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Effect of interspecific protoplast fusants strain (*Trichoderma viride* and *Trichoderma koningii*) on *Arachis hypogaea* L. for zinc tolerance using physiological and biochemical traits

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Abstract
This study was designed to investigate the efficacy of three fusant strain of *Trichoderma koningii* and *Trichoderma viride* on pot culture experiment using *Arachis hypogeal* L. for metal tolerance using physiochemical and biochemical traits against parent *Trichoderma* strain. The zinc toxicity at all concentration (100, 500, 1000, 1500 and 2000 ppm) exhibited significant reduction in chlorophyll, membrane stability index and biomass in groundnut seedling. While fusant strain treated plants under zinc stress showed that increase the chlorophyll, membrane stability index and biomass in groundnut seedling. The enzyme activity in fusant treated plants was always lower compared to parent and control plant. The groundnut seeding treated with fusant strain showed higher biomass, on the other hand non treated plant and parent strain treated plant gained higher biomass. These results indicate that fusant strains possess greater tolerance capacity than parent strain. And this technique feasible for improved biocontrol strain.

Keywords: biomass, zinc toxicity, *trichoderma*, enzyme assay

Introduction
In this era land, air and aquatic ecosystems have been contaminated with heavy metals, non heavy metal and other chemicals through various anthropogenic activities of human beings and as a result of moving up the food chain, have become a major human health hazard. As a result of the increased awareness of the harmful effects of environmental pollution, particularly that caused by heavy metals and hydrocarbons, there has been a marked increase in research to gather information on the effects of heavy metal ions on the microbiota of waste waters and contaminated soils, followed by the exploration of various strategies that may be employed to clean up the environment (Pandey and Jain, 2002) [16].

While some heavy metals on very small quantities are required for good growth of plants, animals but in excess they are highly toxic to plants, animals and human beings (Agrawal *et al*, 2000) [1]. Oil seeds crops play on important role in Indian agriculture as food and industrial commodity. Among oil seeds, groundnut has received the maximum attention of research workers in India. It is an energy rich crop and its macro and micro nutrient contents are relatively high compared with other crops (Nagaraju *et al*, 2015) [13].

Keisling (1977) [9] were the first to provide evidence that Zn toxicity in peanuts (Arachis hypogea L.). They described toxicity symptoms and suggested Zn critical levels of 220 mg kg -] in peanut shoots and 12 mg kg -1 in soils using Mehlich 1 extractant. Zinc toxicity in peanuts has been found in various situations.

Application of zinc fertilizer can recommend practice for corn production in Coastal Plain soils. Since corn commonly precedes peanuts in rotation, Zn levels could build up and become toxic to peanuts. So numerous findings have reported that some microbes are tolerant of heavy metals with an ability to either remove them from the environment, or break down them to a less toxic or comparatively benign forms (Sharma *et al*., 2000) [21].

Bioremediation, which is essentially the use of microbial metabolism, seems to offer aviable, safer, more efficient and less expensive alternative to physiochemical methods for pollution abatement (Pandey and Jain, 2002) [16]. Filamentous fungi have a distinct advantage over bacteria because of their high tolerance for metals and ability to grow under extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations

(Anand *et al.,* 2006) [3]. While inoculating soil with bacteria can promote plant growth and HM accumulation (Ma *et al*., 2011; Sheng *et al*., 2008) [11, 22], growth promoting saprophytic fungi have received less attention.

The present investigation is carried out to study the Effect of Interspecific protoplast fusants strain (*Trichoderma viride* and *Trichoderma koningii*) on *Arachis hypogaea* L. for zinc tolerance using physiological and biochemical traits

Materials and Methods

Peanut (*Arachis hypogaea* L.) seeds, procured from the local market were washed with detergent for 10 min followed by repeated washing with deionized Water. There after the seeds were treated with sterile water, the seeds were disinfected by 0.1% $HgCl_2$ (w/v) treatment for10 min. Adhering $HgCl_2$ was eliminated by rinsing the seeds with sterile deionized water. In pot culture experiment seeds were shown in pre-treated soil (Soil treated with low and higher range of heavy metal with one soil control i.e. heavy metal less soil in greenhouse for 6 months for metal stabilization) and the plant was treated with different concentrations of Zn^{+2} solutions 0 (control), 100, 500, 1000, 1500 and 2000 ppm and observations were made after 25 days of treatment. The leaves and root were taken from plants at 25 days after Zn^{+2} treatment and used for determinations of physiological and biochemical studies. All the experiments were done in triplicates and the data were statistically analyzed and standard errors of mean (SEM) was calculated.

Morphological Characters

On the 25th day, all the plants were carefully harvested, gently washed to remove sand and other debris and spread on filter papers to remove surface moisture if any. All the phenotypic characters viz., root length (cm), shoot length (cm), total fresh root weight (g) and total fresh shoot weight (g) were measured immediately.

Chlorophyll

Total chlorophyll was estimated as described by Hiscox and Israelstam (1979) [7]. 100 mg fresh leaf were cut into small pieces and kept in dimethyl sulfoxide (DMSO) containing tube over night. The extract was filtered through Whatman No.1 filter paper. Filtrate was collected and volume made to 10 ml with DMSO. Absorbance was measured in spectrophotometer at 645 nm and 663 nm for determination of total chlorophyll.

Total Phenol

Total phenol was estimated by following the method of Thimmaiah, S.K. (1999) [24]. The sample 2.0 g was homogenized in 80 % methanol using mortar and pestle and volume was made to 20 ml. The homogenate was centrifuged at 10,000 rpm for 15 minutes and collected the supernatant. The supernatant was used for the assay of phenols. Suitable aliquot (0.2 ml) was taken and made the final volume 1.0 ml with distilled water followed by 0.5 ml of Folin-Ciocalteu reagent was added. After 3 minutes incubation at room temperature, 2.0 ml of 20% Na_2CO_3 was added and the tubes were incubated in boiling water bath for 1 minute and allowed it to cool. The absorbance was measured at 650 nm. Phenol content was calculated from the standard curve prepared from catechol as standard and expressed as mg/100g.

Ascorbic Acid

Ascorbic acid was extracted from groundnut (2.0 g) by adding 10 ml of 4 % oxalic acid solution in a mortar and pestle (Sadasivam and Manickam, 1992) [19]. A homogenized solution was centrifuged and supernatant used as a source of ascorbic acid. The known volume (10 ml) of extracted material was titrated against the 2,4,6-di indophenol dye (Weigh 42 mg sodium bicarbonate into a small volume of distilled water. Dissolved 52 mg of 2, 6 dichloro phenol indophenol in it and made up the volume 200 mL with distilled water). The standard ascorbic acid stock solution was prepared by accurately weighed 100 mg of Ascorbic acid in 100ml 4 % oxalic acid solution in standard flask (1 mg/ml). Transferred the 10 ml from stock solution to another 100 ml volumetric flask and diluted to 100 ml with 4 % oxalic acid. The concentration of working standard was 100 µg/ml. Standard solution 5, 10 and 15 ml in to Erlenmeyer flask containing 10 ml extracting solution (4 % oxalic acid) and titrated rapidly against dye (V1 ml). End point was the appearance of pink colour which persisted for a few minutes. The amount of dye consumed was equivalent to the amount of ascorbic acid. Pipette out 10 ml of sample aliquot in 100 ml conical flask, added 10 ml of 4% oxalic acid and titrated against the dye (V2 ml). Blank was also run with 4 % oxalic acid solution without the sample. Subtracting blank from standard titration, calculated and expressed amount of mg ascorbic acid per 100 g sample.

Membrane Stability Index (MSI)

Leaf membrane stability index was determined according to the method of Premachandra *et al*., (1990) [17] as modified by Sairam R.K (1997) [20]. Leaf strip (0.2 g) of uniform size were taken in test tubes containing 10 ml of double distilled water in two sets. Test tubes in one set were kept at 40^0 C in water bath for 30 min and electrical conductivity of the water containing the sample was measured (C_1) using a conductivity bridge.Test tubes in the other set incubated at 100^0 C in the boiling water bath for 15 min and their electrical conductivity was measured as above (C_2). MSI was calculated using the formulae given below: $MSI = [1 – C_1/C_2] X 100$.

Antioxidant enzymes extraction and assays

A 200 mg of fresh leaves tissue was collected from heavy metal treated and control plants, ground to a fine powder in liquid nitrogen using a pre cooled mortar and pestle. The exact weight of each powdered sample was determined before it was thoroughly homogenized in 1.2 ml of 0.2 M potassium phosphate buffer (pH 7.8 with 0.1mM EDTA) and samples were centrifuged at 15,000 X g for 20 min at 4^0 C and the supernatant was removed and pellet was resuspended in 0.8 ml of the same buffer, and the suspension centrifuged for another 15 min at 15,000 X g. The combined supernatant was stored on ice and used to determine following antioxidative enzymes.

Catalase Assay (CAT)

The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm in a UV spectrophotometer. The 3 ml assay mixture contained 2 ml leaf extract (Diluted 200 times with 50 mM potassium phosphate buffer, pH 7.0) and 1 ml of 10 mM H_2O_2. The extinction coefficient of H_2O_2 (40 $mM^{-1}cm^{-1}$ at 240 nm) was used to calculate the enzyme activity that was expressed in terms of milli moles of H_2O_2 per minute per gram fresh weight.

Peroxidase Assay

The reaction mixtures for peroxidase (EC 1.11.1.7) assay,

Containing 0.1 M potassium phosphate buffer (pH 6.8), 50 µM H2O2, and 50 µM catechol prepared fresh before use. 0.5 ml of enzyme extract was then added to 2.5 ml reaction mixtures. Increase in absorbance was measured at 430 nm at 0.5 min intervals up to 3 min using a UV spectrophotometer. The enzyme activity was expressed as the change in the optical density/mg protein/minute according to Racusen and Foote (1965) [18].

Polyphenol Oxidase Assay

Polyphenol oxidase (EC 1.14.18.1) assay was performed according to the method described by Kar and Mishra (1976) [8]. 5 ml of the assay mixture contained 125 µM of phosphate buffer (pH 6.8), 100 µM of pyrogallol and 1 ml of crude enzyme extract. After incubation at 25° C for 5 min, the reaction was stopped with the addition of 1 ml of 10% H_2SO_4. The colour intensity was read at 430 nm and the enzyme activity was expressed as the change in the optical density/mg protein/h.

Results and Discussion

The results directed towards the hypothetical inference of having significant role of interspecific fusion *Trichoderma* strain in annuling the adverse effect of zinc in groundnut seedling. This experiment had been made evaluate the growth promotion and to quantify the metal uptake in groundnut seedling.

Arachis hypogaea L. seedlings grown over a period of 25 days with increasing concentrations of zinc (0,100, 500, 1000, 1500 and 2000) with combination of *Trichoderma* parental strain *T. viride* and *T.koningii* and their three fusants namely, Tk+Tv 3, Tk+Tv 7, Tk+Tv 10. In this experiment soil with Zn^{+2} solutions but uninoculated with *Trichoderma* strain served as a control.

The concentrations of metals adversely affected root- shoot length, leaf area and biomass of *Arachis hypogea L*. Groundnut seedling when supplied with heavy metal, zinc showed inhibition of growth and concentration dependent decrease in morphological parameter. Nagaraju *et al*. (2015) [13] also observed that inhibition of seedling growth of groundnut was increased with increase concentration of heavy metals. Among six different concentration treatment of zinc toxic metal to groundnut seedling with inoculation of parent and fusant strain of *Trichoderma* showed higher root length at 100 ppm concentration Tk +Tv 10 (36.8 cm), Tk +Tv 8 (36.3 cm) and Tk +Tv 3 (35.4 cm) as compared to control (30.1 cm) and parent strain *T.koningii* (31.4 cm) *and T.viride* (33.3 cm). While lower root length observed at 2000 ppm zinc concentration at control stage (8.12 cm) as compared to *trichoderma* treatment. Shoot length of Tk +Tv 8 (30.8 cm) and Tk +Tv 3 (29.0 cm) showed higher value at 100 ppm as compared to other concentration and control condition (Fig.2). So *Trichoderma* treated plants were able to enhance nutrient uptake, resulting increase plant growth than control plant (Harman, 2006 and Lakhani *et al*, 2016) [5, 10].

It was observed that zinc had significant effect on reduction of root fresh weight in groundnut seedling. Among the different concentration of zinc with the higher toxicity i.e. 0 concentration to 2000 concentration it should be decreased in 0 ppm, but Root fresh weight continually increase up to concentration level (500 ppm) while drastically decrease (1000 to 2000 ppm) (Fig. 3). *Trichoderma* fusant strain Tk +Tv 10 and Tk +Tv 8 showed higher shoot fresh weight compared to the treatments *T. viride*, *T. koningii*, Tk +Tv 3 and control. While in different zinc concentration at control stage showed lower shoot fresh weight (Fig.4). Nongmaithem *et al* (2017) [15] also discussed that *trichoderma* nonamended treatment, cadmium concentration in plant increased with increase in level of cadmium which was equivalent with decreasing biomass of the plants. Heavy metal stress in soil decrease plant biomass production and its used as indicator for environmental stress (Nareshkumar *et al*. 2015) [14].

The variations in chlorophyll content in groundnut seedling were measured under elevated zinc stress and the results obtained have been presented in figure 5. The leaf obtained from Tk+ Tv 8 and Tk+ Tv 10 revealed higher amount of mean total chlorophyll content (3 $mg.g^{-1}$Fr Wt.) which was followed by (Tk+Tv 3) T4 (2.98 $mg.g^{-1}$Fr Wt.) and *T.viride* (T4) plants (2.94 $mg.g^{-1}$Fr Wt.). The mean lowest content was noted for the leaf received from (T1) control (2.03 $mg.g^{-1}$ Fr.Wt.). It was observed that zinc stress had significant effect on reduction of chlorophyll content in leaf. With increase metal contamination in *Trichoderma* non- treated treatment there was a significant reduction in chlorophyll content (Nongmaithem and Bhattacharya, 2017) [15]. The use of tolerant *Trichoderma* spp. helped in enhancing the chlorophyll irrespective of a particular zinc concentration.

The leaf obtained from Tk+ Tv 10 revealed higher amount of mean Membrane Stability Index (97.35 %) which was followed by Tk+ Tv 8 (97.29 %) and Tk+ Tv 3 plants (97.26 %) (Fig.6). The mean lowest content was noted for the leaf received from (T1) control (95.49%). There was a linear decrease in proline content with increasing zinc concentration was observed in groundnut seedling. However, a more pronounce decrease was noticed in Tk+ Tv 10 (0.63 $mg.g^{-1}$ Fr. Wt.) compared to *T. koningii* (0.76 $mg.g^{-1}$Fr Wt.). While increasing proline content at control (T_1) which was followed by T_2 (*T. viride*) (0.80 $mg.g^{-1}$Fr Wt.).

In present investigation, zinc toxicity in groundnut seedling were measured under dual interaction of *Trichoderma* strain and heavy metals and *trichoderma* non- treated plant grown under zinc toxicity stress. The cellular damage brings change in the activities of antioxidant enzymes such as peroxidase, catalase and polyphenol oxidase (Maleva *et al*., 2012) [12].

Groundnut seedling at 2000 ppm concentration showed higher peroxidase value in control condition, while at 0 ppm show minimum value 0.134 U.A. g^{-1} Fresh weight. However decrease peroxidase value at 100 to 2000 ppm zinc concentration with *Trichoderma* inoculation (Table 1). Variation in peroxidase activity under heavy metal stress depends on biotic and abiotic stress (Tamas *et al*., 2008). Among the different zinc toxicity treatments, control leaves exhibited significantly higher catalase (0.20 to 2.41 H_2O_2 mM) as compared to higher zinc toxicity treatments. The catalase activity significantly increased as the zinc toxicity increased from 100 to 2000 ppm. But groundnut seedling inoculated with *Trichoderma* parent and fusant strain exhibited lower catalase activity as compared to control treatment (Table 2). A continuous increase in the activity of PPO in groundnut seedling from 0 to 1000 ppm than drastically decreased from 1500 to 2000 ppm in *trichoderma* treated and control condition (Table 3). Antioxidant enzyme activity in groundnut seedling under the interaction of zinc with non-treated and treated fusant *trichoderma* strain revealed that the enzyme activity in *Trichoderma* non treated plants increased with increase zinc level upto 2000 ppm. In *trichoderma* treated plants antioxidant enzyme activity was always comparatively low irrespective to non treated seedling. Its similarly observed by Nongmaithem and Bhattacharya (2017) [15] in rice plant under the interaction of cadmium and

trichoderma. Harman *et al*. (2004) [6] also observed that plant biomass was increased with inoculation of *trichoderma.*

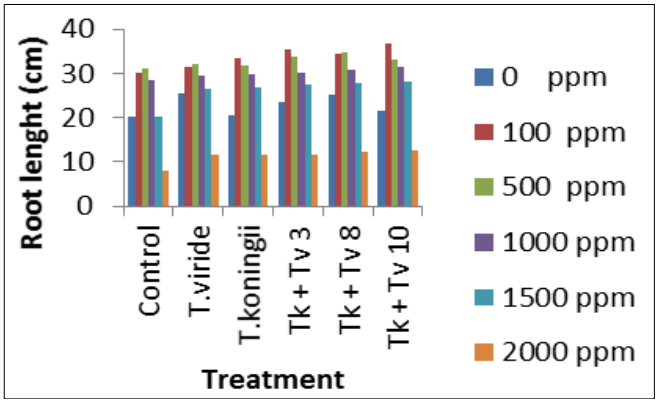

Fig 1: Effect of increasing conc. of zinc on Root length (cm plant^{-1}) of groundnut seedling with *Trichoderma* strain.

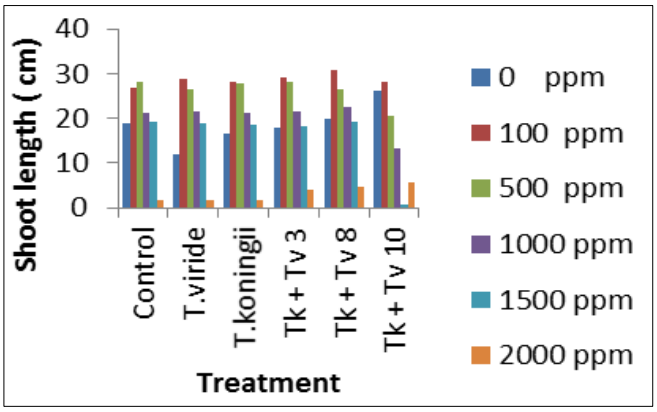

Fig 2: Effect of increasing conc. of zinc on Shoot length (cm plant $^{-1}$) of groundnut seedling with *Trichoderma* strain.

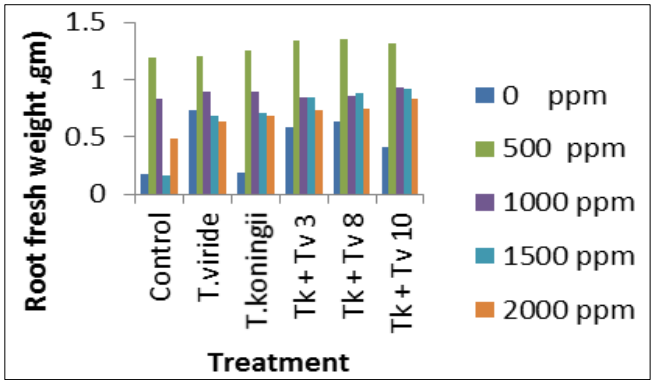

Fig 3: Effect of increasing conc. of zinc on Root fresh weight (gm plant^{-1}) of groundnut seedling with *Trichoderma* strain.

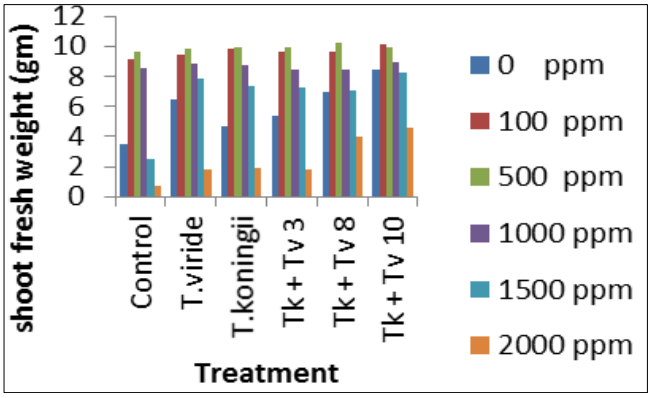

Fig 4: Effect of increasing conc. of zinc on shoot fresh weight (gm plant^{-1}) of groundnut seedling with *Trichoderma* strain.

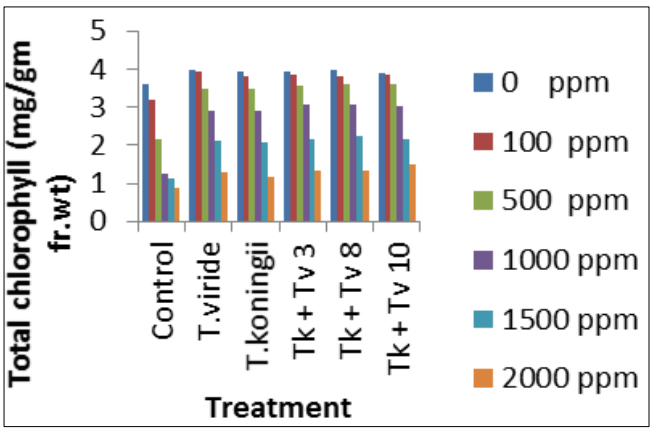

Fig 5: Effect of increasing conc. of zinc on total chlorophyll content (gm plant^{-1}) of groundnut seedling with *Trichoderma* strain.

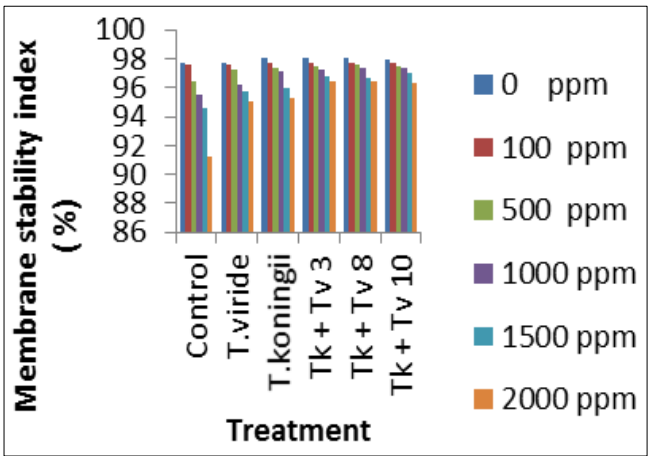

Fig 6: Effect of increasing conc. of zinc on Membrane stability Index of groundnut seedling with *Trichoderma* strain.

Table 1: Effect of different concentration of Zinc on activities of Peroxidase (U.A. g $^{-1}$ Fresh weight) in peanut leaves after 25 DAS treated with parent and fusant of *Trichoderma* strain

Concentration	Treatment						Mean C
	Control (gm)	*T.viride* (gm)	*T.koningii* (gm)	Tk+Tv 3 (gm)	Tk+Tv 8 (gm)	Tk+Tv 10 (gm)	
0 ppm	0.131	1.26	1.38	1.26	1.21	1.26	1.08
100 ppm	2.255	1.24	1.25	1.4	1.45	1.22	1.47
500 ppm	2.244	1.45	1.48	1.46	1.5	1.36	1.58
1000 ppm	4.207	1.46	1.56	1.6	1.46	1.46	1.96
1500 ppm	6.647	2.37	2.40	2.43	2.09	2.15	3.01
2000 ppm	7.808	2.63	2.46	2.56	2.66	2.18	3.38
Mean T	3.882	1.738	1.761	1.79	1.733	1.6	
S.Em±	C	0.025	T	0.025	C x T	0.061	
Cd. @ 5%	C	0.071	T	0.071	C x T	0.175	
C. V. %	5.26						

Table 2: Effect of different concentration of zinc on activities of Catalase (H_2O_2 mM Fresh weight) in peanut leaves after 25 DAS treated with parent and fusant of *Trichoderma* strain.

Concentration	Treatment						Mean C
	Control (gm)	*T. viride* (gm)	*T. koningii* (gm)	Tk+Tv 3 (gm)	Tk+Tv 8 (gm)	Tk+Tv 10 (gm)	
0 ppm	0.206	0.207	0.216	0.178	0.181	0.178	0.194
100 ppm	0.459	0.231	0.236	0.231	0.201	0.229	0.265
500 ppm	0.745	0.281	0.28	0.276	0.268	0.274	0.354
1000 ppm	1.331	0.306	0.309	0.314	0.302	0.304	0.478
1500 ppm	1.192	0.458	0.457	0.464	0.446	0.446	0.577
2000 ppm	2.41	0.692	0.645	0.612	0.619	0.613	0.932
Mean T	1.057	0.362	0.357	0.346	0.336	0.341	
S.Em±	C	0.0347	T	0.0347	C x T	0.085	
Cd. @ 5%	C	0.0984	T	0.0984	C x T	0.241	
C. V. %	4.39						

Table 3: Effect of different concentration of Zinc on activities of Polyphenol oxidase (U/ml Fresh weight) in peanut leaves after 25 DAS treated with parent and fusant of *Trichoderma* strain.

Concentration	Treatment						Mean C
	Control (gm)	*T. viride* (gm)	*T. koningii* (gm)	Tk+Tv 3 (gm)	Tk+Tv 8 (gm)	Tk+Tv 10 (gm)	
0 ppm	0.44	0.36	0.36	0.35	0.37	0.39	0.378
100 ppm	1.09	0.44	0.45	0.47	0.48	0.46	0.565
500 ppm	1.66	0.57	0.56	0.46	0.54	0.46	0.708
1000 ppm	2.07	0.70	0.66	0.56	0.59	0.61	0.865
1500 ppm	3.37	0.93	0.91	1.01	0.92	1.07	1.368
2000 ppm	4.3	1.48	1.47	1.47	1.53	1.57	1.97
Mean T	2.155	0.747	0.735	0.72	0.738	0.76	
S.Em±	C	0.0121	T	0.0121	C x T	0.085	
Cd. @ 5%	C	0.0342	T	0.0342	C x T	0.084	
C. V. %	6.37						

Conclusion

In experiment conclude that heavy metal stress decreased the plant physiochemical and biochemical properties of groundnut seedling. Zinc act as plant nutrient at certain amount but toxic at high level. Zinc toxicity involved in metal uptake and inactivation of enzymes, displacement of essential elements from functional sites (Ambler, 1970) [2], destabilized metabolic functions and not attributed to protective role in plants. The higher tolerance of groundnut seedling in plant biomass and antioxidant enzyme activity observed in fusant strain of *trichoderma* compared to parental strain. *Trichoderma* aided in induction of defence response in plant which helped in production of more biomass with interaction to plants (Gravel *et al*, 2007) [4].

Results demonstrated the significance of the protoplast fusion technique, which could successfully be used to develop superior hybrid strains in filamentous fungi that lack sexual reproduction.

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