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Exploring Microbial Solutions: A Comprehensive Study on Isolating, Characterizing, and Selecting Zinc-Solubilizing Fungi from Rhizospheric Soil

Pukhrambam Helena Chanu [a++*] and Janardan Yadav [a#]

[a] Department of Soil Science and Agricultural Chemistry, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, U.P., 221005, India.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

This study investigates the zinc solubilization potential of fungal isolates and their antagonistic activity against rice pathogens to address zinc deficiency and disease incidence in rice cultivation. Rhizospheric soil samples were collected, and zinc-solubilizing fungi were isolated and purified. Molecular characterization identified *Talaromyces sp, Talaromyces versatilis*, *Talaromyces pinophilus*, and *Aspergillus terreus* as zinc solubilizers. Qualitative and quantitative assays revealed varying solubilization efficiencies among isolates over time, with *Talaromyces versatilis* exhibiting the highest zinc solubilization. In inhibition assays conducted against rice pathogens,

[++] *Ph.D. Scholar;*
[#] *Head and Professor;*
**Corresponding author: E-mail: phelena@bhu.ac.in;*

fungal isolates exhibited antagonistic potential, with *Talaromyces versatilis* demonstrating the highest percentage of inhibition. These findings underscore the potential of fungi as bio-based solutions for improving zinc nutrition and disease management in rice cultivation.

Keywords: Fungal isolates; zinc solubilization; rice pathogens; disease incidence; rice cultivation; zinc nutrition; disease management; micronutrient; zinc deficiency.

1. INTRODUCTION

Zinc, a vital micronutrient for plant vitality and maturation, assumes a pivotal function in diverse physiological mechanisms such as photosynthesis, enzyme triggering, and nucleic acid metabolism [1]. Despite its importance, zinc availability to plants is often limited in soil due to factors such as low solubility and high fixation in mineral complexes [2]. This limitation can lead to zinc deficiency in plants, resulting in stunted growth, reduced yield, and susceptibility to diseases [3].

To address this challenge, various strategies have been proposed to enhance zinc availability in soil, including chemical fertilization and soil amendments [4]. However, these approaches are often costly, environmentally detrimental, and unsustainable in the long term [5]. As an alternative, tapping into the potential of microorganisms, especially fungi, for zinc solubilization has arisen as a promising and environmentally sustainable approach [6].

Fungi possess the ability to produce organic acids, siderophores, and other chelating agents that can solubilize insoluble zinc compounds, thereby making zinc more accessible to plants [7]. This process, known as zinc solubilization, not only improves zinc uptake by plants but also contributes to soil fertility and ecosystem functioning [8]. Additionally, certain fungal species have been reported to exhibit antagonistic activity against plant pathogens, further enhancing plant health and resilience [9].

In the context of rice cultivation, zinc deficiency is a prevalent issue in many rice-growing regions, leading to reduced crop yields and economic losses [10]. Moreover, rice plants are susceptible to various pathogens, including *Bipolaris oryzae* (causing brown spot disease) and *Magnaporthe grisea* (causing blast disease), which further exacerbate yield losses [11]. Therefore, there is a pressing need to explore sustainable and effective approaches to mitigate zinc deficiency and disease incidence in rice production systems.

This study aims to investigate the zinc solubilization potential of fungal isolates and their antagonistic activity against rice pathogens. By elucidating the role of fungi in zinc solubilization and disease suppression, this research seeks to contribute to the development of bio-based solutions for improving zinc nutrition and disease management in rice cultivation. Additionally, the exploration of nano ZnO as a potential tool for enhancing plant growth and disease resistance holds promise for sustainable agriculture practices.

2. MATERIALS AND METHODS

2.1 Rhizospheric Soil Sampling

Rhizospheric soil specimens were gathered from the vicinity surrounding the roots of vigorous plants, recognized for their active microbial community and significant impact on plant well-being. Plants demonstrating robust physiology were specifically chosen, and their root systems delicately removed from the earth. The gathered soil specimens were promptly deposited into sanitized freezer bags and conveyed to the laboratory in a chilled storage container to uphold their integrity. Upon arrival, thorough measures were taken to eliminate any soil adhering to the plant roots using a spatula. The purified soil specimens were subsequently placed in sterilized plastic bags within a refrigerated setting to maintain their microbial constitution until further examination.

2.2 Isolation and Purification of Zinc-Solubilizing Fungi

The isolation of zinc-solubilizing fungi was conducted using the serial dilution method followed by the pour plate technique on Bunt and Rovira agar. Dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were prepared, and the agar medium was supplemented with a composition of glucose (10.0 g), peptone (1.0 g), yeast extract (1.0 g), ammonium sulphate ($(NH_4)_2SO_4$) (0.5 g), potassium hydrogen phosphate (K_2HPO_4) (0.18 g), magnesium chloride ($MgCl_2$) (0.2 g), and distilled water (1000 mL), buffered to pH 7.2. Additionally, 15 g of agar was incorporated into

the mixture along with insoluble zinc oxide. The liquid medium was supplemented with a zinc concentration of 1000 µg Zn mL^{-1}. Fungal colonies exhibiting transparent zones indicative of zinc solubilization were selected, transferred to potato dextrose agar plates, purified, and subsequently preserved in potato dextrose agar slants at 4°C. Among the numerous colonies observed on the agar plates, four isolates displaying halo zones of varying diameters were further subjected to qualitative and quantitative assays for zinc solubilization. The methodology for isolation and purification of zinc-solubilizing fungi was adapted from previous studies [7,12].

2.3 Identifying and Characterizing Zinc-Solubilizing Fungi at the Molecular Level from Soil

Isolates of zinc-solubilizing fungi were initially identified through visual examination, focusing on features like colour and mycelial structure. Following this, molecular identification was conducted, involving the sequencing of specific genetic markers. Partial sequencing of the 18S rRNA gene and full sequencing of Internal Transcribed Sequence 1 (ITS-1) and Internal Transcribed Sequence 2 (ITS-2) were carried out. For amplification, universal primers ITS1 and ITS4 were employed [13,14]

To further characterize the fungal isolates at the molecular level, liquid cultures were prepared using mineralic Czapek's Dox Broth medium. Fresh mycelia were obtained for DNA extraction through centrifugation and subsequent mechanical disruption in liquid nitrogen. Genomic DNA was then extracted from the fungal mycelia using a DNA Purification Kit. The resulting rDNA sequences were submitted to the GenBank database of the National Center for Biotechnology Information (NCBI).

2.4 Qualitative Assay of Zinc Solubilization

Fungal isolates (ZSF-1, ZSF-2, ZSF-3, and ZSF-4) were cultured on Bunt and Rovira agar medium containing 0.1% of various zinc compounds, such as zinc oxide (ZnO), {[Zn(CO3)2] [Zn(OH)2]3}, and Zn3(PO)4 and placed in an optimal environment for fungal growth. After the specified incubation period, the dimensions of the fungal colonies and the extent of the clear zone surrounding each colony were assessed to determine zinc solubilization effectiveness. The appearance of clear zones signified the dissolution of insoluble zinc compounds. Each trial was replicated thrice for each fungal isolate, and the average measurements were computed to guarantee the precision and dependability of the outcomes. This qualitative assay methodology for zinc solubilization was adapted from previous studies [15,16].

2.5 Quantitative Assay of Zinc Solubilization

Fungal isolates were cultured in Bunt and Rovira liquid medium containing zinc of 1000 μg/ml. The cultures were incubated under optimal conditions, with periodic sampling on the 4th, 7th, 10th, and 14th days. The solubilized zinc in the liquid medium was quantified using spectrophotometric methods, and pH changes were monitored as an indicator of microbial activity [12,9].

2.6 Inhibition Assay Against Rice Pathogens

The antagonistic activity of fungal isolates against *Bipolaris oryzae* (brown spot) and *Magnaporthe grisea* (blast) was evaluated using *in vitro* inhibition assays. Fungal isolates were co-cultured with the respective pathogens on agar plates, and the inhibition percentage was determined based on the reduction in pathogen growth compared to control plates without fungal isolates [17,18].

2.7 Statistical Analysis

The statistical analysis entailed computing means, standard errors of the mean (SEm), and critical differences (CD) at the 0.05 significance level. This was accomplished through one-way analysis of variance (ANOVA), followed by post-hoc pairwise mean comparisons, which were conducted manually using MS Excel.

3. RESULTS AND DISCUSSION

3.1 Identifying and Characterizing Zinc-Solubilizing Fungi at the Molecular Level from Soil

The fungal isolates, identified as *Talaromyces sp* (F1), *Talaromyces versatilis* (F2), *Talaromyces pinophilus* (F3), and *Aspergillus terreus* (F4), were characterized based on morphological parameters such as colour and mycelial structure. The genetic profiling of the fungal

isolates involved sequencing a segment of the 18S rRNA gene and fully sequencing Internal Transcribed Sequence 1 (ITS-1). The sequences obtained were deposited in the NCBI GenBank, and their accession numbers are provided in Table 3. Comparative analysis was conducted using the Basic Local Alignment Search Tool (BLAST) on NCBI. (http://ncbi.nlm.nih.gov).

3.2 Analysis of Evolutionary Relationships Using Molecular Phylogenetics

The evolutionary relationships depicted in Fig. 3 were inferred using the Neighbor-Joining method [19]. A bootstrap consensus tree, based on 1000 replicates (Felsenstein, 1985),[20] was employed to illustrate the evolutionary connections among the examined taxa [20]. Branches representing partitions supported by less than 50% bootstrap replicates were condensed. Evolutionary distances were calculated using the Maximum Composite Likelihood method [21] and expressed as the number of base substitutions per site. This analysis included 39 nucleotide sequences, with all ambiguous positions removed for each sequence pair (pairwise deletion option). The final dataset comprised 1391 positions. The evolutionary analyses were conducted using MEGA11 [21].

3.3 Qualitative Assay of Zinc Solubilization

The qualitative assay of zinc solubilization reveals distinct patterns among the fungal isolates (ZSF-1, ZSF-2, ZSF-3, and ZSF-4) when exposed to different zinc compounds. ZSF-1 and ZSF-2 exhibited efficient solubilization of ZnO and $Zn_3(PO_4)_2$, as evidenced by significant halo zone development and high zinc solubilization efficiency percentages of 322.22 % and 344.44 % in ZnO, 172 % and 180 % in $Zn_3(PO4)_2$, 185.37 % and 328 % in $ZnCO_3$ respectively. In contrast, ZSF-3 and ZSF-4 displayed lower solubilization efficiencies, particularly in the case of $ZnCO_3$ (no solubilization), 241.67 % and 225 % in ZnO, 50% and 27.8 % in $Zn_3(PO4)_2$ respectively. The observed variations in solubilization efficiency could be attributed to the inherent metabolic capabilities of each fungal isolate. Parallel observations reported by Khan et al. [7] and Sundaramoorthy et al. [12] emphasizing the strain-specific nature of zinc solubilization in fungi. Furthermore, the substantial increase in colony diameter and halo zone diameter for ZSF-2 indicates a robust capacity for zinc solubilization, aligning with the findings of Kim et al. [16]. However, the absence of solubilization for ZSF-3 and ZSF-4 with $ZnCO_3$ might be attributed to their limited ability to metabolize this specific zinc compound, as suggested by Rajkumar et al. [15]. Hence, the qualitative assay elucidates the diverse zinc solubilization potentials among the fungal isolates, underscoring the importance of strain-specific interactions with different zinc compounds.

3.4 Quantitative Assay of Zinc Solubilization

The quantitative assay of zinc solubilization provides insights into the temporal dynamics of zinc release by the fungal isolates over a 14-day period. Notably, all isolates exhibited a consistent trend of increasing zinc solubilization over time. ZSF-2 displayed the highest zinc solubilization capacity, with a substantial increase from the 4th day to the 14th day. The observed trend aligns with the findings of Sharma et al. [22] who reported a similar temporal increase in zinc solubilization by fungal isolates. Interestingly, ZSF-3 exhibited a slightly lower zinc solubilization rate compared to ZSF-2 but displayed a consistent and significant increase in solubilization over the duration of the assay. This pattern suggests a gradual and sustained zinc solubilization ability, as reported by Basha et al. [23]. Conversely, ZSF-1 and ZSF-4 displayed relatively lower zinc solubilization capacities, with marginal increases observed over the assay period. This observation aligns with the findings of Gupta et al. [24] who noted variations in zinc solubilization efficiency among fungal isolates. So, the quantitative assay underscores the temporal dynamics of zinc solubilization by fungal isolates, highlighting variations in solubilization capacities and temporal patterns among the tested isolates.

3.5 Inhibition Assay Against Rice Pathogens

The *in vitro* antagonistic activity of fungal isolates against rice pathogens *Bipolaris oryzae* and *Magnaporthe grisea* was evaluated. *Talaromyces versatilis* (F2) demonstrated the highest inhibition percentage 62.37% against *Bipolaris oryzae* and 58.30% against *Magnaporthe grisea*, consistent with findings by Khan et al. (2019). F1 (*Talaromyces sp*) also exhibited significant

inhibition of 57.83% against *Bipolaris oryzae and* 55.43% against *Magnaporthe grisea*, which in line with Zhao et al. [25]. Conversely, F3 (*Talaromyces pinophilus*) showed lower inhibition percentages of 53.83% against *Bipolaris oryzae* and 52.93% against *Magnaporthe grisea* and F4 (*Aspergillus terreus*) showed 54.33% against *Bipolaris oryzae* and 53.67% against *Magnaporthe grisea*, as observed by Liu et al. [26-28].

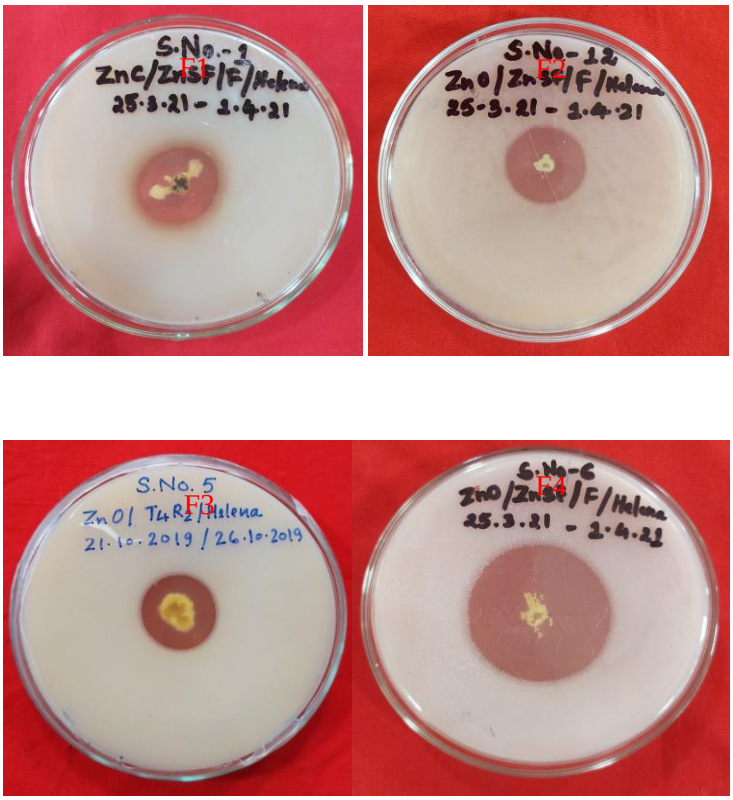

Fig. 1. Isolates (F1, F2, F3, F4) showing halo zone in the Bunt and Rovira agar medium containing 0.1% ZnO

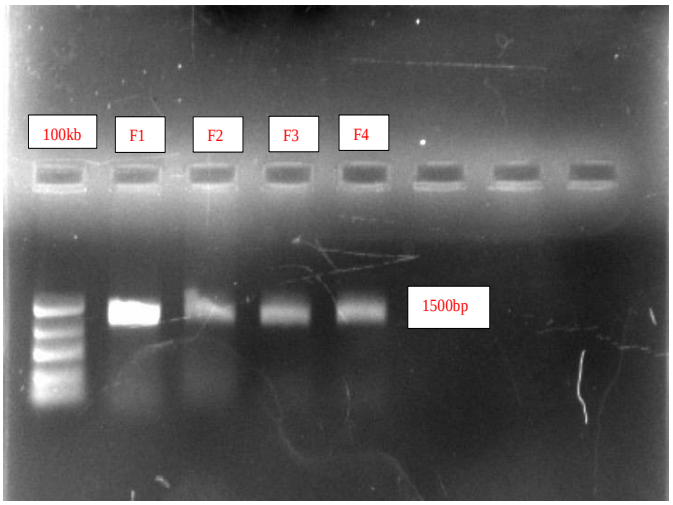

Fig. 2. Amplified DNA of 4 selected fungal strains (100kb ladder) after molecular characterization

(Index: F1 (Talaromyces sp), F2(Talaromyces versatilis), F3(Talaromyces pinophilus), F4 (Aspergillus terreus))

Table 1. Qualitative assay of zinc solubilization in bunt and rovira agar medium supplemented with different zinc compound

Isolates	ZnO solubilization (1000 μg ml^{-1})			$Zn_3(PO4)_2$ solubilization (1000 μg ml^{-1})			$ZnCO_3$ solubilization (1000 μg ml^{-1})		
	Colony diameter (mm)	Halo Zone Diameter (mm)	Solubilization efficiency (%)	Colony diameter (mm)	Halo Zone Diameter (mm)	Solubilization efficiency (%)	Colony diameter (mm)	Halo Zone Diameter (mm)	Solubilization efficiency (%)
ZSF-1	9 ±0.08*	29±0.6	322.22±2.3	6±0.1	10.3±0.8	172±2.3	8.2±0.1	15.2±0.5	185.37±3.2
ZSF-2	9±0.1	31±0.9	344.44±1.1	5±0.2	9±0.6	180±5.1	6.1±0.2	20±1.8	328±3.2
ZSF-3	12±0.4	29±0.9	241.67±5.1	20±0.6	10±1.1	50±6.7	_	_	_
ZSF-4	16±0.54	36±0.8	225±2.7	18±0.5	5±0.2	27.8±2.3	_	_	_

**All the values are the mean of three replications (mean±Sem=3)*

Table 2. Quantitative assay of zinc solubilization in bunt and rovira liquid medium supplemented with Zn at 1000 µg/ml

Isolates	4th day		7th day		10th day		14th day	
	Zn solubilized (mg/L)	pH changed	Zn solubilized (mg/L)	pH changed	Zn solubilized (mg/L)	pH changed	Zn solubilized (mg/L)	pH changed
ZSF-1 (*Talaromyces sp)*	128.8±1.5*	5.5±0.11	151±2.0	5.6±0.22	210.4±1.5	5.4±0.08	200.6±0.0	4.5±0.18
ZSF-2 *(Talaromyces versatilis)*	139.8 ± 1.1	5.8±0.13	168.5±2.2	5.6±0.03	222.1±2.1	5.1±0.04	211.1±2.3	4.5±0.14
ZSF-3 (*Talaromyces pinophilus*)	104.7±1.3	7.1±0.19	140.8±1.4	6.5±0.12	203.8±2.9	6.8±0.26	191.5±2.5	7.0±0.08
ZSF-4(*Aspergillius terreus*)	91.3±2.4	5.8±0.16	124.6±2.1	5.0±0.05	144.6±1.1	5.0±0.13	142.2±1.9	5.0±0.03

**All the values are the mean of three replications (mean±Sem=3)*

Table 3. NCBI gen bank accession numbers along with fungal isolate identities

Fungal Isolates	NCBI GenBank Accession No.	Identity
F1	OR063967	*Talaromyces sp*
F2	OR063966	*Talaromyces versatilis*
F3	OR039063	*Talaromyces pinophilus*
F4	OR063965	*Aspergillius terreus*

Table 4. *In vitro* antagonistic activity of isolates against two major rice pathogens

Isolate	Inhibition per cent	
	***Bipolaris oryzae* (Brown spot)**	***Magnaporthe grisea* (Blast)**
F1 (*Talaromyces sp*)	57.83 ±1.47*	55.43±0.57
F2(*Talaromyces versatilis*)	62.37±0.49	58.30±1.37
F3(*Talaromyces pinophilus*)	53.83±1.56	52.93±0.68
F4 (*Aspergillus terreus*)	54.33±1.68	53.67±2.15
SEm	2.26	0.69
CD (0.05)	1.8	1.9

**All the values are the mean of three replications*

Fig. 3. Phylogenetic tree showing genetic relationship between the isolates *Talaromyces sp, Talaromyces versatilis, Talaromyces pinophilus* and *Aspergillus terreus* and other closely related reference microorganisms

4. CONCLUSION

Our study highlights the diverse zinc solubilization potentials of fungal isolates and their antagonistic activity against rice pathogens. *Talaromyces versatilis* emerged as a promising candidate for both zinc solubilization and disease suppression. These findings aid in the advancement of environmentally sustainable approaches for improving zinc availability and disease resistance in rice cultivation. Further research is warranted to elucidate the underlying

mechanisms driving zinc solubilization and pathogen inhibition by fungal isolates, paving the way for sustainable agriculture practices.

5. FUTURE SCOPE

Future research avenues include exploring the synergistic interactions between fungal isolates and other soil microbes to optimize zinc solubilization and disease suppression. Additionally, investigating the potential of nano ZnO as a tool for enhancing plant growth and disease resistance warrants further exploration. Integration of fungal-based biofertilizers and nano ZnO formulations into agricultural practices holds promise for sustainable and efficient management of zinc deficiency and diseases in rice cultivation. Further studies elucidating the ecological implications and long-term effects of these bio-based interventions are essential for their successful implementation in agricultural systems.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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