-tion of nutrient elements (Wang *et al*., 2009b). Chavan and Banerjee (1980) reported that Zn toxicity appear to be due to Fe deficiency. The absorption and translocation of plant nutrients like Fe, Mg, K, P and Ca depended on Zn concentration in soil (Cayton *et al*., 1985) and high level of Zn might have caused mineral imbalance. The findings indicated that 100ppm of Zn might have compensated the Zn deficiency of soil and resulted increased in growth and biochemical parameters, but at high conc. (300 & 500ppm) Zn have become toxic to the plant and inhibit their physiology. The improvement in plant growth and physiology with AM association at high Zn Conc. as observed in the present study was supported by Lingua *et al*., (2008)

Increase in free amino acid content with increasing Zn conc. can be correlated hydrolysis of protein to amino acid leading to decrease in protein content. Assessment of proline content is an important parameter to evaluate the effect of stress on plants (Mohanty and Patra, 2011). Proline acts as a non-enzymatic free radical scavenger, an osmo-protection (Khan *et al.* 2002) and a redox potential buffer (Molinari *et al*. 2007), thereby protecting the cells from damage. The reduction in the amount of free amino acid with AM association might be due to reduction in protein degradation. The lesser accumulation proline in mycorrhizal than non mycorrhizal plant can be related to stress amelioration effect of AM association under abiotic stress (Borde *et al*., 2011; Damaiyanti *et al*., 2015)

3.4 Antioxidative enzyme activity

Findings of the present study revealed that anti-oxidative enzyme CAT and GPX activity increased in

Table 2

Growth parameters of *E. coracana* under different treatments

Different treatments	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry Weight (g)
T0 (NM)	34.5±1.1	14.8±0.37	2.06±0.08	0.831±0.03
T0(M)	36.3±1.4	16.1±0.40	2.37±0.06	0.852±0.01
T100 (NM)	34.8±1.2	15.9±0.41	2.15±0.04	0.861±0.09
T100(M)	37.1±1.7	17.6±0.47	2.31±0.06	0.881±0.009
T300 (NM)	32.4±1.1	13.1±0.31	1.51±0.03	0.799±0.01
T300(M)	35.8±1.3	14.7±0.44	1.88±0.05	0.82±0.07
T500(NM)	30.1±1.0	10.3±0.27	0.93±0.08	0.733±0.06
T500(M)	33.9±1.1	12.4±0.34	1.23±0.06	0.772±0.06

Table 3

Biochemical parameters of *Eleusine coracana* under different treatments

Different treatments	Total Chlorophyll (mg/g FW)	Carbohydrate content (mg/g FW)	Reducing sugar content (mg/g FW)	Protein content (mg/g FW)
T0 (NM)	0.969±0.023	36.37±0.83	7.13±0.36	9.63±0.43
T0(M)	0.983±0.027	37.82±0.88	8.79±0.49	10.87±0.28
T100 (NM)	0.997±0.026	36.89±0.76	9.53±0.11	11.23±0.33
T100(M)	1.378±0.029	40.11±0.9	10.21±0.13	12.54±0.39
T300 (NM)	0.672±0.026	25.9±0.95	6.43±0.23	6.97±0.21
T300(M)	0.796±0.031	30.19±0.81	7.76±0.16	8.37±0.39
T500(NM)	0.362±0.021	21.04±0.93	4.8±0.21	4.05±0.25
T500(M)	0.562±0.03	24.24±0.89	5.35±0.1	5.01±0.19

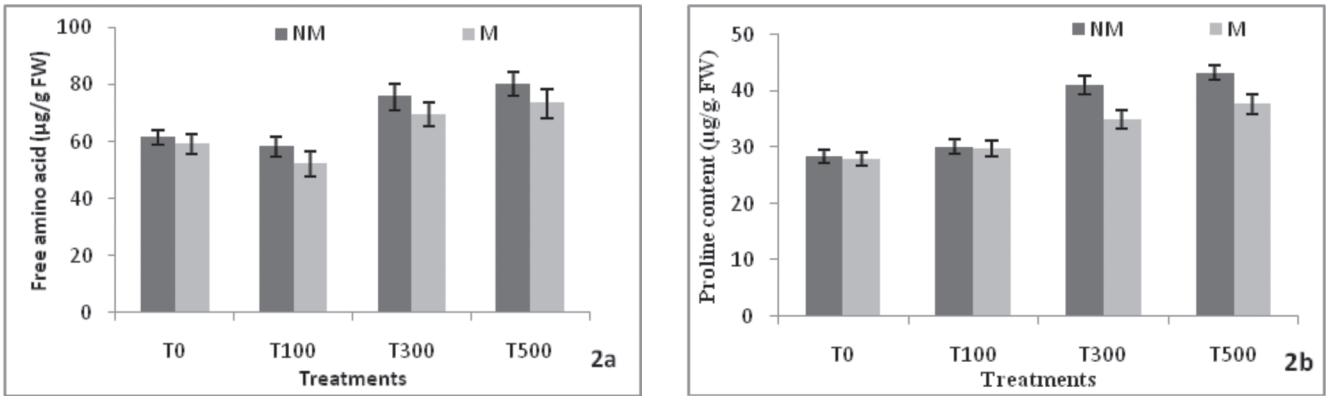

Figure 2: Free amino acid (a) and proline content (b) of *E. coracana* under different treatments

mycorrhizal plants than non mycorrhizal plant in all the treatments (Figure 3 a &b). Plants scavenge ROS generated in response to stress by stimulating antioxidant enzymes (Rout *et al*., 2017). Though Zn is essential for biological system, excess of Zn can promote generation of Fenton-type ROS (Emamverdian *et al*. 2015). AM association is reported to enhance antioxidant enzyme activity in mycorrhizal plant than non mycorrhizal plant in salinity stress (Latef, 2011), and heavy metal lead (Yang *et al*., 2015) and Zn stress (Rout *et al*., 2019). The induction of antioxidant enzymes during appressoria formation attributed to a defense response of plants during the early stage of symbiosis development (Hajiboland *et al*., 2010). The general stimulation SOD, CAT, POD and APX of plant roots associated with AM compared to non-AM roots (He *et al.*, 2007) could be related to enhanced activity of CAT and

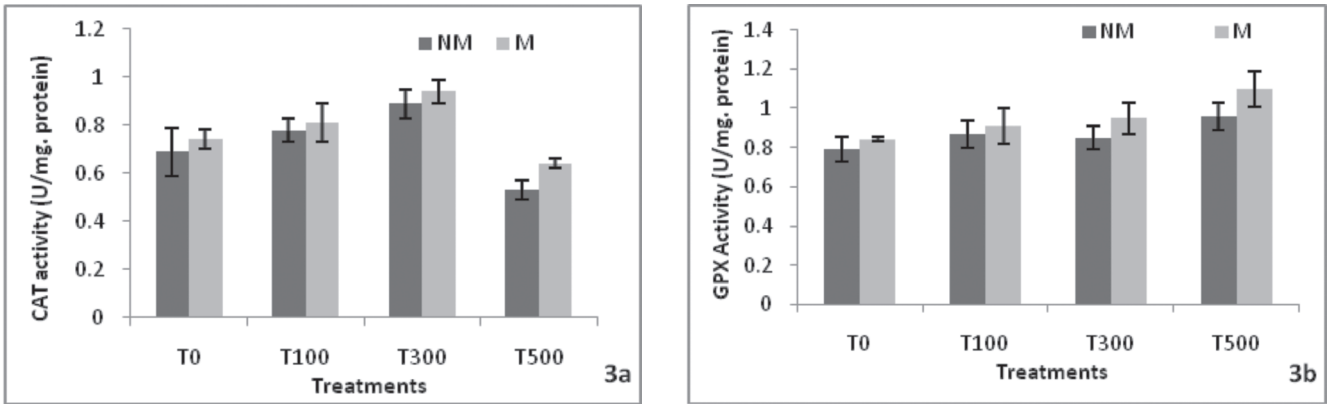

Figure 3(a-b): Antioxidant enzyme CAT (a) and GPX (b) activity in *E. coracana* under different treatments.

GPX of mycorrhizal plant in Zn stress. The CAT activity was highly sensitive to Zn stress than GPX as its activity was drastically reduced at 500ppm Zn.

4. Conclusion

The AM inoculation under Zn stress improves the plant growth in terms of better biomass accumulation, physiology in terms of cholorophyll, carbohydrate, protein and reducing sugar content of *E. coracana.* Enhance antioxidative enzyme activities and proline accumulation may contribute for ROS scavenging activity in mycorrhizal plants. The stress ameliorative effect of AM association is higher at low conc. (100ppm) than high conc. (500ppm) which is positively correlated to percentage of root association. The present study suggested that AM association enhanced the plant tolerance to Zn stress but very high level of stress is inhibitory for AM colonization. Further study is required to understand the AM-heavy metal interaction and mechanism of stress alleviation.

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