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Isolation and identification of zinc solubilizing fungal isolates from agricultural fields

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Zinc is an essential micronutrient required for the normal healthy growth of the plants. It plays a key role as an enzyme cofactor in many important physiological and biochemical functions of the plants. Though it is required in smaller quantity, its unavailability to plants retards its growth. Due to various soil factors such as high pH, low organic matter, high phosphorus content, soil moisture and temperature, zinc content in soil remains low (Alloway 2009). It is documented that around 30% of the world's soils are zinc deficient (http://documents.crinet.com/AgSource-Cooperative-Services/Locations/F-04116-12—Zinc-FS-Lincoln.pdf). Synthetic fertilizer applied to overcome zinc deficiency also becomes unavailable within seven days of application (Nithya 2009). Some soils, despite having fair quantity of zinc cannot support plant growth because of poor availability of zinc. Thus it becomes vital to improve bioavailability of zinc to plants and to reduce the application of zinc fertilizer by the usage of bio-inoculants will be an alternative ecofriendly approach. Rhizospheric microflora plays an important role in the transformation of unavailable form of metals into available form (Lovely 1991). Many fungi are capable of solubilizing metals from insoluble forms thereby increasing their availability to plants (Sayer *et al*. 1999). The release of organic acids that sequester cations and acidify the micro environment near roots is thought to be a major mechanism of solubilization of zinc by fungi (Cunningham and Kuiack 1992).

In the present study, zinc solubilizing fungus (ZSF) were isolated from five (Groundnut field, tomato field, chilli field, maize and sorghum) different agricultural fields of Tirupur District using modified Bunt and Rovira agar medium containing 0.1% of three insoluble sources of zinc (ZnO, $ZnCO_3$, $Zn(PO_4)_3$) by plate count method. The study clearly shown the presence of zinc solubilizing fungi in all the soil samples collected and the population was limited to 7-37% of the total fungal count depending upon the soil type and three insoluble sources of zinc (ZnO, $ZnCO_3$, $Zn(PO_4)_3$). The results also showed that highest population of zinc oxide (ZnO) solubilizing fungus was found in (37.13 %) groundnut field, zinc carbonate ($ZnCO_3$) solubilizing fungus (23.89 %) and zinc phosphate ($Zn(PO_4)_3$) solubilizing fungus (22.99%) in tomato field. Among the collected soils, maximum number of zinc solubilizing fungi (ZSF) was recorded in the soil samples of groundnut field and the minimum numbers of ZSF were recorded in the soils of chilli field (Table 1). About 16 zinc solubilizing fungal strains were isolated and they were designated as ZSF- 1, ZSF-2, ZSF-3…….ZSF-16 based on their morphological variation.

The solubilization efficiency of 16 fungal isolates was tested by means of Plate assay method. The isolates were inoculated on Bunt and Rovira agar medium containing 0.1% of ZnO, $ZnCO_3$, $Zn(PO_4)_3$ and incubated at 30°C for 48h. By measuring the diameter of clear zone and organism's growth Zn solubilization efficiency was tested (Nyugen *et al*. 1992). Zinc solubilizing potential varied with each isolates (Table 1). The ZSF-9 strain produced highest solubilization efficiency 305.56% in ZnO containing medium. The ZSF-16 strain produced maximum solubilization efficiency 143.24% in $ZnCO_3$ containing medium. The ZSF-10 strain produced highest solubilization efficiency 178.33% in $Zn(PO_4)_3$ containing medium.

Based on the plate assay, five strains were selected and were used for further studies such as identification using molecular markers (18S rRNA), quantitative estimation of zinc and IAA, determining pH change of the medium and analysis of gluconic acid production using HPLC method.

Amplification and sequencing of target regions within the ribosomal RNA gene complex has emerged as a useful adjunctive tool for the identification of fungi and does not depend on mold sporulation for identification (Schwarz *et al*. 2006). The internal transcribed spacer (ITS) regions the highly conserved 18S ribosomal subunit genes in the rRNA operon are known to have sufficient sequence variability to allow identification to the species level for many fungi (Brandt *et al*. 2005 and Schwarz *et al*. 2006). Thus five fungal strains were identified using 18S rRNA universal

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Table 1 Total zinc solubilising fungi in soil samples

Soil sample collected from agricultural fields	Zinc source in the medium	Total fungi (CFU×10^3/g)	ZSF (CFU×10^3/g)	% of ZSF
Ground nut field	ZnO	10.67 ± 1.53	4.00 ± 1.00	37.13 ± 4.22
	$ZnCO_3$	11.67 ± 4.93	2.33 ± 1.15	19.37 ± 2.44
	$Zn(PO_3)_4$	16.67 ± 7.23	3.00 ± 1.73	17.36 ± 2.38
Tomato field	ZnO	16.67 ± 7.23	4.33 ± 1.53	25.69 ± 7.31
	$ZnCO_3$	23.33 ± 2.31	5.67 ± 2.08	23.89 ± 6.38
	$Zn(PO_3)_4$	17.33 ± 0.58	4.00 ± 1.00	22.99 ± 5.09
Chilli fields	ZnO	17.00 ± 6.08	3.00 ± 1.73	16.84 ± 3.50
	$ZnCO_3$	12.00 ± 1.00	1.33 ± 0.58	10.92 ± 3.88
	$Zn(PO_3)_4$	13.00 ± 4.36	2.67 ± 1.15	20.14 ± 2.01
Maize	ZnO	18.00 ± 1.73	5.33 ± 1.53	29.73 ± 8.01
	$ZnCO_3$	14.67 ± 5.51	2.00 ± 1.00	13.59 ± 4.98
	$Zn(PO_3)_4$	21.33 ± 5.51	1.67 ± 0.58	7.68 ± 0.86
Sorghum	ZnO	13.33 ± 5.13	4.67 ± 2.08	34.48 ± 2.04
	$ZnCO_3$	10.67 ± 1.15	2.33 ± 0.58	21.67 ± 2.89
	$Zn(PO_3)_4$	16.67 ± 6.43	1.67 ± 1.15	9.31 ± 2.82
SEd				3.59592
CD (P<0.05)				7.34393

Values are mean ± SD of three samples in each group

primer as *Emericella rugulosa* (ZSF-2), *Penicillium citrinum* (ZSF-5), *Aspergillus candidus* (ZSF-7), *Aspergillus terreus* (ZSF-9), *Aspergillus niger* (ZSF-16) (Fig 1).

The five best isolates were screened for their Zn solubilizing efficiency by growing them in 50ml of Bunt and Rovira broth medium supplemented with 0.1% ZnO, $ZnCO_3$ and Zn $(PO_3)_4$. To this, 0.5mm fungal disc from 7 days old culture was inoculated into the medium. Appropriate uninoculated controls were maintained. All the treatments were replicated. The fungal cultures were withdrawn after 6, 8 and 10 days of incubation at room temperature for the estimation of soluble Zn. The fungal cultures were centrifuged at 10000 rpm for 20min and filtered through Whatman No. 42 filter paper. The supernatant was passed through 0.2µm membrane filter so as to obtain the culture filtrate containing only the soluble forms of metal (Francis *et al*. 1988). The concentration of soluble zinc in the culture filtrate was analysed by Atomic absorption spectrometer (AAS). The higher zinc solubilization was recorded by the isolate *Aspergillus terreus* (ZSF-9) in all the three insoluble sources of Zn (ZnO-27.94 mg/l), ($ZnCO_3$-19.52 mg/l) and ($ZnPO_4)_3$-27.04 mg/l). *Aspergillus niger* (ZSF-16) recorded the low solubility of ZnO (7.48 mg/l), whereas *Aspergillus candidus* (ZSF-7) showed minimum solubilization of $ZnCO_3$ (7.69 mg/l) and Zn $(PO_4)_3$ (7.52 mg/l) (Table 2). This might be due to the potential of fungal organism to produce organic acids which could solubilize complex metal cations (Naz *et al*. 2005).

The effect of zinc solubilizing fungi on the pH of the medium was estimated using Elico pH meter. Among the five isolates *Aspergillus terreus* (ZSF-9) showed the lowest pH in ZnO (2.9) and $ZnCO_3$ (3.2), whereas *Aspergillus niger* (ZSF-16) showed the pH of 3.5 in Zn $(PO_4)_3$ after 10 days of incubation. Decrease in the pH is due to the acidity of the medium proportional to acidic pH of the culture broth (Desai *et al*. 2012).

Table 2 Zinc solubilizing potential of the fungal isolates in broth culture

Isolates	Zinc source in the medium	Amount of zinc present in the cultural filtrates (mg/l)		
		6 DAI	8 DAI	10 DAI
ZSF-2	ZnO	12.29 ± 0.16	14.26 ± 0.53	18.50 ± 1.32
	$ZnCO_3$	10.96 ± 0.49	11.52 ± 0.02	12.22 ± 0.49
	Zn $(PO_3)_4$	12.45 ± 0.44	13.45 ± 0.45	13.74 ± 0.30
ZSF-5	ZnO	14.07 ± 0.48	14.92 ± 1.81	14.94 ± 0.52
	$ZnCO_3$	8.42 ± 0.18	11.30 ± 0.30	11.10 ± 0.45
	Zn $(PO_3)_4$	6.50 ± 0.20	7.03 ± 0.45	7.53 ± 0.21
ZSF-7	ZnO	10.42 ± 0.49	17.86 ± 1.21	20.63 ± 3.40
	$ZnCO_3$	5.75 ± 0.09	6.38 ± 0.09	7.69 ± 0.53
	Zn $(PO_3)_4$	6.26 ± 0.10	7.91 ± 0.32	7.52 ± 0.33
ZSF-9	ZnO	17.50 ± 1.49	21.09 ± 1.64	27.82 ± 4.05
	$ZnCO_3$	14.24 ± 0.77	18.66 ± 1.85	19.52 ± 0.24
	Zn $(PO_3)_4$	17.31 ± 0.94	24.01 ± 4.16	27.04 ± 3.94
ZSF-16	ZnO	6.54 ± 0.22	6.64 ± 0.10	7.48 ± 1.05
	$ZnCO_3$	7.53 ± 0.10	9.62 ± 0.21	10.81 ± 0.23
	Zn $(PO_3)_4$	11.97 ± 0.25	15.33 ± 0.53	16.14 ± 0.77
SEdCD (P<0.05)			1.10539	
			2.19607	

*DAI-Days after Incubation. Values are mean ± SD of three samples in each group

The gluconic acid produced by zinc solubilizing fungi in the presence of ZnO was determined by using HPLC. 20µl culture filtrate of the isolates was injected in HPLC

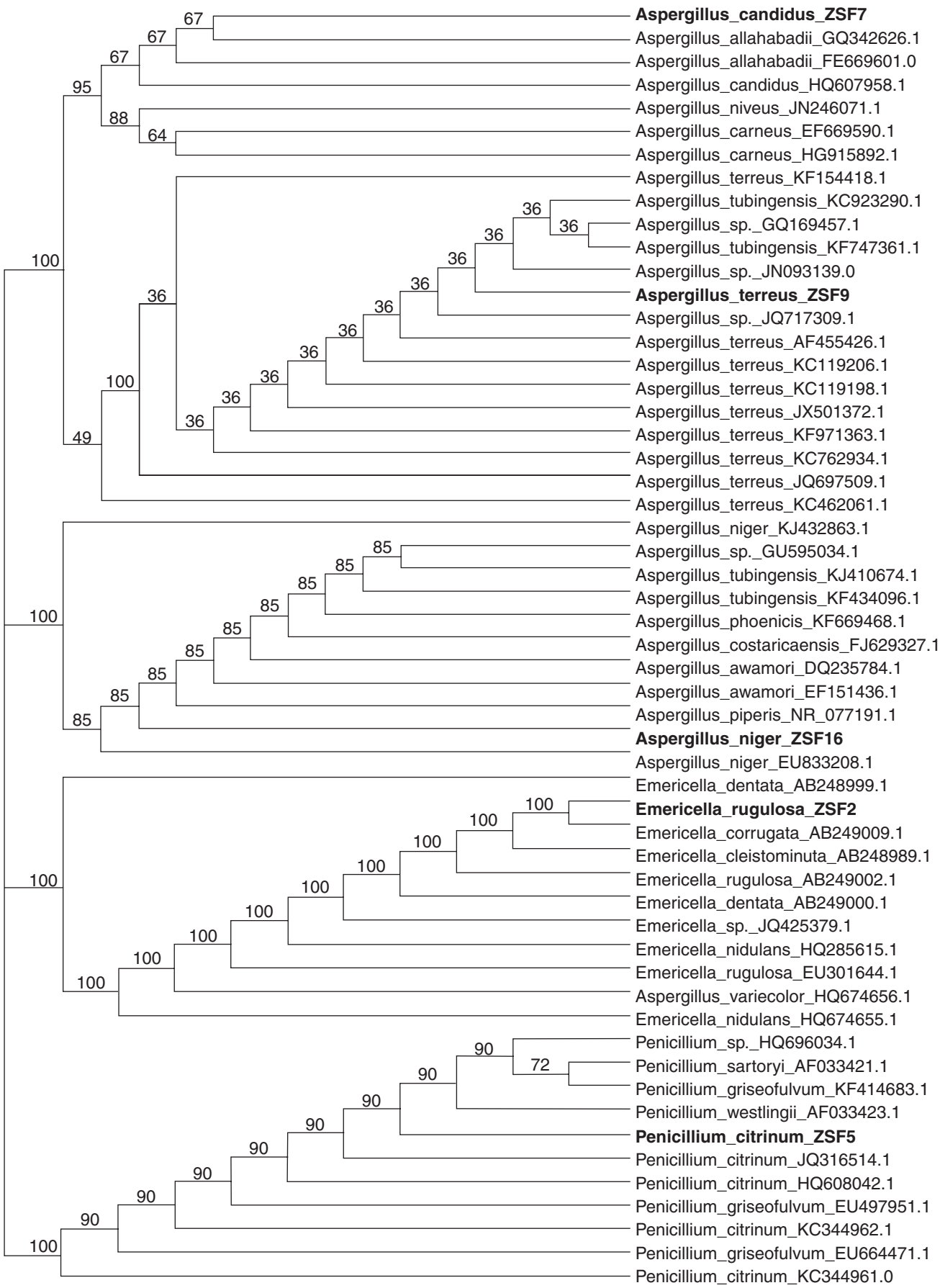

Fig 1 Phylogenetic tree based on 18S rRNA gene sequences comparison showing the position of zinc solubilizing fungi (ZSF)

using a separon SGX C18 column as described by Roukas (2000). Elution was performed with an isocratic consisting of acetonitrile: water (30:70 v/v) with a flow rate of 1.0 ml/min at 210nm. Presence of gluconic acid present in the culture filtrate was determined by comparing the retention times and peak areas of the sample with the standard of gluconic acid. All the isolates produced gluconic acid in the growth medium incorporated with insoluble zinc. Peak height and peak area of the chromatograph of *Aspergillus terreus* (ZSF-9) isolate was found more than other isolates. The production of gluconic acid by the isolates indicated their potential in the solubilization of zinc salts (Di simine *et al*. 2012).

The selected isolates were tested further for their ability to produce IAA by inoculating it into the flasks containing 50ml of Bunt and Rovira medium supplemented with 0.1% ZnO. Another set devoid of Zn material was also inoculated. All the treatment were amended with 0.1% tryptophan and incubated for seven days. The quantity of IAA produced by the organisms was estimated by the method of Brick *et al.* (1991). The results indicated that all the isolate produced IAA when supplied with tryptophan in the medium. The addition of zinc as zinc oxide in the medium stimulated the IAA production. Among the five zinc solubilizing fungi, *Aspergillus terreus* (10.03 mg/l) and *Penicillium citrinum* (7.6 mg/l) aproduced more IAA in the presence of zinc than other isolates (Table 3). The result indicated that fungi produce more IAA in the presence of zinc as source which forms the precursor for the production of IAA.

Table 3 Production of IAA by zinc solubilizing fungi (ZSF)

Treatment	IAA (mg/l)
Control	0
ZSF-4+ZnO	7.10 ± 0.36
ZSF-4 alone	6.13 ± 0.32
ZSF-5+ZnO	7.60 ± 0.26
ZSF-5 alone	6.00 ± 0.40
ZSF-7+ZnO	6.00 ± 0.30
ZSF-7 alone	5.00 ± 0.20
ZSF-9+ZnO	10.03 ± 0.15
ZSF-9 alone	7.07 ± 0.40
ZSF-16+ZnO	7.00 ± 0.50
ZSF-16 alone	5.03 ± 0.35
SEdCD (P<0.05)	0.2644
	0.5483

Values are mean ± SD of three samples in each group

SUMMARY

Zinc forms an important enzyme cofactor which is involved in the metabolic functions of the plants. Though large amount of zinc is present in the soil plant exhibit Zn deficiency due to the unavailability to plants. In the present study, 16 zinc solubilizing fungus (ZSF) were isolated from five (Groundnut field, tomato field, chilli field, maize and sorghum) different agricultural fields of Tirupur District using modified Bunt and Rovira agar medium containing 0.1% of three insoluble sources of zinc (ZnO, $ZnCO_3$, $Zn(PO_4)_3$) by plate count method and they were designated as ZSF-1, ZSF-2…….ZSF-16. Based on their solubilizing efficiency, five strains were selected and were identified as *Emericella rugulosa* (ZSF-2), *Penicillium citrinum* (ZSF-5), *Aspergillus candidus* (ZSF-7), *Aspergillus terreus* (ZSF-9), *Aspergillus niger* (ZSF-16) using molecular marker-18S rRNA. The best isolates were subjected to further experiments such as quantitative estimation of zinc and IAA, determining pH change of the medium and analysis of gluconic acid production using HPLC method. In all the experiments *Aspergillus terreus* (ZSF-9) recorded highest Zn solubilizing efficiency and indicated that it can be used as a potent bio-inoculant for various agricultural crops as an alternative to synthetic zinc sources.

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