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Isolation and identification of zinc solubilizing fungal isolates from cumin of semi-arid region of Rajasthan

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Abstract
Zinc is an essential element necessary for plant, humans and microorganisms required in little quantities to compose a complete array of physiological functions. Rhizospheric microbes are known to influence plant growth by various direct and indirect mechanisms and have some additional properties such as multiple metal solubilization. Current investigation aimed to isolate and identify potential zinc solubilizing fungi from arid and semi-arid soils for environment friendly biofertilizer development. Five fungal isolates were Screened out and identified as *Aspergillus spp*. and *Penicillium spp*. from arid and semi-arid soil based on zinc solubilization index, morphological studies. Subsequently, fungal isolates having excellent zinc solubilization efficiency were selected by their potential in broth containing insoluble ZnO. Interestingly, isolate DCU-402 (*Penicillium spp.*) have marked highest zinc solubilization ability followed by DCU-452 (*Aspergillus spp.*). The maximum, solubilization index (2.5) and the maximum solubilized Zn at 5 Days after inoculation (142 µg mL^{-1}) were associated with DCU-402 (*Penicillium spp.*) whereas the maximum solubilized Zn at 15 Days after inoculation (365 µg mL^{-1}) was associated with DCU-401 (*Aspergillus spp.*) In addition, there was inverse proportion between the pH and phosphate solubilizing capacities. These excellent properties of isolates suggested that they have a great potential for agricultural utilization as environmentally sound biofertilizer. In this study, phosphate solubilization by filamentous fungi is reported for the first-time in and semi-arid region of India.

Keywords: Zinc, *Penicillium spp.*, *Aspergillus spp.*, biofertilizer

Introduction
Cumin (*Cuminum cyminum*) is a member of apiaceae family and an annual plant, which is widely cultivated in arid and semi-arid regions. Cumin is the second most important spice in the world, after black pepper (*Pepper nigrum*), according to (Sowbhagya, 2013; Mnif *et al*., 2015) [24, 20]. India is the largest producer, consumer and exporter of cumin in the world. It is widely grown in Gujarat, Rajasthan, Punjab and Uttar Pradesh. Gujarat and Rajasthan are the major producers of cumin in India. They account for 92% of total Indian production. Gujarat state alone contributes around 65% of the total national production. Apparently, enhancing the available Zn pool in the soil by application of Zn containing synthetic fertilizers or organic manures becomes Unfortunately, exogenous application of chemical fertilizers alone cannot help in combating soil Zn deficiency in the longterm since 96.0–99.0% of the applied Zn is once again converted to unavailable Zn pools by precipitation to carbonates or oxides or phosphates etc. (Ma and Uren, 1997; Zhang *et al*., 2017) [19, 27]. Hence, decreased use efficiency of chemical Zn fertilizers, remains an issue, especially in the long-term. Micronutrient deficiency has become a limiting factor for crop productivity in many parts of the world. Among the micronutrient deficiencies, Zn deficiency is considered to be the most ubiquitous abiotic stress in countries like Afghanistan, Australia, Bangladesh, Brazil, China and India, Iran, Iraq, Pakistan, Philippines, Sudan, Syria, Turkey, and many parts of Europe, USA and Africa (Alloway, 2009; Cakmak *et al*., 1999) [3, 9]. This is because Zn is the only micronutrient relevant to all classes of enzymes present in biological systems (Broadley *et al*., 2007) [7] and almost 2800 proteins need Zn for their structural integrity and activity (Andreini *et al*., 2009) [4]. However, Zn deficiency in millions of hectares of agricultural soils has not only reduced crop yields but also severely hampered the nutritional quality of the crop produce causing critical nutritional and health problem in one-third of the world's human population (Hotz and Brown, 2004; Myers *et al*., 2015) [14, 21]. Based on zinc solubilizing solubilizing efficiency, fungal isolates 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 isolate *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 (Anitha, *et al*. 2015) [5].

Materials and Methods

The experiment was done at Microbiology laboratory, Division of crop production, ICAR-National Research Centre on Seed Spices, Tabiji, Ajmer, Rajasthan during the year 2017-18 and 2018-19.The samples were collected in winter from cumin rhizosphere soil of Ajmer District, located at 26º 03' 29" N, 74 o 46' 10" E to 26º 21' 28" N, 74 o 37' 61" E. Its climate is semi-arid, in winters the minimum temperature (12^0) and maximum temperature (27^0), rainfall (529.83). Soil and plant samples were collected from ten different locations using sterile auger. One-hundred-gram soil was taken from each sampling point and it makes a total of 500 g composite sample (five points from each location make one composite sample). The samples were transferred to laboratory in sterile sealed polythene bag under aseptic condition and stored at room temperature. Then microbiological study was done as early as possible. For isolating zinc solubilizing fungi zinc solubilizing agar medium was used. Zinc solubilizing agar medium consisted of 10.0 g Dextrose, 1.0 g Ammonium sulphate, 0.2 g Potassium chloride, 0.1 g Dipotassium hydrogen phosphate, 0.20 g Magnesium sulphate, 1.0 g Zinc oxide, 15.0 g agar and 1000 mL distilled water. In this medium Zinc oxide (ZnO) was used as a source of insoluble zinc. The medium was autoclaved at 121 °C for 15 min. About 20 ml of the sterilized medium poured into each petri dish and allowed to solidify before inoculation. Chloramphenicol was also used to avoid bacterial growth. Isolation of zinc solubilizing fungi using serial dilution plate technique. One-gram soil sample was diluted in to 10 ml of sterile water. It was vigorously shaken until to get homogenous suspension and serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. From each dilution, 200 µL was plated on Zinc solubilizing agar medium. The zinc solubilizing fungi were identified by the presence of a clear halo around the colonies after 7 days incubation at 25 °C (Rao, 1982). The experiment was performed in triplicate. Zinc solubilizing fungi of the soil samples were isolated and purified by transferring into new plates. The pure cultures were preserved on potato dextrose agar slants at 4°C for further study. Zinc solubilisation index was measured using the following formula (Birhanu *et al*., 2017) [6]:

SI = [Colony diameter + Halo zone diameter] / colony diameter

Quantitative estimation of zinc solubilization

The zinc solubilizing fungal isolates, those showed halo zone formation were further tested for their ability to release soluble Zn from insoluble zinc oxide (ZnO) using Atomic Absorption Spectrometer (Varian AAS 240 FS). The isolates were grown in 50.0 ml zinc solubilizing broth containing 0.1% zinc oxide (ZnO) at 28˚C for different interval of days (5th, 10th and 15th day) with four replicates in incubator cum shaker at 120 rpm along with their controls. At 5th, 10th and 15th days after incubation, cultures were withdrawn and transferred aseptically to centrifuge tubes. They were centrifuged at 8,000 rpm for 15 min at 4˚C. The supernatant was collected in test tubes. Then 1.0 ml aliquot from the supernatant was transferred to 50.0 ml standard flask and the volume was made up to 50.0 ml using distilled water. The soluble Zn was estimated from standard curve by plotting readings drawn from standard solution against ppm of Zn taken.

Results

Screening and identification of zinc solubilizing fungi

A total of 5 fungal isolates showed zinc solubilizing activities. The isolates were 2 *Penicillium spp*. and 3 *Aspergillus spp*., identified based on colony morphology and microscopic observation (Table 1). Apart from Zn solubilization, DCU-401 (*Penicillium spp.)* have also the ability to solubilize potassium and phosphorous from substance potassium alumino silicate and tri calcium phosphate, respectively (Table 1).

Table 1: List of fungal strains isolated from cumin of semi-arid region of Rajasthan

S.N.	Isolates	Probable genera	P Solubilization	K Solubilization	Zn Solubilization
1.	DCU-401	*Aspergillus sp.*	+	+	+
2.	DCU-402	*Penicillium sp.*	-	-	+
3.	DCU-403	*Aspergillus sp.*	-	-	+
4.	DCU-404	*Penicillium sp.*	-	-	+
5.	DCU-452	*Aspergillus sp.*	-	-	+

Qualitative zinc solubilization

Five fungal isolates showed significant zinc solubilization in zinc solubilizing agar medium using zinc oxide (ZnO) as the substrate. The zinc solubilization index (ZnSI) ranged from 1.4 to 2.5 (Table 2). Isolate DCU-402 (*Penicillium spp*.) produced highest ZnSI; 2.5 (Table 2), whereas; the smallest ZnSI of 1.4 was achieved from DCU-404 (*Penicillium spp*.). Both fungal isolates, DCU-402 and DCU-404 were found somewhat same in colony color. The maximum Zn solubilization zone recorded 20 mm (Figure 1) and the minimum Zn solubilization zone (13 mm) were recorded in DCU-452 (*Aspergillus spp*.) and DCU-404 (*Penicillium spp*.), respectively. Zinc Solubilization index (ZnSI) of the culture medium exhibited the positive changes. It increased with the increased of zone of clearance in the medium.

Table 2: Qualitative assay for zn solubilization by rhizofungal isolates

S.N.	Isolates	Probable genera	Solubilization zone (mm)	Solubilization index (SI)
1.	DCU-401	*Aspergillus sp.*	15	1.7
2.	DCU-402	*Penicillium sp*	16	2.5
3.	DCU-403	*Aspergillus sp.*	14	1.6
4.	DCU-404	*Penicillium sp*	13	1.4
5.	DCU-452	*Aspergillus sp.*	20	1.9
	SEm		1.283	0.087

	CD (5%)		2.625	0.188
	CV		4.833	3.642

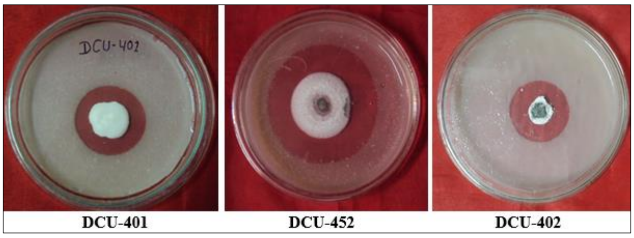

Fig 1: Clear halo formation by representative fungal isolates in zinc solubilizing agar plates (A: *Aspergillus spp.*, B: *Aspergillus spp.*, and C: *Penicillium spp.*)

Quantitative Zinc solubilization

Zinc solubilizations by the isolated fungi were analyzed in Zinc solubilizing broth medium using substrate of zinc oxide (ZnO) compounds. The Zn-solubilizing ability of fungal isolates varied with incubation period. The best period of observation was selected considering their mean Zn solubilization, which 15 days for ZnO. The strongest Zinc solubilization effect was found in the medium containing ZnO at 15 days of inoculation (DI) followed by 10 and 5 DI (Table 3, Figure 2). The solubilized Zn ranged between 117-142 µg /mL, 196-238 µg /mL and 301-365 µg /mL at 5, 10 and 15 Days of inoculation, respectively. At Among the isolates the highest amount of zinc was solubilized by *Aspergillus spp.* followed by *Penicillium spp.* Finally, DCU-401 *Aspergillus spp.* was considered as outstanding isolate because solubilized Zn was higher than sum of the mean and standard deviation of solubilized by 5 isolates. At 10 days after inoculation, the highest amount of soluble Zn was 238 µg /mL and At 15 days after inoculation, the highest amount of soluble zinc was 365 µg /mL were associated with DCU-401 *Aspergillus spp.* (Table 3) showed outstanding performance at both 10 and 15days of solubilization but in case at 5 days, it was close to the outstanding. pH of the culture medium exhibited the opposite changes. It decreased with the increased amount of soluble Zn in the medium.

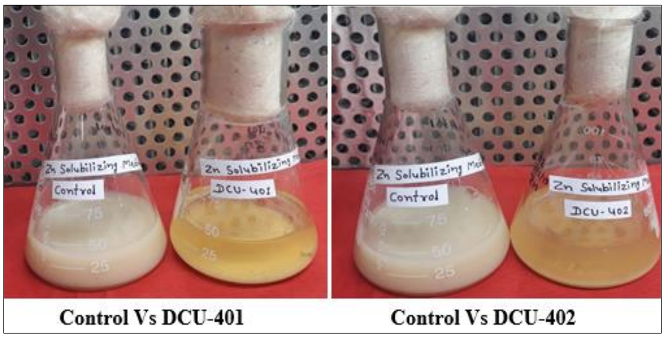

Fig 2: Zinc solubilization by fungal isolates in zinc solubilizing broth medium (A: *Aspergillus spp.*, B: *Penicillium spp.*)

Table 3: Quantitative estimation of released zinc in broth containing zinc oxide by rhizofungal isolates

S.N.	Isolates	Probable genera	5 Days		10 Days		15 Days	
			Soluble zn (µg /ml)	pH	Soluble zn (µg /ml)	pH	Soluble zn (µg /ml)	pH
1.	DCU-401	*Aspergillus sp.*	127	5.56	238	4.69	365	4.83
2.	DCU-402	*Penicillium sp*	142	5.35	231	5.21	342	5.31
3.	DCU-403	*Aspergillus sp.*	137	4.58	196	4.21	329	3.87
4.	DCU-404	*Penicillium sp*	135	5.82	207	4.86	301	4.50
5.	DCU-452	*Aspergillus sp.*	117	5.43	212	4.43	338	4.53
	S.Em		3.998		3.793		6.251	
	CD (5%)		8.528		7.635		12.732	
	CV		5.274		5.696		6.845	

Discussion

The five zinc solubilizing fungal strains were isolated from cumin crop grown under semi-arid region (Ajmer) of Rajasthan, India. The isolates were belonging to the genera of *Aspergillus*. and *Penicilium*. Similar work presented by Anitha *et al*. (2015) [5] isolated sixteen zinc solubilizing fungus (ZSF) from five (Groundnut, tomato, chilli, maize and sorghum) using modified Bunt and Rovira agar medium containing 0.1% of three insoluble sources of zinc (ZnO, $ZnCO_3$, Zn_3 $(PO_4)_3$) and they were designated as ZSF-1 to 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. *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.

Aspergillus spp. (DCU-452) showed an excellent zinc degradation ability on halo assay whereas the *Penecillium spp*. (DCU-402) found potent as maximum solubilization index. *Aspergillus spp*. (DCU-452) have the bigger colony size as compare to *Penecillium spp.* (DCU-402), that is why, DCU-452 had less solubilization index as compare to DCU-402. According to Jain *et al*. (2014) [15], Alam *et al.* (2002) [2], Elias *et al*. (2016) [10] and Jain *et al*. (2017) [16] solid medium using agar plates performed better insoluble mineral degradation ability than those in liquid media). *Aspergillus spp*. are widely used for the production of fermented foods, organic acids and enzymes (Wongwicharn *et al*., 1999) [26]. Especially, *A. niger* has a long history of industrial usage, which means many strains already have a GRAS ("generally regarded as safe") status (Wongwicharn *et al*., 1999) [26]. It has been used for commercial production of many enzymes, e.g. pectinase, glucose oxidase, glucoamylase, hemicellulase, glucanases, acid proteinase, catalase (Aguilar and Huitron, 1993; Liu *et al*., 1999; Garhartz, 1990) [1, 18, 12] and citric acid (Friedrich *et al.*, 1989; Gokhale *et al*., 1991; Lee *et al*., 1989) [11, 13, 17].

In this study, DCU-401 (*Aspergillus spp.*) showed the highest efficiency in zinc solubilization by decreasing pH of the culture medium, which indicated higher amount of organic acid production. Apart from zinc solubilization DCU-401 (*Aspergillus spp*.) have the ability to solubilize potassium and phosphate from their insoluble form. Singh *et al*. (2017) [23] reported that Zn solubilizing isolate ZnSF-1 showed maximum solubilization zone of 85 mm followed by ZnSF-2 with 34 mm for ZnO. The solubilization zone is ranged 6-25 mm on agar plate assay containing zinc phosphate ($ZnPO_4$). Williams *et al*. (1996) reported that the production of gluconic and its 2 and 2,5-keto-derivatives, are produced by fungal isolates *viz*., *Penicillium luteum* and *Aspergillus niger* and bacteria belonging to *Pseudomonas* as a result of an external oxidative pathway effective on glucose and other aldose sugars. Burgstaller *et al*. (1994) [8] reported fungal isolate *Penicillum simplicissimum* which able to producing organic acids, those are important in solubilization of zinc in media containing ZnO. They observed that this compound stimulated the efflux of citric, oxalic and gluconic acids because of its buffering capacity, and that high molarity biological buffers were able to mimic the effect of Zinc oxide.

Conclusions

Isolates DCU-401 (*Aspergillus spp*.) and DCU-402 (*Penicillium spp*.) have unique capabilities to solubilized insoluble zinc compounds (zinc oxide) and may become an important bio resource for soil fertility management as well as sustainable crop production and pollution free environment. They may beneficial to solubilize zinc in arid and semi arid region of Rajasthan as well as all over country.

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