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# Selection and characterization of novel zinc-tolerant *Trichoderma* strains obtained by protoplast fusion

**Y.S. Mazrou[1,2], B. Neha[3], U.K. Kandoliya[3], G. Srutiben[4], L. Hardik[5], A. Gaber[6], M. F. Awad[6,7] and M.M. Hassan[6,8]***

[1]Department of Business Administration, Community College, King Khaled University, 62529, Saudi Arabia
[2]Department of Agric. Economics, Faculty of Agriculture, Tanta University, 3111, Egypt
[3]Department of Biotechnology, Junagadh Agricultural University, Junagadh-362 001, India
[4]Department of Biotechnology, Saurashtra University, Rajkot-360 005, India
[5]ATMA Project, Farmer training centre, Junagadh-362 001, India
[6]Department of Biology, Faculty of Science, Taif University, 21944, Saudi Arabia
[7]Department of Botany and Microbiology, Faculty of Science, Al-azhar University, Assuit, 71511, Egypt
[8]Department of Genetics, Faculty of Agriculture, Menoufia University, 32514, Egypt

***Corresponding Author Email :** khyate_99@yahoo.com

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## Abstract

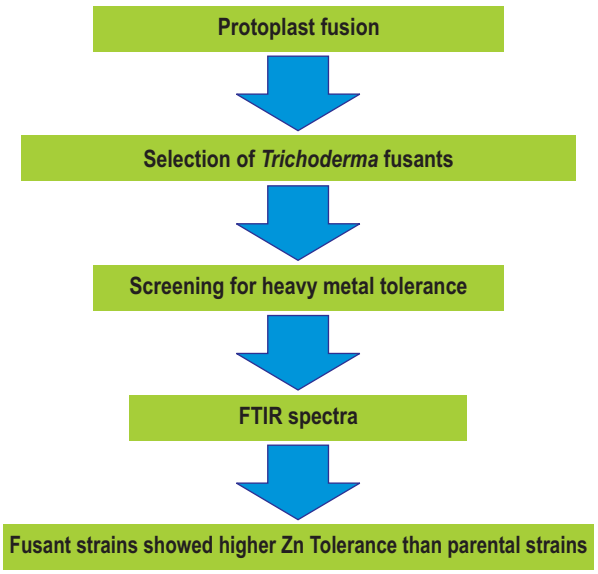
**Aim:** To develop new *Trichoderma* strains, capable of removing toxic heavy metal ions from polluted environments, via protoplast fusion.

**Methodology:** *Trichoderma* parental strains (*T. viride* and *T. koningii)* along with their ten fusants (Tk+Tv 1, Tk+Tv 2, Tk+Tv 3, Tk+Tv 4, Tk+Tv 5, Tk+Tv 6, Tk+Tv 7, Tk+Tv 8, Tk+Tv 9 and Tk+Tv 10) were obtained from the Department of Plant Pathology, Junagadh Agricultural University, Junagadh. The strains obtained by protoplast fusion were examined for their ability to remove toxic heavy metal ions, especially zinc ion. Fourier-transform infrared spectroscopy (FTIR) was conducted to detect the zinc uptake mechanism of *Trichoderma* parental and their fusant strains.

**Results:** FTIR results demonstrated the Zn ion uptake capacity of fusant strains was found to be higher than that of the parental strains (12.8 to 10.7 mg $g^{-1}$ on a dry weight basis at 1300 ppm). The highest Zn ion mobility observed was 62.1 mg. $kg^{-1}$ and the highest Zn ion mobility observed per strain was 12.4% in Tk+Tv 3, followed by 11.86 % in Tk + Tv 7, 11.84% in Tk + Tv 9 and 11.28% in Tk + Tv 10. Parental and fusant strains Tk + Tv 3, Tk + Tv 8 and Tk + Tv 10 confirmed the involvement of different functional groups for different concentrations of zinc during adsorption by the fungus.

**Interpretation:** FTIR results identified greater metal removal capacity in the fusant strains, particularly for soil Zn ion. Zinc tolerance was higher in the fusant strains than in the parental strains. Thus, protoplast fusion is an effective and feasible method for constructing new strains that can be used for bioremediation of contaminated environments.

**Keywords:** Heavy metals, Protoplast fusion, *Trichoderma* spp., Zn tolerance

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## Introduction

Increased development caused by industrialization, and new technological improvements have resulted in deterioration of environmental quality on a global scale. Heavy elements, which are natural components of the earth's crust, have been found to exist in low concentrations in the soil, in sediments, in water and even in living organisms. Therefore, additional production and release of heavy metals, from persistent inorganic matter that cannot be broken down in water, pollutes the environment. Unlike organic contaminants, heavy metal pollutants are non-biodegradable and are capable of entering the food chain via bioaccumulation (Bodar *et al.,* 2018). According to Lopez and Vazquez (2003), certain metals, like Cu, Fe, Mn and Zn are micronutrients for most organisms (though not for all) and play vital roles as metalloenzymes. Thus, researchers have resorted to the use of alternative methods of treatment, such as utilizing fungi, to remove these contaminants. A promising way to combat pollution, as suggested by Gadd (1986), is to use microbial biomass to remove toxic heavy metals as an alternative method to physio-chemical techniques or traditional waste disposal ways. Microbial (fungal) biomass in water shows higher affinity for heavy metal ions as compared to visual biomass.

Fungi can tolerate and detoxify heavy metals due to their capacity to degrade toxic substances as they can easily make large microbial biomass (Doblera *et al*., 2000); they possess several mechanisms for metal resistance, such as cell membrane metal efflux (Kamizomo *et al*., 1989), intracellular chelation by metallothionein proteins (Presta and Stillman, 1997), glutathione-derived peptides called phytochelatins (Tripathi *et al*., 2013), and metal compartmentalization in vacuoles (Pradhan *et al*., 2007). Fungal strains belonging genus *Trichoderma* possess effective soil colonization, with high biodegradation potential (Harman *et al*., 2004; Lorito *et al*., 2010). The genus *Trichoderma* is genetically diverse with a number of capabilities among different strains with agricultural and industrial importance. They are tolerant to a wide range of recalcitrant pollutants including heavy metals, pesticides and polyaromatic hydrocarbons (Lorito *et al*., 2010; Tripathi *et al*., 2013; Lakhani *et al*., 2016).

Genetic engineering and biotechnology are currently being applied in phytoremediation and are important for the design and use of green technology for heavy metal pollution control (Rupassara *et al*., 2002; Marchiol *et al*., 2007). Protoplast fusion is one of the important approaches in the fungal strain improvement technique including *Trichoderma* species (Hassan *et al*., 2011; Hassan, 2014). Fungal protoplasts are important tools in physiological and genetic research, as well as genetic manipulation which can be successfully achieved through fusion of protoplasts in filamentous fungi that lack the capacity for sexual reproduction (Srinivasan *et al*., 2009; Kushwaha and Verma, 2014). Protoplast fusion facilitates the transfer of fungal mitochondrial genomes between taxonomically related species (Zujun *et al*., 2014; Lakhani *et al*., 2016). It can be viewed as one of the recombinant DNA technology that provides the tool for increasing gene dosage and gene expression from strong promoters, deletion of unwanted genes from the fungal genome, manipulation of metabolic pathways and developing fungal strains for the production of heterologous proteins. Further, construction of mutants by fusion of protoplast is feasible and an effective technique to enhance heavy metal tolerance in fungal strains for phytoremediation techniques (Deng and Cao, 2017).

In view of the above, the aim of this study was to improve the characteristics of *Trichoderma* strains to increase their capacity of Zn metal tolerance and bioaccumulation of heavy elements using protoplast fusion technology.

## Materials and Methods

**Sample collection :** *Trichoderma* parental strains *T. viride* and *T. koningii* plus their ten fusants Tk+Tv 1, Tk+Tv 2, Tk+Tv 3, Tk+Tv 4, Tk+Tv 5, Tk+Tv 6, Tk+Tv 7, Tk+Tv 8, Tk+Tv 9 and Tk+Tv 10 were obtained from the Plant Pathology Department, Junagadh Agricultural University, Junagadh. *The* strains were then sub-cultured and preserved on fresh Potato Dextrose Agar (PDA) medium for further studies. All cultures were maintained on PDA slants at 5 °C.

**Fungal growth and determination of metal tolerance index :** Zinc is ubiquitous in the environment (Falih 1997); therefore, it was selected for this study. $Zn^{+2}$ stock solutions were prepared by dissolving zinc sulfate ($ZnSO_4$. $7H_2O$) in distilled water. The solid medium was prepared by pouring 4 ml of $Zn^{+2}$ stock solution, followed by 16 ml of sterilized PDA, into a glass bottle to obtain the desired heavy metal concentrations of 200 mg $l^{-1}$, 500 mg $l^{-1}$, 800 mg $l^{-1}$, 1100 mg $l^{-1}$ and 1300 mg $l^{-1}$. Screening of fungal growth was conducted according to Hart *et al*. (1998). Inoculated plates were incubated at 28 °C for 48 hr. Cultures without zinc served as control. Radial growth was evaluated based on four measurements (in cm) that passed through the center of the inoculated portion. The initial diameter of the portion was subtracted from the growth diameter. The mean of perpendicular diameter measurements was recorded for each plate following incubation for 48 hr. Tolerance index (TI), an indication of the organism's response to metal stress, was calculated as: growth of the strain exposed to the metal divided by growth in the control plate.

**Toxicity tests for selected fungal isolates:** Ten milliliters of stock solution of Zn was added to the medium in each 250 ml conical flask to prepare the required concentration (200, 500, 800, 1100 and 1300 mg $l^{-1}$) in a volume of 100 ml. The medium was later inoculated with a pure culture of *Trichoderma* parental strain and fusants, taken from the edge of an actively growing PDA culture (Makun *et al*., 2009). The initial concentration of zinc ions in each conical flask was checked using an Inductively Coupled

Plasma Mass Spectrometer ICPEMS (ICP-OES Model Optima 2000 DV, Perkin Elmer, United States) prior to fungal inoculation. Cultures were incubated at room temperature for seven days (28 ºC± 2 ºC, 12 hr daylight and 12 hr darkness).

**Removal at different concentrations of heavy metals :** After seven days of incubation, fungal biomass treated with different concentrations (0, 200, 500, 800, 1100 and 1300 mg $l^{-1}$) of zinc were harvested and filtered through Whatman No. 1 filter paper. Biomass samples were rinsed several times with distilled water and oven dried at 50 ºC until a constant weight was achieved, which was defined as dry biomass (g $l^{-1}$) (Doblera *et al*., 2000). Removal of zinc ions by selected fungi was calculated according to the method proposed by Lopez and Vazquez (2003). The amount of zinc uptake (q) was calculated by the following equation:

$$q = [(Ci - Cf)/m] V$$

where, q (mg $g^{-1}$) is the metal ion uptake per gram of biomass; Ci (mg $l^{-1}$) is the initial metal concentration; Cf (mg $l^{-1}$) is the final metal concentration; m (g) is the amount of dry biomass; V (l) is the volume of medium

**Effects of *Trichoderma* strains on the mobility of Zn ions in the soil :** Soil samples were prepared in laboratory with different concentrations of Zn to emulate heavy metal-contaminated soil. Samples were air-dried and sieved (1.5 mm) to remove plant residuals, soil macrofauna and stones. Soil pH was 4.5 with a $Zn^{+2}$ concentration of 500 mg $kg^{-1}$ water-soluble metals, and the samples comprised of 11% sand, 78 % silt, 11 % clay and silt loam soil (Sung *et al*., 2019). The washed soil inoculated with 0.1 g of fungal biomass (parent strain plus their ten fusant strains) was added to 1 ml of sterile water and placed on a rotator shaker (150 rpm) at 30 °C for 1 week. Deionized water was then added to each tube, and the tubes were incubated at 150 rpm, 30 °C for 4 hr to extract the water-soluble metals. The soil suspension was centrifuged at 1610 rpm for 15 min. Zn concentration in the supernatant was measured with Inductively coupled plasma mass spectrometry (ICP-MS) (Sung *et al*., 2019).

**Analysis of functional groups responsible for metal absorption by Fourier transform infrared spectroscopy (FTIR) :** Functional groups present in the fungal cell wall were studied using FTIR. Samples were prepared based on the methods of Chew *et al*. (2012). For FTIR spectroscopy, the lyophilized fungal mycelium (lyophilized at -85 ºC under high vacuum conditions using a freeze-dryer) was mixed with potassium bromide, oven-dried overnight at 100 ºC, in 1:100 ratio. FTIR spectrum of the samples was recorded with a SHIMADZU-100 FTIR instrument with a diffuse reflectance (DRS8000) accessory. All measurements were performed in the range of 500–4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$.

**Statistical analysis :** Data from three replicates were analyzed by ANOYA using SPSS Inc. 16.0 software (IBM, USA). Significant differences were calculated according to Duncan's least significant difference test or a multiple range test (Steel and Torrie, 1980). Means within a column followed by same letter were not significantly different at p = 0.05 level.

## Results and Discussion

The number of *Trichoderma* spp. are ubiquitous distributed in almost all types of crop rhizosphere and in metal-polluted ecosystems (Paremeswari *et al*., 2010). In the present study, *Trichoderma* parental strains (*T. viride* and *T. koningii)* plus their ten fusants ($Tk+Tv_1$, $Tk+Tv_2$, $Tk+Tv_3$, $Tk+Tv_4$, $Tk+Tv_5$, $Tk+Tv_6$, $Tk+Tv_7$, $Tk+Tv_8$, $Tk+Tv_9$, and $Tk+Tv_{10}$) were identified and selected for further study, based on their cultural variability and growth rates. The effects of different concentrations of Zn on mycelial growth of parental strains and ten fusants were tested (Table 1). The maximum growth rate was recorded at 200 ppm zinc, which decreased with increase in Zn concentration. The maximum radial growth occurred in the fusant $Tk+Tv_7$, followed by $Tk+Tv_8$, $Tk+Tv_3$, $Tk+Tv_9$, $Tk+Tv_6$, $Tk+Tv_{10}$ and $Tk+Tv_1$ at 1300 ppm Zn. Similar changes were also observed by Shafiquzzaman *et al*. (2013), where higher mycelial growth on Zn-amended PDA medium was seen for *T. virens* strain T128, which also displayed the highest tolerance for zinc and nickle at 1200 mg $l^{-1}$ concentration. Accumulation and uptake capacity were determined by the maximum removal of Zn, Cu, and Ni ions by *T. harzianum*. According to Gadd (1986), exposure of filamentous fungi to heavy metals could lead to physiological adaptation or selection of mutants, which may be subsequently associated with increased metal absorption capacity. Importantly, some filamentous fungi are able to grow in the presence of higher concentrations of heavy metals (Tripathi *et al*., 2013). At 100 mg $l^{-1}$ – 500 mg $l^{-1}$ concentrations of zinc, mycelial growth for *T. harzianum* strain T32 was higher than that for *T. aureoviride* strain T 121; however, this reaction peaked at this point, and mycelial growth further decreased on increasing concentrations further to 700 mg $l^{-1}$–1200 mg $l^{-1}$. A previous study had reported *T. viren* strain T128 to be the best filamentous fungus for tolerating and reducing toxicity over a wide range of concentrations of metal solutions (Carrillo-Gonzalez *et al*., 2012; Siddiquee *et al*., 2013).

Tolerance index for parental strains (*T. viride* and *T. koningii)* and their selected ten fusants were tested (Table 2). TI decreased with higher concentrations of Zn, suggesting it to be inversely proportional to Zn toxicity. The highest TI of 1.0 was achieved by $TK+TV_3$ and $TK+TV_{10}$ whereas the lowest TI of 0.53 was found in $TK+TV_9$. Mehran *et al*. (2015) had also reported the TI for each fungus, however, they found a different order of tolerance: *Aspergillus versicolor* and *Trichoderma* spp. showed the maximum cadmium tolerance, followed by *Paecilomyces* spp., *Paecilomyces* spp., and *Aspergillus fumigatus*, and *Cladosporium* spp. and *Microsporum* spp. thereafter. Rasool

**Table 1 :** Growth rate of *Trichoderma* parental strain (*T. viride and T. koningii*) and fusant strains on zinc amended PDA media at 48 hr

| Concentration of zinc | Growth rate | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. viride* | *T.koningii* | TK+TV$_1$ | TK+TV$_2$ | TK+TV$_3$ | TK+TV$_4$ | TK+TV$_5$ | TK+TV$_6$ | TK+TV$_7$ | TK+TV$_8$ | TK+TV$_9$ | TK+TV$_{10}$ | Mean C |
| 0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 |
| 200 | 8.50 | 8.81 | 8.91 | 8.61 | 9.00 | 8.95 | 8.80 | 8.71 | 8.87 | 8.94 | 8.95 | 8.98 | 8.83 |
| 500 | 8.07 | 8.52 | 8.62 | 8.13 | 8.65 | 8.49 | 8.36 | 8.54 | 8.86 | 8.76 | 8.91 | 8.46 | 8.53 |
| 800 | 7.50 | 7.10 | 7.71 | 8.05 | 8.40 | 8.21 | 7.01 | 7.94 | 7.71 | 7.87 | 7.47 | 7.87 | 7.73 |
| 1100 | 6.40 | 6.48 | 6.91 | 7.82 | 7.91 | 7.80 | 6.95 | 7.89 | 7.58 | 6.96 | 6.91 | 7.10 | 7.22 |
| 1300 | 6.10 | 6.15 | 6.24 | 6.81 | 6.84 | 6.10 | 6.70 | 6.71 | 6.87 | 6.84 | 4.75 | 6.48 | 6.38 |
| Mean T | 7.59 | 7.67 | 7.89 | 8.07 | 8.3 | 8.09 | 7.8 | 8.13 | 8.14 | 8.06 | 7.66 | 7.98 | 9 |
| SEM± | C | 0.1 | T | 0.007 | CXT | 0.026 | | | | | | | |
| CD at 5% | C | 0.029 | T | 0.021 | CXT | 0.072 | | | | | | | |

**Table 2 :** Tolerance index of *Trichoderma* parental strains and their selected fusant strains at different concentrations of zinc on PDA

| Concentration of zinc | Tolerance index | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. viride* | *T.koningii* | TK+TV$_1$ | TK+TV$_2$ | TK+TV$_3$ | TK+TV$_4$ | TK+TV$_5$ | TK+TV$_6$ | TK+TV$_7$ | TK+TV$_8$ | TK+TV$_9$ | TK+TV$_{10}$ | Mean C |
| 200 | 0.98 | 0.98 | 0.99 | 0.96 | 1.00 | 0.99 | 0.98 | 0.97 | 0.99 | 0.99 | 0.99 | 1.00 | 0.98 |
| 500 | 0.90 | 0.95 | 0.96 | 0.90 | 0.96 | 0.94 | 0.93 | 0.95 | 0.98 | 0.97 | 0.99 | 0.94 | 0.94 |
| 800 | 0.83 | 0.79 | 0.86 | 0.89 | 0.93 | 0.91 | 0.78 | 0.88 | 0.86 | 0.87 | 0.83 | 0.87 | 0.85 |
| 1100 | 0.71 | 0.72 | 0.77 | 0.87 | 0.88 | 0.87 | 0.77 | 0.88 | 0.84 | 0.77 | 0.77 | 0.79 | 0.8 |
| 1300 | 0.68 | 0.68 | 0.69 | 0.76 | 0.76 | 0.68 | 0.74 | 0.75 | 0.76 | 0.76 | 0.53 | 0.72 | 0.7 |
| Mean T | 0.82 | 0.82 | 0.85 | 0.87 | 0.9 | 0.87 | 0.84 | 0.88 | 0.88 | 0.87 | 0.82 | 0.86 | |
| SEM± | C | 0.007 | T | 0.004 | CXT | 0.015 | | | | | | | |
| CD at 5% | C | 0.019 | T | 0.012 | CXT | 0.042 | | | | | | | |

(2014) investigated the concentration of copper in the contaminated soil and water samples collected from Multan and Gujranwala. *Aspergillus versicolor* showed the highest TI of 2.88 at 100 ppm of copper present reduced in Gujranwala water, exhibiting 2 times more growth than the control group. Conversely, *Aspergillus flavus* showed a minimum TI of 0.5 at 100 ppm of copper present in the Multan water, showing less growth than the control group. Bioremediation strategies of heavy metals and other inorganic pollutants by *Trichoderma* can be divided into the following four categories; Biosorption, Bioaccumulation, Biovolatilization and Phytobial remediation (Harman *et al*., 2004; Tripathi *et al*., 2013). Moreover, El-Kassas and El-Taher (2009) investigated the removal of Cr(VI) by non-pathogenic marine fungus. *T. viride* in batch system. However, Ann *et al*. (2012) demonstrated that metal binding capacity of *Trichoderma* permits the removal of toxic metal from contaminated water through fungal biomass and heavy metal can be adsorbed onto the functional group present on the surface biomass, at binding sites present in the cellular structure.

Dry biomass of the parent strains *T. viride* and *T. koningii* and their selected fusants were obtained following 25 days of inoculation with zinc amended PDA medium. Table 3 shows that the level of dry biomass decreased with increasing initial concentration of zinc in the PDA medium. Similar observations on fungal dry biomass related to higher concentrations of heavy metals has been reported by Yazdani *et al.* (2010) and Asha *et al.* (2013). Shafiquzzaman *et. al*. (2013) observed that the dry biomass of *T. harzianum, T. virens* and *T. aureoviride* decrease with increasing initial concentrations of heavy metals over 7 days at room temperature. The current study demonstrated that the increasing concentrations of zinc to decreased the dry biomass values for each fungus. The results, therefore, indicated that the highest biomass values for each fungus occurred at a controlled concentration, while the lowest biomass values were recorded at 1300 mg $l^{-1}$ of Zn. This active process of metal removal by living cells is referred to as bioaccumulation which employs an energy dependent metal influx mechanism (Ting and Choong, 2009). Certain species of *Trichoderma* have been reported to tolerate and accumulate several heavy metals such as copper, zinc, cadmium and arsenic *in-vitro* conditions (Harman *et al.,* 2004; Le *et al*., 2006; Zeng *et al.,* 2010).

Resulted in decrease of dry biomass with increasing in zinc concentration, the fungal uptake capacity increased in all the strains. The maximum uptake of zinc from the solution occurred at 1300 mg $l^{-1}$, with a value of 12.8 mg $gm^{-1}$ for the fusant strain TK+TV$_3$, followed by TK+TV$_{10}$, TK+TV$_1$, and TK+TV$_9$ while the

**Table 3 :** Dry biomass (g $l^{-1}$) values of *Trichoderma* parental and fusant strains in different concentrations of zinc in PDB liquid medium

| Concentration of zinc | Dry biomass | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. viride* | *T.koningii* | TK+$TV_1$ | TK+$TV_2$ | TK+$TV_3$ | TK+$TV_4$ | TK+$TV_5$ | TK+$TV_6$ | TK+$TV_7$ | TK+$TV_8$ | TK+$TV_9$ | TK+$TV_{10}$ | Mean C |
| 0 | 0.42 | 0.46 | 0.38 | 0.39 | 0.51 | 0.53 | 0.49 | 0.43 | 0.39 | 0.41 | 0.46 | 0.54 | 0.45 |
| 200 | 0.32 | 0.37 | 0.32 | 0.30 | 0.42 | 0.46 | 0.35 | 0.34 | 0.29 | 0.30 | 0.34 | 0.42 | 0.35 |
| 500 | 0.30 | 0.34 | 0.30 | 0.29 | 0.38 | 0.40 | 0.28 | 0.29 | 0.26 | 0.32 | 0.31 | 0.39 | 0.32 |
| 800 | 0.26 | 0.30 | 0.27 | 0.26 | 0.34 | 0.38 | 0.26 | 0.24 | 0.22 | 0.27 | 0.29 | 0.36 | 0.28 |
| 1100 | 0.18 | 0.29 | 0.19 | 0.23 | 0.26 | 0.25 | 0.19 | 0.20 | 0.19 | 0.23 | 0.15 | 0.29 | 0.22 |
| 1300 | 0.06 | 0.05 | 0.08 | 0.11 | 0.16 | 0.14 | 0.12 | 0.13 | 0.09 | 0.06 | 0.08 | 0.10 | 0.09 |
| Mean T | 0.25 | 0.3 | 0.25 | 0.26 | 0.34 | 0.36 | 0.28 | 0.27 | 0.24 | 0.26 | 0.27 | 0.35 | |
| SEM± | C | 0.008 | T | 0.006 | CXT | 0.02 | | | | | | | |
| CD at 5% | C | 0.023 | T | 0.016 | CXT | 0.056 | | | | | | | |

**Table 4 :** *In-vitro* zinc uptake by *Trichoderma* parental and fusant strains in different concentrations of zinc in PDB liquid medium

| Concentration of zinc | Zinc uptake (mg $g^{-1}$) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. viride* | *T.koningii* | TK+$TV_1$ | TK+$TV_2$ | TK+$TV_3$ | TK+$TV_4$ | TK+$TV_5$ | TK+$TV_6$ | TK+$TV_7$ | TK+$TV_8$ | TK+$TV_9$ | TK+$TV_{10}$ | Mean C |
| 200 | 2.4 | 3.8 | 3.9 | 3.2 | 4.9 | 3.1 | 3.5 | 3.4 | 3.5 | 3.2 | 4.4 | 4.1 | 3.61 |
| 500 | 3.2 | 4.1 | 4.3 | 4.5 | 4.7 | 3.9 | 4.6 | 4.9 | 4.7 | 5.9 | 6.1 | 5.9 | 4.73 |
| 800 | 5.6 | 6.8 | 6.9 | 6.0 | 7.10 | 5.7 | 5.9 | 6.2 | 6.9 | 7.2 | 8.4 | 8.2 | 6.74 |
| 1100 | 7.5 | 9.7 | 10.6 | 9.9 | 9.8 | 8.2 | 8.4 | 8.7 | 8.6 | 9.4 | 10.3 | 10.1 | 9.26 |
| 1300 | 10.7 | 11.5 | 12.2 | 11.6 | 12.8 | 10.6 | 11.8 | 11.3 | 11.6 | 11.1 | 12.1 | 12.4 | 11.64 |
| Mean T | 5.88 | 7.18 | 7.58 | 7.04 | 7.86 | 6.3 | 6.84 | 6.9 | 7.06 | 7.36 | 8.26 | 8.14 | |
| SEM± | C | 0.11 | T | 0.07 | CXT | 0.25 | | | | | | | |
| CD at 5% | C | 0.2 | T | 0.2 | CXT | 0.71 | | | | | | | |

uptake of zinc by the parental strains *T. koningii* and *T. viride* was 11.5 and 10.7 mg $gm^{-1}$, respectively. Further, exposure of *Trichoderma atroviride* to zinc, at different concentrations ranging from 0–6000 mg $l^{-1}$, was studied by Yazdani *et al*. (2010) and it was found to exhibit a higher tolerance to Zn. The uptake capacity of *T. atroviride* ranged from 18.1–26.7 mg $g^{-1}$ in liquid medium with Zn concentrations from 500–1000 mg $l^{-1}$. *T. atroviride showed* 47.6–64% adsorption in the liquid medium and 30.4–45.1% absorption for zinc. Based on this study, 5.7–7.4% of zinc removal was considered to be the result of biomass washing. The high adsorption, relatively low absorption, and high uptake capacity of Zn suggested *T. atroviride* to be a potential bioremediator of zinc. Siddiquee *et al*. (2013) had reported an increase in metal uptake capacity for *T. harzianum, T. virens* and *T. aureoviride*. The maximum uptake of zinc from the metal solution occurred at 500 mg $l^{-1}$ with a value of 3.17 g $g^{-1}$ for *T. harzianum*, 2.17 g $g^{-1}$ for *T. virens* and 1.50 g $g^{-1}$ for *T. aureoviride.*

Inoculation of parental strains *T. viride* and *T. koningii* and their fusants improved the availability of water-soluble zinc in the soil (Table 5). The highest $Zn^{+2}$ mobility observed was 12.4% in TK+$TV_3$, followed by 11.86% (TK + $TV_7$), 11.84% (TK+TV 9) and 11.28% (TK+$TV_{10}$). Deng *et al.* (2014) had reported that protoplasts from different metal-resistant endophytic fungi could participate in interspecific fusion of protoplasts, thereby enhancing bioremediation of the contaminated soils. Three stable fusants, with resistance to Zn, were constructed by interspecific fusion of inactivated protoplasts from the endophytic *Mucor* spp. CBRF59 and Zn-resistant endophytic *Fusarium* spp. CBRF14. The fusants increased the concentration of water-soluble Zn in the soils, promoted rape growth, and the increased metal concentrations in rape. In addition to several cross peaks between 650 and 1750 $cm^{-1}$ related to variation of carbohydrates, lipids and proteins, significant cross peaks related to –OH groups were also present both in synchronous and asynchronous spectra. These findings showed that heavy metals have double toxic mechanism involving quantitative and structural modifications within living cells. Richard *et al*. (2002) reported that Cu, Zn and Pb ions seemed to bind to certain groups present on the cell surface, however, lead precipitated in an insoluble form localized to the cell membrane or cell surface (Levinson *et al*., 1996). This was even seen in the present study where black precipitate was formed in the presence of lead salts. This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl and phosophryl) of the bacterial cell wall adsorb metal cations through various mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or due to combination of such processes (Chojnacka *et al*., 2005).

**Table 5 :** Water-soluble zinc in soil treated with parental and fusants strains of *Trichoderma*

| Treatment | Water-soluble Zn (mg $kg^{-1}$) | Ratio to total Zn (%) |
|---|---|---|
| Control (water+ soil) | 40.1 | 8.02 |
| *T. viride* + soil | 48.4 | 9.68 |
| *T. harzanium* + soil | 54.2 | 10.8 |
| $TK + TV_1$ | 50.4 | 10.08 |
| $TK + TV_2$ | 49.5 | 9.90 |
| $TK + TV_3$ | 62.1 | 12.4 |
| $TK + TV_4$ | 54.8 | 10.96 |
| $TK + TV_5$ | 53.2 | 10.64 |
| $TK + TV_6$ | 56.0 | 11.02 |
| $TK + TV_7$ | 59.3 | 11.86 |
| $TK + TV_8$ | 58.6 | 11.72 |
| $TK + TV_9$ | 54.2 | 10.84 |
| $TK + TV_{10}$ | 56.4 | 11.28 |
| CD | 1.922 | 0.871 |
| SEM± | 0.657 | 0.298 |
| CV | 2.123 | 4.81 |

**Table 6 :** Frequency assignment of FTIR spectra of control *Trichoderma* parent and fusant strains after 15 days

| *T. viride* | *T. koningii* | $TK+TV_3$ | $TK+TV_8$ | $TK+TV_{10}$ | Tentative frequency assignment |
|---|---|---|---|---|---|
| 629 | 640 | - | 641 | - | S; =C-CHO in-plane deformation vibration , N-C=O deformation vibration prim. Aliphatic amide, O-H out-of-plane bending, often broad phenol |
| 1030 | 1033 | 1031 | 1033 | 1037 | S; C-O stretching vibration of c-o-h carbohydrate, s=o streching sulfoxide, nuclei acids; C-N+-C symetric stretch |
| 1242 | 1246 | 1055 | 1023 | 1054 | S-M; C-H deformation vibrationM; C-N stretching vibration, CH2 wagging vibration, c-n streching amineS; N-H and C-H deformation vibrations; one H-atom |
| 1450 | 1442 | 1444 | 1443 | 1450 | M; C-H and O-H deformation vibration; any bandsw; CH2 deformation vibration. carbohydrates |
| 1654 | 1652 | 1652 | 1655 | 1652 | V; also called imidazole I band,w-m; C=C stretching vibration, w; asym. -NH3+ deformation vibration.free amino acid |
| 2895 | 2923 | 2854 | 2853 | 2926-2854 | S-M; C-H str. vib.carboxylic acid, free amino acidM-W ; mostly broad, O-H stretching vibrationm; asymmetrically $CH_2$ stretching vibration, |
| - | 3354 | 3348- 3406 | 3353 | 3347- 409 | M; O-H stretching vibration, broad. W; N-H stretching vibration.sec.aliphatic amine |

FTIR spectra of parental and fusant strains $TK+TV_3$, $TK+TV_8$ and $TK+TV_{10}$ were highest change observed in functional groups after zinc absorption so , they selected to complete other analysis . The functional groups that were observed to be associated with adsorption of Zn by the *Trichoderma strains*, before and after treatment with Zn ions are detailed as follows: Peaks around 3600–3100 $cm^{-1}$ represented OH bond stretching vibrations as well as the acetamido group of chitin fraction. Peaks at 2925 $cm^{-1}$ and 2855 $cm^{-1}$ were attributed to asymmetric and symmetric stretching vibrations of $CH_2$, respectively. C=O stretching vibration and NH deformation (amide I) were seen at 1646 $cm^{-1}$. The peak at 1545 $cm^{-1}$ was assigned to the combination of –NH bending (amide II) and –CN stretching vibrations of protein. Peaks at 1230 $cm^{-1}$ and 1078 $cm^{-1}$ reflected $SO_3$ groups and C-O and C-N stretching vibrations. Peaks at 925 $cm^{-1}$ and 821–869 $cm^{-1}$ regions represented –OH and aromatic –CH stretching, respectively. The fingerprint region 500 $cm^{-1}$ to 700 $cm^{-1}$ represented phosphate or sulfur functional groups. Durve and Chandra (2014) reported that (FTIR) spectroscopic techniques could applied to determine the overall structural and compositional changes in some microorganism cells after heavy metal treatment. FTIR analysis showed changes in several peak positions in the spectrum pattern when microorganisms were grown in the medium containing heavy metals such as Hg, As, Zn, Pb and Cd indicating that there are functional groups as carboxyl, hydroxyl, phosphate, amino and amide, present on the cell surface of microorganisms. These functional groups may facilitate heavy metal binding on the cell surface of microorganism cells (Wenning and Scherer, 2013).

The bonding mechanism between metal ion and biomass of *Trichoderma* spp. can be determined by interpreting the infrared absorption spectrum. FTIR spectroscopy is used to analyze the changes in carbohydrates, proteins, cell walls, and their role in metal adsorption (Ann *et al*., 2012). The FTIR spectrum presented distinct peaks at 4000 to 500 $cm^{-1}$. Little

**Table 7 :** Frequency assignment of FTIR spectra of zinc (1300 ppm) loaded *Trichoderma* parental and fusant strains after 15 days

| *T. viride* | *T. koningii* | TK+$TV_3$ | TK+$TV_8$ | TK+$TV_{10}$ | Tentative frequency assignment |
|---|---|---|---|---|---|
| 630.74 | 629.78 | 630.74 | 632.0 | 629.1 | s; =C-CHO in-plane deformation vibrations; N-C=O deformation vibration prim. Aliphatic amides; O-H out-of-plane bending, often broad phenol |
| 1038.7 | 1032.92 | 1038.7 | 1037 | 1031 | s;zn cu xanthates; C-O stretching vibrations: s=o streching sulfoxide |
| 1243 | 1247 | 1243 | 1246 | 1246 | w-m; C-H asym. deformation vibration ZN—CH3s-m; C-H deformation vibrationm; C-N stretching vibrationm; CH2 wagging vibration |
| 1450 | 1441 | 1450 | 1450.52 | 1450 | s-m; aromatic ring in-plane stretching vibration P—Ars-m; C=O and C=C stretching vibrations (4 bands) BETA DIKETONE (metal chelate) |
| 1659 | 1653 | 1659 | 1650 | 1651 | s; C=O str. vib. (alpha form ) of polypeptides; C=C stretching vibration conjugateds; Amide I (C=O stretching vibration)s; Amide I (C=O stretching vibration) polypeptidev; C=C stretching vibration; A= heavy element,group with a heavy element directly attached to C=C |
| 2902 | 2919 | 2902 | 2910 | 2915 | m; NH stretching vibration p—NHs-m; C-H stretching vibration- sym Ar—CH3.V; chelate H bridge |
| 348.50 | 3350.46 | 3348.54 | 3342 | 3353 | m; N-H stretching vibration second amine |

changes in peak position of the spectrum of *Trichoderma spp*. biomass, either untreated or treated with zinc, indicated binding of zinc to amino and hydroxyl groups (Table 1, 2). Pradhan *et al*. (2007) had studied the broadband range of 3200–3550 $cm^{-1}$, which corresponded to the presence of hydroxyl groups associated with N–H bond of amino groups. Decrease in the wave-number for zinc treated biomass (1729 $cm^{-1}$) in comparison to that of untreated biomass (1743 $cm^{-1}$) highlighted the role of C=O stretching of carboxylates during metal uptake. Band position at 1630 $cm^{-1}$ in the untreated biomass belonged to N-acetyl glucosamine (polymer of the protein-peptide bond) and manifested significant variation following metal treatment. Likewise, NH of amide-II at the fingerprint region in raw biomass (1547 $cm^{-1}$) was likely supported by electrostatic bindings with negatively charged chromate ions, as evidenced by shift in band to a lower wave-number (1516 $cm^{-1}$) in the metal-treated biomass. The band at 1371 $cm^{-1}$ revealed the presence of amide-III or sulfonamide. Shifting of stretching vibrations of several other functional groups like C-C, C-O, C-O-P, and C-O-C of polysaccharides at 1234, 1150, and 1020 $cm^{-1}$ to various modes of higher and lower stretching frequencies indicated binding of zinc. The bands at < 1000 $cm^{-1}$ corresponded to the fingerprint zone of phosphate and sulfur groups. Based on the changes in bands, peak values were considered to be suggestive of metal chelation (Ann *et al*., 2012). These signify the involvement of hydroxyl groups in the binding of Zn ions. A slight change in the frequency of peaks confirm the existence of bioadsorption. In addition, the functional groups like hydroxyl, carbonyl, carboxyl, sulfonate, amide, imidazole, phosphonate and phosphodiester play important role in bioadsorption process (Pradhan *et al*., 2007). Some of these groups are present in the biomass of *Trichoderma* and have high potential to interact with heavy metal ions.

FTIR spectra has proved to be a powerful tool to comply with the purpose of comprehensive characterization. The unique characteristic of functional group presented by the spectrum shows material properties, its behavior as well as specific components represented by their functional groups. This combination provides several advantages and is being followed for quality control in sevral area such as for determining nitrogen in soil by visible and near-infrared reflectance spectroscopy (Moron and Cozzolino, 2004), monitoring crystallization procedure and solid-state examination of crystalline product (Pollanen *et al*., 2005), and measuring the kinetics reaction of adsorption processes (Zhang *et al*., 2005).

The FTIR spectra indicated that the zinc uptake capacity of *Trichoderma* fusant strain was higher than parent strain according to their functional group characteristics, peak position and band influence by metal ions. Therefore, the results of the present study highlights that genetic manipulation by protoplast fusion of *T. viride* and *T. koningii* have to be exploited to improve the phytoremediation efficiency as it provides green alternative solution for heavy metal removal from the environment, based on its growth pattern and metal tolerance index.

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