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Isolation and identification of heavy metal tolerant plant growth promoting rhizospheric *Aspergillus niger* WMzF10

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ABSTRACT:

Soil microorganisms are capable of solubilizing insoluble soil phosphate to release soluble P and making it available to plants. The potential heavy metal tolerant fungi, WMzF10 was isolated from the maize fields of Warangal district, Telangana state. Solubilized (410µg/ml) of tricalcium phosphate under optimum conditions. WMzF10 showed its plant growth promoting potential by producing indole acetic acid (47µg/ml), which was further confirmed by enhanced growth of Chilli seedlings with seed vigor index (1140) when compared to uninoculated control seed vigor index (615). WMzF10 also solubilized TCP under Heavy metals stress such as lead, chromium, nickel and cadmium in concentration ranging from 100-400µg/ml. Solubilization of P shows decrease in pH of the medium. This potential isolate WMzF10 was identified as *Aspergillus niger* by ITS regions and sequence was deposited in the GenBank, under the accession number MN907460.

Key words: Rhizosphere, Phosphate solubilization, Heavy metal, Tricalcium phosphate, *Aspergillus niger*

1.INTRODUCTION:

The world population will reach at least 9.8 billion by 2050 according to the United Nations Department of Economic and Social Affairs, projections [1] and which raises toughest challenge to Agrarian community to feed the growing population and maintain the sustainability of soil. Large quantities of fertilizers and pesticides are regularly added to soils, to combat the challenge of increased food production. These applied agrochemicals contains toxic metals (Cr, Cd, Cu, Zn, Ni, Mn, Pb and As) as impurities, an indirect source of heavy metals in soil. As these metals cannot be degraded they persist for a long period in soil [2] and hence reach many organisms that were not the intended target. An elevated level of heavy metals not only decrease soil microbial activity and crop production, but also threaten human health through the food chain [3].

Several methods have been accomplished to remediate heavy metals pollution, among them Physico-chemical (conventional) methods such as ion exchange, redox, electrochemical techniques, membrane filtration, and precipitation, are most commonly utilized [4, 5]. The disadvantages of the conventional methods are the inability of these methods to detoxify heavy metals permanently [6] in addition to the cost-effectiveness and the hazardous by-products produced by the elimination process. Microorganisms exert various kinds of

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tolerance mechanisms against heavy metals encoded by chromosomal genes or plasmids **[7].** Hence soil microbes which not only grow under heavy metal stress but also possess plant growth promotion characteristics such as P solubilization is of particular importance **[8].**

Phosphate-solubilizing fungi (PSF) are able to enhance the solubilization of insoluble phosphate (P) compounds. They also have the capacity to mobilize and increase nutrient uptake, produces IAA, organic acids and increases the efficiency of phosphate fertilizers, such as superphosphate and rock phosphate. PSF in soils, particularly filamentous fungi such as *Aspergillus* spp., and *Penicillium* spp., have been widely studied Genus **[9]**. Fungi are also known to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake **[10]**.

Therefore, the present study is focused to isolate heavy metal tolerant, phosphate solubilizing fungi, which not only increases availability of P to plant, also improved metal accumulation.

2. MATERIALS AND METHODS:

2.1 Isolation of Fungi:

Rhizospheric soil samples were collected from the agricultural fields of Maize, Sorghum and Bajra in sterile polythene bags from Warangal district of Telangana State, India. To isolate fungi, 0.1ml aliquot (10^{-2}– 10^{-4}) were spread on potato dextrose agar (PDA) media plates, supplemented with streptomycin. Plates were incubated for 72 h at 28 ±2 ºC. **[11]**

2.2 Qualitative Screening for Heavy metal tolerant phosphate solubilizing fungi:

For the selective screening of heavy metal tolerance, 5 mm disk of each fungal isolate was inoculated on National Botanical Research Institute Phosphate (NBRIP) agar medium **[12]** incorporated with 200µg/ml of lead. [7].Plates were incubated at 28±2ºC for 72h, colonies showing clear zone of phosphate solubilization was subculture and preserved. Morphologically distinct colonies were identified by cultural and microscopic studies **[13,14].**

2.3 Quantitative Estimation of phosphate solubilization:

NBRIP broth was inoculated with 5 days old 5.0 mm, mycelial disk of fungal isolates **[15]** and the flasks were incubated on rotary shaker at 150 rpm at 28±2ºC for 7 days. **[16]**The cultures were harvested by centrifugation at 8000 rpm for 15 minutes **[17].**All experiments were done in triplicates; an uninoculated flask was served as control. After incubation solubilized phosphate was estimated by Ames method**. [18]**

2.4 Quantitative estimation of IAA:

Quantitative analysis of IAA was performed by the method described by Pattern and Glick **[19].** 50ml of Potato dextrose broth (PDB) supplemented with 1mg/ml tryptophan was inoculated with 5mm disk covered with actively growing mycelium and incubated at 28±2ºC on rotator shaker, 150rpm for 5 days. After incubation each isolate sample were centrifuged and IAA was estimated in the supernatant by using colorimetric assay **[20, 21].**

2.5 Effect of PSF inoculants on *Chilli* plant in cup experiment:

PSF isolates were grown on PDA plates for 5 days and the spores were scraped and seeds were surface sterilized by dipping in 95% ethanol solution for 5 minutes, 0.2% $HgCl_2$ solution for 3 minutes and washed thoroughly with distilled water. The seeds were then left

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immersed with fungal spore suspension containing 1% carboxymethyl cellulose for 24 hours. The seeds treated with uninoculated Ringers solution, containing 1% CMC served as control. **[22].** Germination percentage was assessed as per International Seed Testing Association **[23]**

2.6 TCP solubilization under Heavy metal stress:

To evaluate metal tolerance in PSF's, 100ml NBRIP broth was prepared, supplemented with 4 different heavy metals (Pb, Cr, Ni and, Cd) with concentration of 100µg, 200µg, 300µg and 400µg/ml**.** [24] Inoculum preparation, incubation conditions and estimation of P was done as mention above**.**

2.7 Molecular Identification:

Potential PSF isolate which showed improved growth of Chilli seedlingd and solubilizes TCP under various heavy metal concentrations, was identified by 18S rRNA sequencing at Triyat Genomics, Nagpur, Maharashtra.

3. RESULTS AND DISCUSSION:

3.1 Isolation of heavy metal tolerant fungi:

Phosphorus is the second important macro nutrient after nitrogen required for overall plant physiological activities and plays vital role in crop yield, therefore termed as king-pin in agriculture system of India **[25]** Major portion of phosphorous in soil exists as insoluble inorganic phosphates that plant can't take up. Currently agricultural microbiologists are focused on soil microflora, which have the efficiency to solubilize, insoluble inorganic P of soil, making it available to plants.

Heavy metals are natural constituents of the Earth's crust but human activities have altered the balance of biochemical and geochemical cycles of some of them. **[26]** In this regard, Microbial remediation technology is one of the most promising technologies for existing-soil remediation. It has superiorities in terms of non-pollution, simple operation, low cost, sustainability, etc. Some specific functional microorganisms (phosphate-solubilizing bacteria and fungi) have been widely used in the remediation of toxic heavy metal pollute **[27].** Therefore, the role of phosphate solubilizing microbes in the plant growth promotion and heavy metal detoxification is significantly important

In contrast to other metals like zinc, copper, and manganese, lead is an exceedingly poisonous heavy metal that interferes with a variety of physiological processes in plants. which results in severe environmental and human health hazards. **[28]**

In the present study, on potato dextrose agar plate, 23 fungal colonies were isolated from Warangal District of Telangana state. Out of which seven showed the solubilization of TCP, under lead acetate stress in NBRIP agar plate assay, were sub cultured and preserved for further study.

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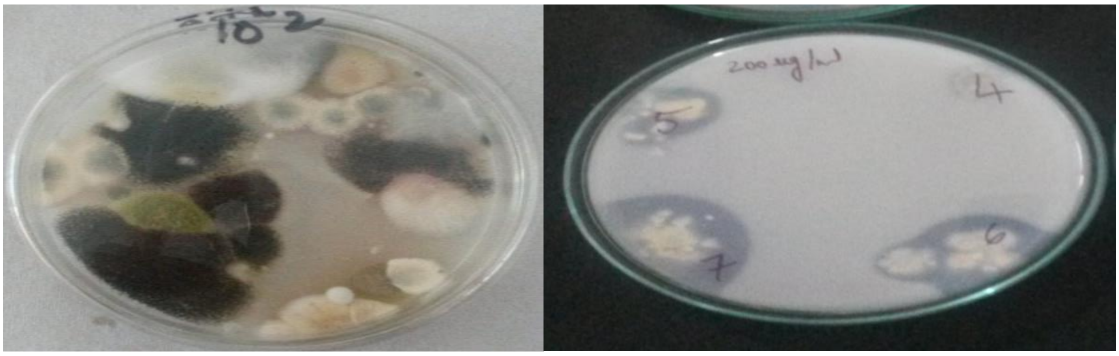

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(a) (b)

Fig 1: Screening of a) fungi from the agricultural fields of Warangal b) TCP Solubilization under lead stress

3.2 Phenotypic identification:

Based on the colony characters exhibited by the seven fungal isolates on PDA medium and on microscopic observation by wet mount staining, all isolates were identified from the genus *Aspergillus*, showing black, green, brown and cream color conidial formation.(Table 1). Due to the efficiency of *Aspergillus* spp., in root colonization, is the most frequently occurring P-solubilizing fungi in the soil. [29]

Table1: Colony characteristics of heavy metal tolerant, P solubilizing fungal isolates on PDA.

Isolates	**Growth on PDA**		**Conidial shape**	**Microscopic Observation**
	Conidial color	**Reverse**		
WMzF1	Deep brown	Colorless	Globose	*Aspergillus* sp.,
WMzF10	Black	Pale yellow	Globose	*Aspergillus* sp.,
WSgF4	Dull blue green	Yellow	Columnar	*Aspergillus* sp.,
WSgF7	Black	Pale yellow	Globose	*Aspergillus* sp.,
WBjF3	Wood brown	Dull brown	Sub Globose	*Aspergillus* sp.,
WBjF4	Blue green	Yellow	Columnar	*Aspergillus* sp.,
WBjF5	Black	Pale yellow	Globose	*Aspergillus* sp.,

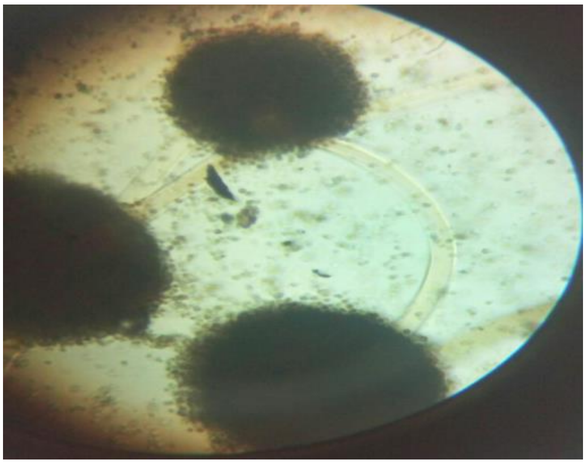

Fig 2: Microscopic observation of *Aspergillus* sp.,WMzF10

3.3 Quantitative Estimation of Phosphate solubilization:

The soil phosphate solubilizing fungi constitute about 0.1-0.5% of the total fungal population, but their biochemical stability to solubilize phosphate, traverses long distance in soil and produces more organic acids which make fungi, better P solubilizer than bacteria. **[30]** Tricalcium phosphate is used as insoluble phosphate source and all seven heavy metal tolerant isolates solubilized TCP, maximum solubilization was observed with WMzF10 410±0.22µg /ml followed by WSgF4 368±0.91 µg/ml. Isolate WBjF3 solubilized least TCP i,e 190±1.12 µg /ml. PSM are reported to dissolve insoluble phosphates by the production of organic or inorganic acids by decrease in pH. Isolate WMzF10 exhibits highest decrease in pH, 3.61±0.11 followed by WSgF4 3.92±.0.76. Our results are similar to the study of Srividya et al 2009**[31]** which suggested that, *Aspergillus* spp., showed much higher drop in pH and high P solubilization. Studies of Yasser et al **[32]**, also suggested that any microorganism that acidifies its environment will exhibit, some level of P solubilization.

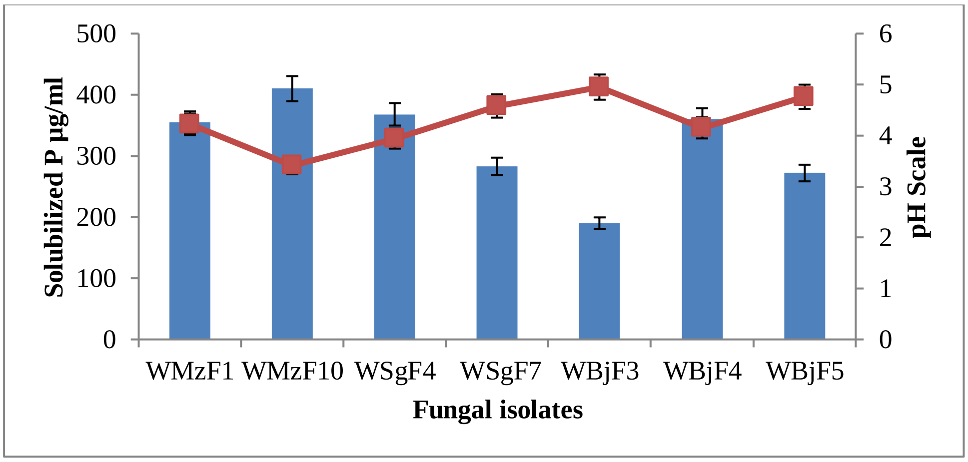

Fig 3: TCP solubilization and change in pH

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3.4. IAA production:

All fungal isolates showed the IAA production, highest IAA was produced by WMzF10, i,e 47µg/ml followed by WSgF4 38 µg/ml. Yadav et al [22] also reported the production of IAA by *Aspergillus, Trichoderma* and *Penicillium.* Koti Ratnam studies also stated, the *Aspergillus* spp., produced IAA in the rhizospheric region of Chilli plants, contributes to the plant growth and development.[33] Most root-promoting microbes synthesize IAA, which regulates various processes of plant development and growth, including enhancement of the root system that increases nutrients uptake. Ali et al., studied the effects of auxin on the growth of spinach in Cd stressed soil. The study reported that the combined inoculation of bacterial strains along with auxin enhanced the uptake of Cd up to 5.36 folds and increased fresh and dry weight of the plant by 261 and 45% respectively. [34]

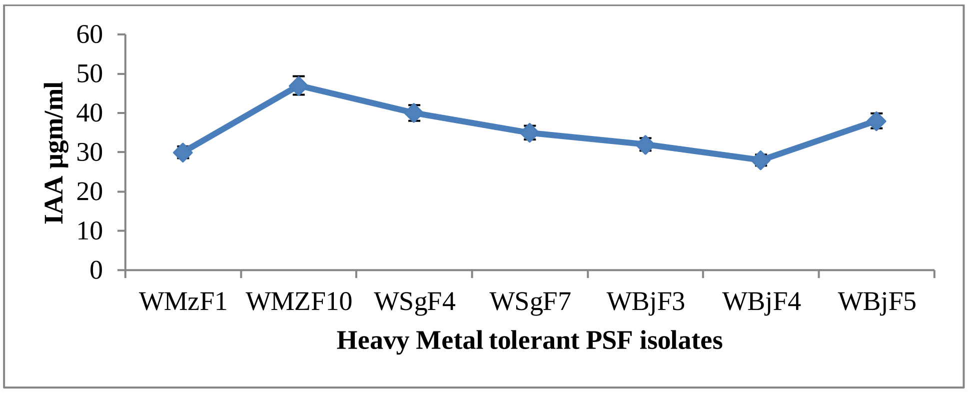

Fig 4: IAA production by Fungal isolates

3.5 Evaluation of isolates on chilli plant growth in cup experiment:

Seed or soil inoculation with PSM is known to improve solubilization of fixed soil P and applied phosphates, resulting in higher crop yield.[35]. A cup experiment was done to know the effect of isolates on Chilli (*Capsicum annum L*) plant; an improved seed vigor was observed with all isolates compare to control. Highest seed vigor index was observed with *Aspergillus* spp., WMzF10, 1140±2.3, followed by WSgF4 999±2.17, compare to control 615±2.32 (Table-2). Filamentous fungi improves growth of many other economically important crop such as Chick pea, Soya bean and Wheat[16,22,36] .Our studies are very well corroborated with the work of many researchers. Increasing attention is being paid to the use of growth promoting soil microbes for bioremediation of heavy metal pollution in soils, and to the nature and benefits of specific plant-microbe interactions. [37]

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Table 2: Effect of Heavy metal tolerant isolates on Chili plant

Isolate	Root Length(cm)	Shoot Length(cm)	Germination	Seed Vigor Index
Control	3.7±2.32	3.8±2.15	82±1.10	615±2.32
WMzF1	4.2±1.11	4.7±2.39	85±2.47	756±2.70
WMzF10	6.0±2.21	6.4±2.52	92±2.18	1140±2.34
WSgF4	5.4±2.52	5.7±1.89	90±2.43	999±2.17
WSgF7	4.9±3.29	5.2±3.12	83±2.11	838±2.63
WBjF3	5.2±2.42	5.4±2.10	91±2.45	964±2.77
WBjF4	5.1±1.37	5.6±1.72	89±2.73	952±2.54
WBjF5	5.3±1.92	5.4±2.12	90±2.79	963±2.12

Fig 5: Effect of fungal isolates on chilli plants,root development (a) Control b) WMzF10

3.6 P solubilization under heavy metal stress:

Microorganisms have developed the mechanism to cope with variety of toxic metals for their survival in the environment enriched with such metals. [38]. In present study, our fungal isolates, WMzF10 and WSgF4 exhibited potential to solubilize TCP, in NBRIP broth with four different heavy metals (lead, chromium, nickel and cadmium).*Aspergillus* sp.,WMzF10 solubilizes more TCP i,e 95, 70, 80 and 70µg/ml *Aspergillus* sp.,WSgF4, solubilized TCP 55, 45, 35 & 32 µg/ml under 400µg/ml of each metal concentration. We

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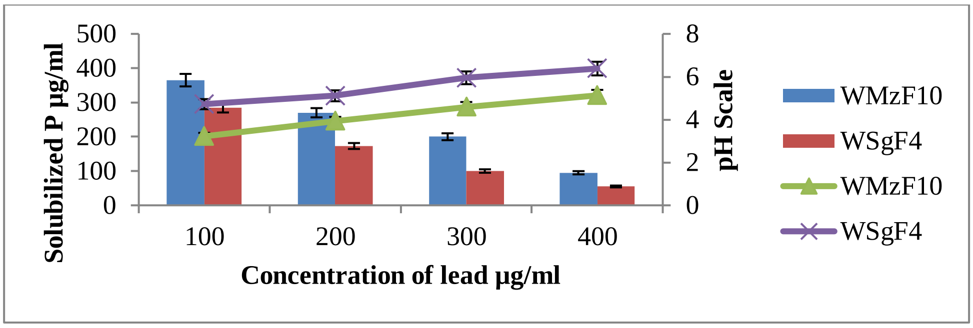
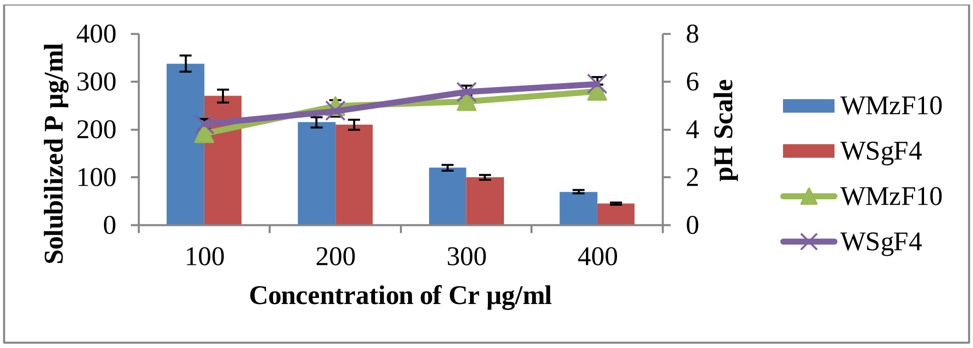
observed the reduction in pH value, with both of our isolates, during P solubilization under different metal stress. At highest metal stress, i, e 400 µg/ml less TCP solubilized, hence less reduction in pH, our findings are very well supported through the work of Oniya et al [39]. However, for WSgF4 under Nickel and Cadmium stress, final pH was observed as 6.2, however solubilized P was estimated as 42 and 30 µg/ml, (Fg 6) these results are very well corroborated with the findings of Pradhan and Shukla **[40]** it indicates that decreasing pH due to organic acid production **[16]** is not the single factor for P solubilization. H+ translocation, chelating agents, production of inorganic acids etc., also take their share of solubilizing P in soil. Various mechanisms have been proposed for the phosphate-induced immobilization of metals by microbes, which include direct metal adsorption by P compounds, phosphate anion-induced metal adsorption, direct precipitation of metals with P in solution as metal phosphates, precipitation through the liming action of rock phosphate, and rhizosphere modification through acidification [41].



(a)



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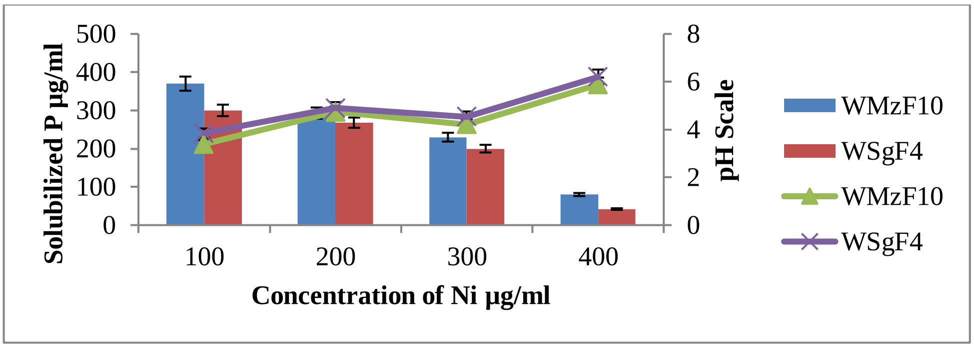

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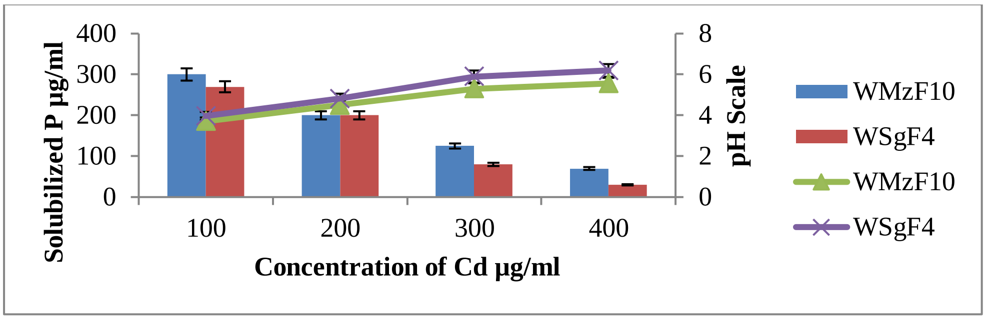

(d)

Fig 6: Solubilization of TCP, by *Aspergillus* spp., WMzF10 and WSgF4 under various Heavy metal stress(a) Lead (b) Chromium (c) Nickel and (d) Cadmium

3.7 Molecular Identification:

Based on the solubilization of TCP under various heavy metal stressed conditions and plant growth promoting traits, potential isolate, namely, WMzF10 (*Aspergillus* spp.) was selected for molecular identification [42] by 18S rRNA sequencing, showed highest similarity to *Aspergillus niger*, sequence is submitted in NCB1 GenBank with accession number MN907460 (Fig. 7).

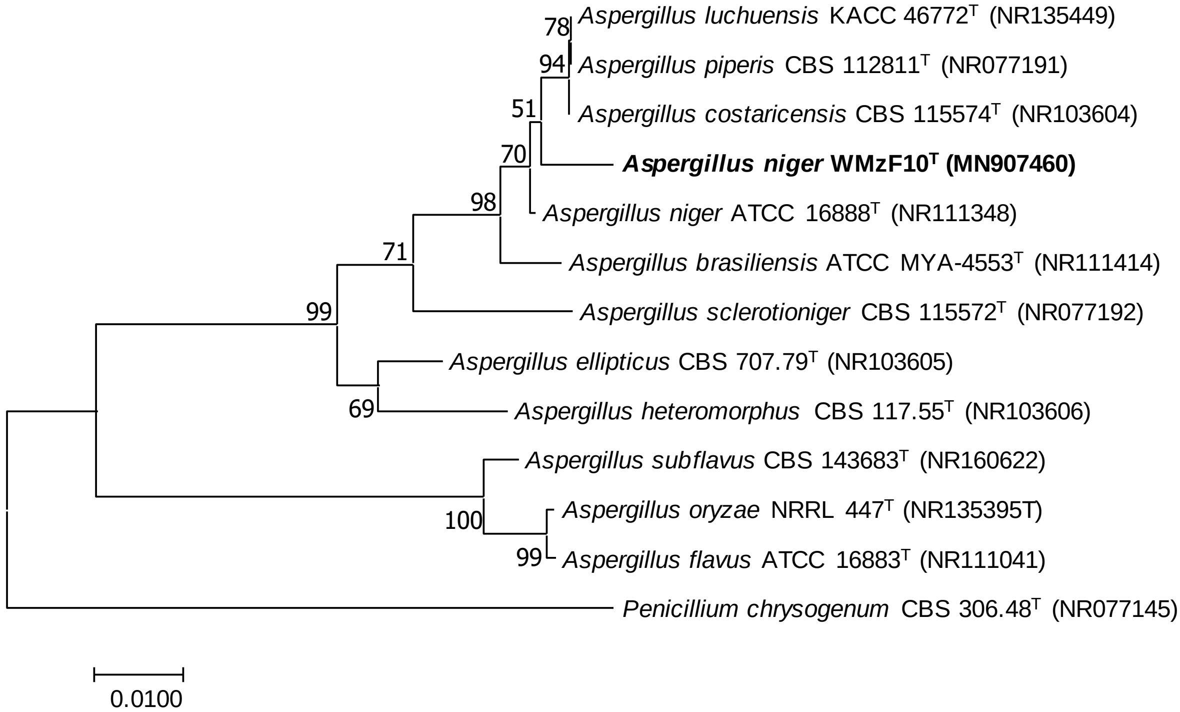

Fig 7: Phylogenetic tree of *Aspergillus niger* WMZF10

4. CONCLUSION:

. *A. niger* WMzF10, isolated from the agricultural fields of Maize, from Warangal District of Telangana state, is a potential TCP solubilizer, under various heavy metal stress, can be further evaluated as bio-inoculants, which will increase the available phosphorus, reduces heavy metal pollution and promotes sustainable agriculture.

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