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Isolation and characterization of potential Zn solubilizing bacteria from soil and its effects on soil Zn release rates, soil available Zn and plant Zn content

R. Dinesh[a,*], V. Srinivasan[a], S. Hamza[a], C. Sarathambal[a], S.J. Anke Gowda[a], A.N. Ganeshamurthy[b], S.B. Gupta[c], V. Aparna Nair[c], K.P. Subila[a], A. Lijina[a], V.C. Divya[a]

[a] ICAR-Indian Institute of Spices Research, P.O. Box 1701, Marikunnu P.O., Calicut 673012, Kerala, India
[b] ICAR-Indian Institute of Horticultural Research, Hessaraghatta lake post, Bengaluru 560089, Karnataka, India
[c] College of Agriculture, Faculty of Agriculture, Indian Gandhi Krishi Vishwavidyalaya, Raipur 492012, Chhattisgarh State, India

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ABSTRACT

In this study, experiments were designed to isolate, characterize and evaluate an array of bacteria isolates for their Zn solubilization potential. Out of the six promising Zn solubilizing bacteria (ZnSB), ZnSB2 (*B. megaterium*, KY687496) was found to be the most potential strain owing to its enhanced Zn solubilization in vitro. In the quantitative study, the net Zn solubilized by ZnSB2 was significantly higher than those solubilized by the other ZnSB at all days of sampling. Similar effects of ZnSB2 was observed in the soil per se, wherein the rate of release of available Zn by ZnSB2 was markedly higher at all days of incubation (25.6%–40.7% of added Zn), with a peak on the 8th day. Such enhanced rates of Zn release by ZnSB2 were attributed to marked decrease in pH owing to enhanced gluconic acid production. In fact, gluconic acid production by ZnSB2 was 1884.7 ± 413.4 μg mL^{-1}, which was 35.3–69.7% greater than the other shortlisted ZnSB isolates. Further evaluation of ZnSB2 was done in the green house using turmeric as the test crop. ZnSB2 was applied either alone or in combination with chemical Zn (75% and 100% of recommended Zn). The results revealed that soil available Zn level in the treatment with 75% Zn + ZnSB2 (12.69 ± 2.96 mg kg^{-1}) was on par with the level in the treatment with 100% Zn (12.74 ± 2.63 mg kg^{-1}) at 120 days after planting, while at harvest the treatment with 75% Zn + ZnSB2 maintained 65.0% higher available Zn levels than 100% Zn. The positive effect of ZnSB2 was also manifested on rhizome yield, which was at par in the treatments with ZnSB2 + 75% Zn (154.2 g ± 36.0 pot^{-1}) and 100% Zn (177.2 ± 36.7 g pot^{-1}). Besides, the Zn concentration in the rhizome was significantly higher ($P < 0.05$) in the treatment with ZnSB2 + 75% Zn (40.5 ± 3.5 mg kg^{-1}), which was at par with 100% Zn, but was greater by 98.5% compared to control. The study indicated that ZnSB2 strain was a potential candidate for enhanced Zn dissolution in soil, which would allow reduced inorganic Zn application rates. Nonetheless, in vitro interaction studies (dual culture) suggested that this strain was seriously lacking in disease suppressing traits. But its compatibility with several plant growth promoting rhizobacteria enhanced the possibility of co-inoculation or applying ZnSB2 in a consortium mode especially in condition wherein both soil Zn solubilization and disease suppression becomes imperative.

1. Introduction

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). This is because Zn is the only micronutrient relevant to all classes of enzymes present in biological systems (Broadley et al., 2007) and almost 2800 proteins need Zn for their structural integrity and activity (Andreini et al., 2009). 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). Acid, calcareous, saline and sodic soils and coarse-textured soils prone to high weathering, besides soils subjected to intensive cropping and poor

* Corresponding author at: Division of Crop Production & Post Harvest Technology, ICAR-Indian Institute of Spices Research, P. O. Box 1701, Marikunnu P.O., Calicut 673012, Kerala, India.
E-mail address: rdinesh2005@gmail.com (R. Dinesh).

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drainage exhibit Zn deficiency (Singh et al., 2005). Also, factors like high available P and Si, drying of upper horizons, sub-soil constraints and sometimes high fertilizer cost subscribe to Zn deficiency (Alloway, 2009).

Apparently, enhancing the available Zn pool in the soil by application of Zn containing synthetic fertilizers or organic manures becomes imperative. Unfortunately, exogenous application of chemical fertilizers alone cannot help in combating soil Zn deficiency in the long-term 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). Hence, decreased use efficiency of chemical Zn fertilizers, remains an issue, especially in the long-term.

Nevertheless, a redeeming feature is that the worldwide occurrence of Zn scarcity issues in crops is not due to low levels of total Zn but is due to low solubility of Zn in soils (Cakmak, 2008). In fact, the total Zn content in soils is substantially high and exists in fixed forms such as smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe_2O_4$), wellemite (Zn_2SiO_4), and hopeite ($Zn_3(PO_4)_2{\cdot}4H_2O$), which are only sparingly soluble. Values in the literature indicate that available Zn level in soils is very low (4.0–270.0 $\mu g\,L^{-1}$) in relation to the mean total Zn level of 64.0 $mg\,kg^{-1}$ (Alloway, 2009). Reports suggest that Zn deficiency due to low amounts of bioavailable Zn is rampant in at least one-third of the cultivated soils globally (Sillanpää and Vlek, 1985). Apparently, low bioavailability not only hampers crop productivity but also markedly lowers Zn density in the harvested produce (seeds, grains, rhizomes etc.) thereby impairing nutritional quality (Cakmak and Hoffland, 2012).

Hence, a feasible alternative would be to exploit the innate capacity of certain soil microorganisms, especially bacteria and fungi, to solubilize these fixed forms of Zn to labile Zn forms for enhanced availability and subsequent uptake by plants. However, the ability to solubilize immobilized Zn (ZnO, $ZnCO_3$ or $ZnPO_4$) is not a common characteristic of cultivable bacteria and fungi in soils, though there are in vitro studies on a few genera of bacteria like *Pseudomonas* sp., *Gluconacetobacter* sp., *Thiobacillus* sp., *Bacillus* sp., *Acinetobacter* sp. etc. (Di Simine et al., 1998; Fasim et al., 2002; Hafeez et al., 2013) and fungi like *Beauveria caledonica* (Fomina et al., 2004), *Lecanicillium psalliotae* (Senthil Kumar et al., 2018) etc. capable of solubilizing Zn. Several Zn solublising bacteria (ZnSB) have been isolated from soils of tropical and temperate regions and strains of genera *Acinetobacter*, *Bacillus*, *Burkholderia*, *Gluconacetobacter*, *Pseudomonas*, *Thiobacillus* have been reported from mostly plate assays (Bapiri et al., 2012; Saravanan et al., 2007a, 2007b; Saravanan et al., 2011; Vidyashree et al., 2016).

The major objective of this study was to isolate an array of bacteria isolates and characterize them for their Zn solubilization potential and to study the Zn release mediated by the promising ZnSB in soil per se. In addition to the Zn solubilizing potential, the shortlisted ZnSB were also screened for their multi-tasking abilities that included solubilization of P, K, Si, production of IAA, NH_3, HCN, siderophore, cell wall degrading enzymes (pectinase, protease α-amylase & cellulase) etc. The effects of the most promising ZnSB on soil Zn availability and Zn uptake by turmeric were also studied. Turmeric (*Curcuma longa* L.) was used as the test crop since there are no reports on the effect of ZnSB on rhizome Zn content, though there are reports involving ZnSB on Zn biofortification in soybean (Ramesh et al., 2014), rice (Krithika and Balachandar, 2016), maize (Mumtaz et al., 2017), wheat (Shaikh and Saraf, 2017), green gram (Sharma et al., 2012) etc. Turmeric is an annual crop with duration of 7–8 months and is grown under both rainfed and irrigated conditions. It is used worldwide as a condiment, flavouring and colouring agent in the food industry besides its extensive use in the drug industry owing to its anti-viral and anti-cancer activities (Srinivasan et al., 2016).

Table 1
Physico-chemical properties of the soil samples collected from the rhizosphere of wild black pepper and wild cardamom.

	Wild black pepper (50)[a]		Wild cardamom (20)[a]	
	Range	Mean ± SD[b]	Range	Mean ± SD[b]
pH (1:2.5 H_2O)	3.3–5.2	4.3 ± 0.4	3.8–5.3	4.4 ± 0.5
Organic C ($g\,kg^{-1}$)	5.3–33.9	19.3 ± 7.2	12.0–31.0	23.0 ± 6.0
Available P ($mg\,kg^{-1}$)	3.1–34.5	10.9 ± 8.7	3.8–33.0	10.0 ± 8.7
Exchangeable K ($mg\,kg^{-1}$)	57.0–228.0	128.9 ± 36.9	73.0–195.0	112.8 ± 41.7
Available Fe ($mg\,kg^{-1}$)	21.1–61.4	33.6 ± 10.7	3.1–36.5	27.8 ± 6.5
Available Mn ($mg\,kg^{-1}$)	4.6–31.9	20.6 ± 5.4	15.4–24.8	18.6 ± 2.3
Available Zn ($mg\,kg^{-1}$)	0.6–4.7	2.1 ± 1.0	0.4–4.6	1.8 ± 0.9
Available Cu ($mg\,kg^{-1}$)	0.54–2.51	1.25 ± 0.47	0.48–3.08	1.58 ± 0.61

[a] Total number of soil samples collected from the rhizosphere of wild black pepper and wild cardamom.
[b] SD - Standard Deviation.

2. Materials and methods

2.1. Soil sampling and analyses

Soil samples (70 nos) were collected from the rhizosphere of wild pepper (*Piper nigrum* L. - 50 nos) and cardamom (*Elettaria cardamomum* (L.) Maton - 20 nos) growing in the forest areas of Idukki district (Latitude: 9° 50′ 60.00″ N; Longitude: 76° 58′ 0.01″E) of Kerala State (India). These two crops were chosen because we wanted to isolate prospective bacterial species from the rhizosphere of domesticated crops present in undisturbed soils of virgin forest sites. Virgin sites were selected because it would provide opportunity to explore undisturbed soils with more diverse bacterial communities. The soil within the confines of the space occupied by plant roots and those strongly clinging to the roots were considered to belong to the rhizosphere (Garcia et al., 2005). The soil samples were transported to the laboratory in an ice box and subsequently the plant and root debris were discarded, moisture content estimated and a chunk of each sample required for estimating the microbial parameters was stored at 4 °C. The physico-chemical properties of the soil samples are presented in Table 1.

The soil pH was measured in 1:2.5 soil: water suspension. Organic C was determined by the dichromate-oxidation method (Nelson and Sommers, 1982) and available P using the dilute acid–fluoride extractant (Kuo, 1996). Available Zn, Cu, Fe & Mn were extracted using DTPA (Lindsay and Norvell, 1978) and the concentration of these nutrients in these extracts was measured using atomic absorption spectrophotometer (AAS, Varian AA240FS).

2.2. Isolation of bacteria

This was done by serial dilution plate count wherein 1.0 g soil was transferred to a dilution tube containing 9.0 mL sterilized water (10^1) and shaken for 10 min. After shaking, the dilution tubes were kept undisturbed for 30 min to allow the suspension to stabilise. Later, 1.0 mL of the bacterial suspension from 10^1 dilution was transferred to another dilution tube containing 9.0 mL sterilized water (10^2), shaken for 10 min and allowed to stabilise for 30 min. This was continued up to 10^{10} dilution, pour-plated on Nutrient Agar (NA) and incubated at 28 °C for 72 h. The population of bacteria [colony forming units (CFU) per gram soil] was estimated from the most suitable dilution and individual bacterial colonies were sub-cultured on NA (Dinesh et al., 2015). About 70 bacterial isolates were obtained, which were cryopreserved at

−80 °C for subsequent assays. The preliminary identification of the isolates was done using the Bergey's Manual of Determinative Bacteriology.

2.3. Qualitative assay for Zn solubilizing potential

Basal medium (glucose-10.0 g, $(NH_4)_2SO_4$–1.0 g, KCl-0.2 g, K_2HPO_4–0.1 g, $MgSO_4$–0.2 g, double distilled water-1000 mL and buffered to pH 7.0; Saravanan et al., 2003) was augmented with 0.2% insoluble Zn, separately from three sources viz., ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$. After autoclaving at 125 °C for 15–20 min, it was transferred to sterilized petri plates. One loop full (10 μL) of overnight matured culture of bacterial isolates was inoculated on to the petri plates and incubated at 28°C for 48 h. The zone of solubilization/clearance was determined after 24 h up to 7 days and expressed in mm.

2.4. Identification of promising Zn solubilizing bacteria

The shortlisted ZnSB isolates was identified using 16S rRNA gene amplification. The standard protocol of Sambrook and David (2000) was used to extract bacterial DNA. Universal primer set pA (5′-AGAG TTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCC GCA-3′) in 25 μL of reaction mixture containing 1 × buffer (0.01% gelatine, 50 mM KCl, 10 mM Tris pH 9), 3 mM $MgCl_2$,100 μm dNDP's mix, 10 μg BSA, 5 pM each primer, 0.5 units of Taq DNA polymerase and 100 ng template DNA was employed (Woese, 1987; Stackebrandt and Goebel, 1994). The thermo cycling condition comprised of initial denaturation (94 °C for 1 min 10 s, 48 °C for 30 s, 72 °C for 2 min 10 s) followed by final polymerization step (72 °C for 6 min) with Eppendorf master thermal cycler. The final PCR product was resolved in 0.8% agarose gel in Tris acetate EDTA buffer at 4 V cm^{-1}, excised and purified with GenElute™ Gel Extraction Kit (Sigma, India).

2.5. 16S rRNA gene sequencing and phylogenetic tree construction

The 16 S rRNA gene sequence was determined by direct sequencing of the PCR product (Chromous Biotech Pvt. Ltd., Bengaluru, India). Sequence analysis was performed with BLAST and run against registered sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic trees and evolutionary distances (Knuc) of 16S rRNA sequences were measured by the neighbour-joining method with Kimura 2 + G parameter model supported by boot strapping using 1000 replicates. Briefly, the sequence of the best Zn solubilizing strain viz., ZnSB2 and sequences of other Zn solubilizing bacteria reported in the literature were aligned using MUSCLE integrated in MEGA 7.0 (Kumar et al., 2016b). A distantly related genus (*Pseudomonas putida* AF094747) was used as an out-group to better reflect the association among participating candidates.

2.6. Quantitative assay for Zn solubilization

The ZnSB isolates that showed halo zone formation were further tested for their ability to release inorganic Zn in liquid medium. For the quantitative assay, 20.0 mL of liquid mineral salts medium containing (g L^{-1}) dextrose: 10.0; $(NH_4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$: 0.2; pH: 7.0 and insoluble Zn compounds (Zn_3 $(PO_4)_2$, ZnO and $ZnCO_3$: 100 mg L^{-1} each; Agar: 15.0 g; Saravanan et al., 2007a) was transferred to 50 mL Erlenmeyer flask, autoclaved at 121.0 °C for 20 min. Subsequently, 1.0 mL suspension of individual bacterial culture was transferred to the flask and kept in an incubator-shaker at 120 rpm. Each treatment had 4 replications along with an uninoculated control. After 24 h (1st day) of incubation, the mixture was transferred aseptically to centrifuge tubes and centrifuged @8000 rpm for 15 min at 4 °C. The supernatant was collected in test tubes and 1.0 mL of the supernatant was transferred to 50.0 mL volumetric flask, volume made up to 50.0 mL using distilled water and soluble Zn was estimated using AAS. The procedure was repeated for further time intervals (2nd day, 3rd day, 5th day and 10th day).

2.7. Phenotypic and biochemical characterization

Basic characters like gram staining, cell size/form, spore formation, motility, colony pigmentation, methyl red, VP, citrate, oxidase, catalase, casein hydrolysis, starch hydrolysis were studied using the method described by Tindal et al. (2007).

2.7.1. IAA production

The isolates were tested for their ability to produce IAA by the method of Sawar and Kremer (1995). Briefly, 50.0 μL of cell suspension of the bacterial isolate in 5.0 mL of the sterile tryptone/peptone yeast extract broth (peptone/tryptone - 10 g, beef extract - 3.0 g, NaCl - 5.0 g, L-tryptophan - 0.204 g, distilled water - 1.0 L; pH - 7) was transferred into 15 mL culture tubes and incubated for 72 h at 28 °C in the dark. Subsequently, 1.5 mL of this broth was centrifuged at 12850 g for 10 min, followed by addition of 1.0 mL of Salkowski reagent (50.0 mL 35% of $HClO_4$, 1.0 mL 0.5 M $FeCl_3$ solution) to the 1.0 mL of the supernatant in 2.0 mL Eppendorf tube. The culture tubes were then incubated at 37 °C in the dark for 1.0 h. Formation of red colour in the medium indicated IAA production by the organism.

2.7.2. NH_3 production

For measuring NH_3 production, peptone broth (4%) was used to inoculate the bacterial cell suspension (50.0 μL), incubated at 25 °C for 72 h followed by addition of 1.0 mL of Nessler's reagent. The development of yellow-brown precipitate showed the production of NH_3 (Cappuccino and Sherman, 1992).

2.7.3. HCN production

For measuring HCN production, the qualitative method of Kloepper et al. (1991) with few modifications was followed. King's B broth (5.0 mL) supplemented with glycine (4.4 g L^{-1}) was used to inoculate the log phase cultures (25.0 μL) of the shortlisted bacteria in 30 mL sterile glass vials. Filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3 in 1.0 L distilled H_2O) were inserted in half of the vials and tightened with screw cap. Vials were sealed with parafilm and incubated for 72 h in a mechanical shaker. Change in colour of the filter paper strips from yellow to brown to red indicated HCN Production.

2.7.4. Siderophore production

Siderophore production was estimated using the procedure of Schwyn and Neilands (1987) with modifications suggested by Louden et al. (2011). Briefly, 50.0 mL of MM9 salt solution (15.0 g KH_2PO_4, 25.0 g NaCl, and 50.0 g NH_4Cl in 500.0 mL of double distilled water) was added to 375.0 mL of double distilled water. To this 16.12 g of PIPES [piperazine-N,N′-bis (2-ethanesulfonic acid)] buffer was added and pH was brought to 6.8 by adding conc. NaOH. Then 7.5 g of Bacto agar was added and autoclaved. After cooling the autoclaved media to 50 °C, 15.0 and 5.0 mL of 10% casamino acid solution (3.0 g of casamino acid in 27.0 mL of double distilled water, extraction with 3% 8-hydroxyquinoline in chloroform to remove any trace iron & filter sterilize) and 10.0 mL of sterile 20% of glucose solution were added to MM9/PIPES mixture respectively. Then 50.0 mL of CAS dye solution (chrome azurol S (CAS) - hexadecyltrimethylammonium bromide (HDTMA) blue dye) was added slowly along the glass wall with continuous agitation. CAS agar medium was then poured into plates and spot inoculated with overnight grown cultures and incubated for 72 h at 28 °C. Blue colour is produced when the CAS/HDTMA complexes tightly with ferric iron. Subsequently, in the presence of siderophore the blue colour changes to orange due to removal of iron from the dye complex.

2.7.5. Solubilization of P, K, and Si

Solubilization of P was tested by spot inoculating a loop full of the 24 h broth culture on Pikovskaya's agar medium (Glucose - 10 g; $Ca_3(PO_4)_2$ - 5.0 g; Yeast extract - 0.5 g; $(NH_4)_2SO_4$ - 0.5 g; KCl - 0.2 g; NaCl - 0.2 g; $MgSO_4$ - 0.1 g; $FeSO_4$. $7H_2O$ - trace; $MnSO_4$ - trace; agar agar 15.0 g; distilled water 1000 mL; pH adjusted to 7.0 ± 0.2 before sterilization; Pikovskaya, 1948), incubated at 28 °C for 96 h and the zone of clearance around the bacterial colony was observed (Gaur, 1990). For K solubilization, the clearance zone was observed by inoculating the cultures in Aleksandrov medium containing mica (Hu et al., 2006) and incubation at 30 °C for 3 days. In case of Si solubilization, basal media containing 5.0 g potassium alumina silicate ($KAlSi_3O_8$) was poured into petri plates, a 0.5 cm diameter well was made after solidification and 0.1 mL of the bacterial suspension was introduced into the well followed by incubation at 28 °C for 4–5 days. The plates were observed for zone of clearance (mm) around the wells (Barker et al., 1998).

2.7.6. Hydrolytic enzymes production

Production of cell-wall-degrading enzymes (α-amylase, cellulase, pectinase and protease) was measured by the method of Cappuccino and Sherman (1992). For determining the production of α-amylase and protease enzymes, one loopful of the bacterial cell suspension was streaked on starch agar plate (peptone - 5.0 g, beef extract - 3.0 g, soluble starch - 10.0 g, agar - 15.0 g, distilled water - 1000 mL) and skim milk agar plate (skim milk - 100.0 g, peptone - 5.0 g, agar - 15.0 g, water - 1000 mL), respectively. After incubation at 28 °C for 48 h, clear zone around the streak was observed.

For determining pectinase and cellulase production, the media was prepared by adding 1.0% pectin and cellulose in basal medium ($NaNO_3$ - 1.0 g, K_2HPO_4 - 1.0 g, KCl - 1.0 g, $MgSO_4$ - 0.5 g, yeast extract - 0.5 g, glucose - 1.0 g, distilled water - 1000 mL, agar - 15.0 g; Dinesh et al., 2015). One loopful of the bacterial cell suspension was streaked on the medium and incubated for 5 days. Gram's iodine solution was poured in the pectin agar and zone of clearance was observed against the dark blue back ground. The cellulose medium was flooded with 0.01% congo red solution for 15 min and the plates were destained using 1% NaCl solution for 5 min. A clear zone against the red back ground indicated that the bacteria were positive for pectinase and cellulase production.

2.8. ZnSB mediated Zn release in soil

An incubation study was conducted to study the release pattern of Zn in soil treated with shortlisted ZnSB isolates. A reference strain (*Gluconacetobacter diazotrophicus*) known for its high Zn solubilization capacity (Saravanan et al., 2007b) was included for comparison. This particular strain was obtained from Department of Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. For the study, 0.02 g of ZnO was added to 20.0 g soil (i.e. 1000 mg kg^{-1}; soil sieved to < 2.0 mm; sterilized), mixed well and transferred to 100 mL polypropylene tubes. To this mixture, 5.0 mL of 24 h grown culture of ZnSB isolate was inoculated and the tubes were maintained at room temperature and field capacity moisture regime. Appropriate sets of uninoculated controls were also maintained. Available Zn was estimated on 2, 4, 8, 10 and 12th day by destructive sampling. Each treatment for each sampling day had four replications. For estimation of available Zn, 40 mL of DTPA extract was added to each tube, shaken for 2 h, filtered through Whatman 42 filter paper followed by estimation of available Zn using AAS. An identical set for each treatment for each sampling day was maintained to measure the pH. The soil used was Ustic Humitropept with low available Zn content of 0.65 mg kg^{-1}.

2.9. Gluconic acid production

The short-listed bacteria were grown in Bunt and Rovira medium [K_2HPO_4 - 0.4 g; $(NH_4)_2HPO_4$ - 0.5 g; $MgSO_4$. $7H_2O$ - 0.05 g; $MgCl_2$ - 0.1 g; $FeCl_2$ - 0.01 g; $CaCl_2$ - 0.1 g; peptone - 1.0 g; yeast extract - 1.0 g; soil extract - 250 g (Prepared by autoclaving 1000 g soil with 1000 mL H_2O for 15 min and filtering); Tap water - 750 mL; agar - 15.0 g; Bunt and Rovira, 1955] supplemented with ZnO (0.1%) and grown in a rotary shaker for 5 days. It was then centrifuged at 10,000 × *g* for 20 min and passed through 0.22 μm membrane filter. About 20.0 μL of the culture filtrate was injected into a HPLC system and gluconic acid was quantified using a SGX C18 column (3 × 150 mm; 5 μm) and PDA detector. Mobile phase consisted for aqueous phosphate buffer (20 mM at pH 2.0) and acetonitrile: water [30/70 (v/v) @ 1.0 mL/ min flow rate]. The retention time and peak area of the standard gluconic acid at different concentrations (0.5–50 μg mL^{-1}) was used to plot a linear calibration curve, which was used to determine the gluconic acid produced by the different culture filtrates.

2.10. Green house evaluation

For the green house study, only the most promising Zn solubilizer viz., ZnSB2 was used. The treatment details were:

- T1 - Control (without Zn)
- T2 - 100% Zn applied as $ZnSO_4$ (6.0 kg ha^{-1})
- T3 - 75% Zn applied as $ZnSO_4$ (4.5 kg ha^{-1})
- T4 - ZnSB2 (*B. megaterium*) only
- T5 - ZnSB2 (*B. megaterium*) + 75% Zn applied as $ZnSO_4$ (4.5 kg ha^{-1})

For the study, earthen pots (20 kg capacity) were filled with 15 kg sieved soil (< 2 mm). The soil, which was a clay loam Ustic Humitropept had pH – 5.10; organic C – 14.5 g kg^{-1}; Bray P – 5.2 mg kg^{-1}; exchangeable K – 86 mg kg^{-1}; available Zn - 0.65 mg kg^{-1}. The soil was not sterilized since we wanted to evaluate the performance of ZnSB2 under natural conditions. Besides, an important criterion during screening of such beneficial microorganisms is their potency in the soil conditions/type wherein they are expected to be deployed (Rana et al., 2011). The bacteria strains in the initial soil sample used for the greenhouse study were found to belong to 5 genera viz., *Pseudomonas* sp., (9) *Bacillus* sp., (8) *Arthrobacter* sp., (5), *Klebsiella* sp., (4) and *Micrococcus* sp. (4).

During planting, the rhizomes (20–30 g) of turmeric (variety Prabha) with at least two sprouted buds were placed 3.5–5.0 cm deep in each pot and covered with soil. All the pots including control were given a uniform dose of N, P & K (60–50–120 kg ha^{-1} in the form of urea, rock phosphate and muriate of potash) in two equal splits at 30 days after planting (DAP) and 60 DAP.

For application of ZnSB2, the freshly grown culture was inoculated in 500 mL nutrient broth and incubated in a rotary shaker at 28 °C for 48 h. Thereafter, the culture suspension was diluted to 5000 mL and this diluted culture (~ × 10^8 cfu mL^{-1}) was applied @250 mL pot^{-1} at 35 DAP. The rhizomes were harvested at 240 DAP, freed from the adhering soil and other organic debris, weighed and oven-dried at 60 °C and powdered using a Wiley mill to pass a 0.5 mm sieve.

2.10.1. Estimation of Zn content in plant and soil

A known quantity of the powdered rhizome sample was digested using triacid mixture (HNO_3: H_2SO_4: $HClO_3$ in the ratio of 9:2:1) and the Zn content in the digested mixture was determined using AAS. The effect of treatments on available Zn content in soil at 120 DAP and in the post-harvest soil samples was determined by extraction with DTPA (Lindsay and Norvell, 1978) and measuring the Zn content in the extract using AAS.

2.11. Data analysis

Each data point related to soil physico-chemical parameters are expressed on an oven-dried basis (105 °C). In all the studies related to

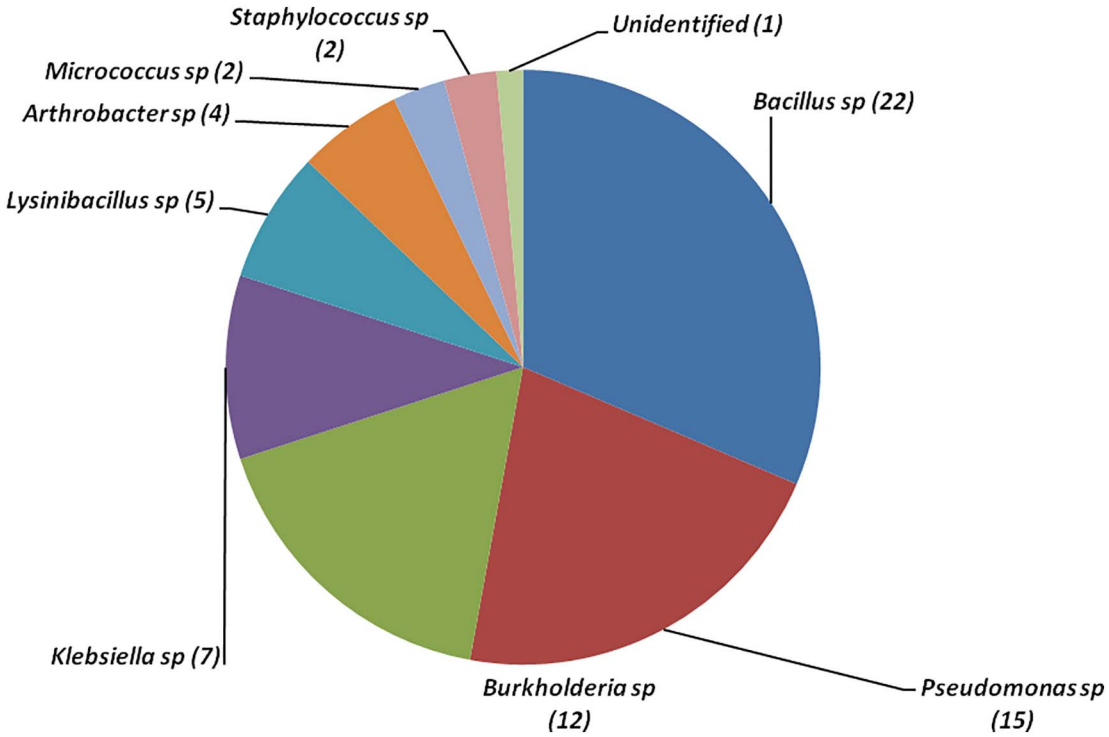

Fig. 1. Major genera of bacteria isolated from rhizosphere of wild black pepper and wild cardamom.

Zn solubilization including the green house study, one-way ANOVA was employed to test the significance of treatments. In case the F values were significant, Least Significance Test (LSD) at the 0.05 probability level was employed for post hoc comparisons of means.

3. Results

About 70 soil samples were collected from the rhizosphere of wild cardamom and wild black pepper. Texture was clay loam, while the soil pH was very acidic across the sites (mean 4.3–4.4; Table 1). Mean organic C was slightly higher in the cardamom rhizosphere (mean 23.0 ± 6.0 g kg^{-1}) compared to black pepper rhizosphere (19.3 ± 7.2 g kg^{-1}). Available P and exchangeable K levels varied little between black pepper (10.9 ± 8.7 and 128.9 ± 36.9 mg kg^{-1}, respectively) and cardamom (10.0 ± 8.7 and 112.8 ± 41.7 mg kg^{-1}, respectively) rhizospheres. Likewise, mean available-Fe, -Mn, -Cu and -Zn levels also did not vary appreciably among the two rhizospheres.

With regard to bacteria, around 70 strains were isolated from these soil samples, which were found to belong to 9 genera (Fig. 1) viz., *Bacillus* (22), *Pseudomonas* (15), *Burkholderia* (12), *Klebsiella* (7), *Lysinibacillus* sp. (5), *Arthrobacter* (4), *Micrococcus* (2), *Staphylococcus* (2). One strain could not be identified.

3.1. Qualitative assay for Zn solubilization

All the 70 bacterial strains were screened for their Zn solubilization ability in vitro. Only seven out of the 70 bacterial isolates showed halo formation. The results (Fig. 2) showed that the efficiency to solubilize insoluble Zn compounds varied between the bacterial strains and the source of Zn applied. Irrespective of the sources of Zn used, maximum zone of clearance was observed with ZnSB6 (20.7 mm, 14.6 mm and 12.4 mm with $ZnCO_3$, ZnO and $Zn_3(PO_4)_2$, respectively), closely followed by ZnSB2 (19.3 mm, 15.3 mm and 11.8 mm with $ZnCO_3$, ZnO and $Zn_3(PO_4)_2$, respectively).

The identity of the isolates identified using 16S rRNA is ZnSB1 - *Burkholderia lata*, ZnSB2 - *Bacillus megaterium*, ZnSB3 - *Lysinibacillus* sp., ZnSB4 - *Bacillus* sp., ZnSB5 - *Burkholderia latens*, ZnSB6 - *Staphylococcus warneri* and ZnSB8 - unidentified. The isolate ZnSB6 (*Staphylococcus warneri*), was excluded from further experimentation since, it is a human and animal pathogen.

3.2. Morphological, biochemical, growth promotion and nutrient solubilization traits of the short-listed ZnSB isolates

Among the 6 isolates, 3 were Gram negative and non-spore formers, while the rest were Gram positive and spore forming. All were negative for IAA production, while only ZnSB2 was positive for Voges-Proskauer, citrate utilization and glucose utilization and only ZnSB5 was positive for methyl red (Table 2). With regard to hydrolytic enzymes, all the isolates were positive for protease, ZnSB1, ZnSB2, ZnSB4 and ZnSB5 for α-amylase, ZnSB1, ZnSB2 and ZnSB8 for oxidase and ZnSB1 and ZnSB3 for catalase (Table 3). In case of growth promotion traits (Table 4), all the isolates were positive for NH_3 production. Nutrient mobilization traits were absent in all the isolates, expect for ZnSB2 which was positive for P solubilization. With regard to bicontrol traits, except for ZnSB2, all the other isolates were positive for HCN production. Likewise, except for ZnSB3, all the other isolates were negative for siderophore production.

3.3. Quantitative assay for Zn solubilization

The six isolates were further tested for their Zn solubilization potential at different time intervals in liquid medium (broth assay) ZnSB2 consistently registered significantly higher ($P < 0.05$) available Zn at all days of sampling (Fig. 3). Maximum available Zn registered by ZnSB2 was 65.11 mg L^{-1} on the 3rd day, which peaked to 126.80 mg L^{-1} during the 5th day, followed by a decrease to 54.57 mg L^{-1} during the 10th day. The net Zn solubilized (difference between available Zn content in ZnSB2 broth and available Zn content in control) was, therefore, 54.57, 116.95 and 43.86 mg L^{-1}, on the 3rd, 5th and 10th day, respectively. Apparently, this meant that 18.2%, 38.9% and 14.6% of the added Zn was solubilized by ZnSB2 on the 3rd, 5th and 10th day, respectively. There were, however, no significant differences in the amount of Zn solubilized by the other bacterial

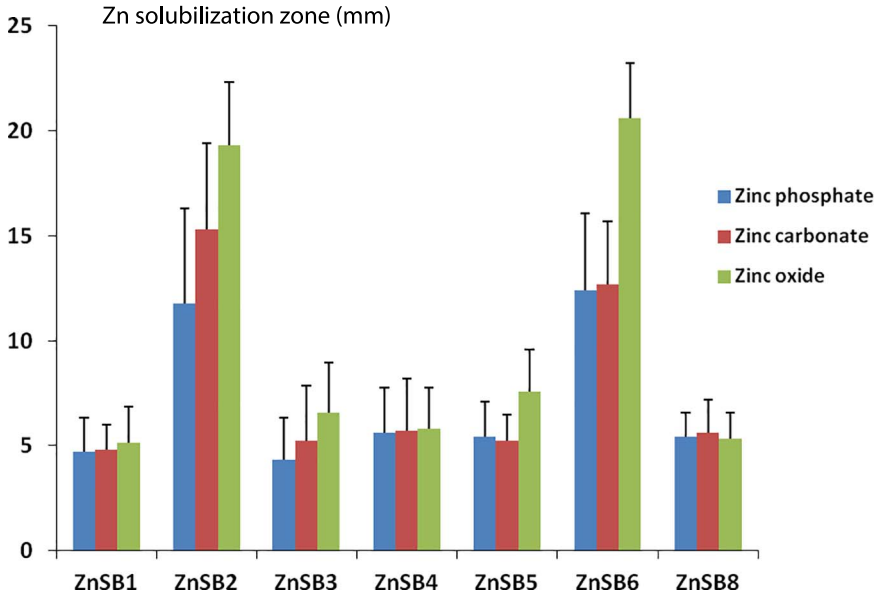

Fig. 2. Zn solubilization zone (mm) of promising Zn solubilizing bacteria (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified) in solid medium supplemented with different Zn sources [$Zn_3(PO_4)_2$; $ZnCO_3$ & ZnO] [Vertical bars indicate standard deviation; LSD ($P < 0.05$) for $Zn_3(PO_4)_2$ – 1.32; $ZnCO_3$ – 1.56 & ZnO- 1.43].

strains, irrespective of the days of sampling, so much so that the Zn levels by were even lower than the control at some days of sampling. Compared to control, there was a slight decrease in pH of the medium in treatments with ZnSB (Fig. 4) and the decrease was perceptible in the treatment with ZnSB2 where the pH ranged from 4.20 (day 5) to 6.41 (day 10). In fact, the highest level of soluble Zn (126.80 mg L^{-1}) was registered by ZnSB2 when the pH was 4.20 during the 5th day. The pH in other ZnSB treatments ranged between 6.40 (ZnSB5) to 7.71 (ZnSB8) (Fig.4).

3.4. ZnSB-mediated Zn release in soil

This study was done to investigate the rate of Zn release in soils treated with the short-listed ZnSB isolates and compared with a reference strain *G. diazotrophicus* at different days of incubation (2nd, 4th, 8th, 10th and 12th day). Zn was added as ZnO at 1000 mg Zn kg^{-1}. The results revealed an increase in available Zn levels in the treatments with ZnSB up to the 8th day of incubation followed by a gradual decrease (Fig. 5). Consistent with the results of the quantitative assay, among the short-listed isolates, markedly higher available Zn was registered by ZnSB2 at all days of incubation (255.5–406.5 mg kg^{-1}; i.e. 25.6%–40.7% of added Zn), with a peak on the 8th day. However, the available Zn release by ZnSB2 was relatively lower than *G. diazotrophicus* up to the 10th day, while being higher on the 12th day.

The net available Zn levels (difference between available Zn in ZnSB treatment and that in the control) in the soil with ZnSB2 were 35.5, 83.3, 196, 145.9, and 138.6 μg g^{-1} at 2, 4, 8, 10 and 12th day of incubation, respectively. This suggested that the rate of release of available Zn levels in treatment with ZnSB2 was greater by 16.1% on day 2, by 37.5% on day 4, by 93.1% on day 8, by 72.9% on day 10 and by 65.1% on day 12 compared to control. Corresponding rates of Zn release in case of *G. diazotrophicus* was slightly higher at 42.0%, 50.5, 109.9%, and 90.5% on day 2, 4, 8 and 10, respectively in relation to control. However, on the 12th day, the net available Zn release by ZnSB2 was greater by 26.5% compared to *G. diazotrophicus*. The levels of soil available Zn among the other ZnSB, though slightly higher than control, did not vary significantly at all days of incubation. Enhanced

Table 3
Hydrolytic enzyme production by the short-listed Zn solubilizing bacteria.

Isolate[a]	Protease	Cellulase	Pectinase	α-Amylase	Oxidase	Catalase
ZnSB1	+	−	−	+	+	+
ZnSB2	+	−	−	+	+	−
ZnSB3	+	−	−	−	−	+
ZnSB4	+	−	−	+	−	−
ZnSB5	+	−	−	+	−	−
ZnSB8	+	−	−	−	+	−

[a] ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified.

Table 2
Colony morphology and biochemical properties of shortlisted Zn solubilizing bacteria.

Isolate	Morphological and biochemical traits	Identity
ZnSB1	G-, irregular, undulate, flat, moderate, smooth, dull, non-pigmented (white)and opaque colonies, non-spore forming, indole (−), methyl red (−), Voges-Proskauer (−), citrate (−), glucose (+), adonitol (−), arabinose(−), lactase (−), sorbitol (−), mannitol (−), rhamnose (−), sucrose (−)	*Burkholderia lata*
ZnSB2	G+, irregular, undulate, convex, moderate, smooth, glistening, non-pigmented (cream) and opaque colonies, spore forming,, indole (−), methyl red (−), Voges-Proskauer (+), citrate (+), glucose (+), adonitol (−), arabinose(−), lactase (−), sorbitol (−), mannitol (−), rhamnose (−), sucrose (−)	*Bacillus megaterium*
ZnSB3	G+, irregular, undulate, flat, moderate, smooth, glistening, non-pigmented (cream) and opaque colonies, spore forming,, indole (−), methyl red (−), Voges-Proskauer (−), citrate (−), glucose (−), adonitol (−), arabinose(−), lactase (−), sorbitol (−), mannitol (−), rhamnose (−), sucrose (−)	*Lysinibacillus* sp.
ZnSB4	G+, small, circular, entire, smooth, moistened, translucent and creamy white colonies, spore forming,, indole (−), methyl red (−), Voges-Proskauer (−), citrate (−), glucose (−), adonitol (−), arabinose(−), lactase (−), sorbitol (−), mannitol (−), rhamnose (−), sucrose (−)	*Bacillus* sp.
ZnSB5	G-, large, irregular, undulate, raised, flat, opaque, smooth, moistened and creamy white colonies, non-spore forming,, indole (−), methyl red (+), Voges-Proskauer (−), citrate (−), glucose (+),adonitol (−), arabinose(−), lactase (−), sorbitol (−), mannitol (−), rhamnose (−), sucrose (−)	*Burkholderia latens*
ZnSB8	G-, punctiform, circular, entire, flat, opaque, smooth, moistened and white colonies, non-spore forming,, indole (−), methyl red (−), Voges-Proskauer (−), citrate (−), glucose (−), adonitol (−), arabinose(−), lactase (−), sorbitol (−), mannitol (−), rhamnose (−), sucrose (−)	Unidentified

Table 4
Growth promotion, nutrient solubilization and biocontrol traits of short-listed Zn solubilizing bacteria.

Isolate[a]	Growth promoting traits		Nutrient solubilization traits			Biocontrol traits	
	IAA	NH_3	P	K	Si	HCN	Siderophore
ZnSB1	−	+	−	−	−	+ + +	−
ZnSB2	−	+ +	+ +	−	−	-	−
ZnSB3	−	+	−	−	−	+	+
ZnSB4	−	+	−	−	−	+ +	−
ZnSB5	−	+	−	−	−	+ +	−
ZnSB8	−	+	−	−	−	+ +	−

[a] ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified.

rate of Zn release in both ZnSB2 and *G. diazotrophicus* treatments showed concomitant decreases in soil pH (Fig. 6). In fact, soil pH values in case of ZnSB2 ranged from 4.45–5.25, while in case of *G. diazotrophicus* it ranged from 4.17–5.20. The pH of the soil in the other ZnSB treatments (range 6.99–7.43) did not register as much decrease as observed with ZnSB2 and *G. diazotrophicus*.

3.5. Gluconic acid production by promising ZnSB

The decrease in pH in both broth assay and in soil was validated by measuring the gluconic acid production by the shortlisted bacteria (Fig. 7). It ranged from 1110.4–1884.7 μg mL^{-1} and significantly higher ($P < 0.05$) gluconic acid production was registered by ZnSB2, which was 35.3–69.7% greater than the other shortlisted ZnSB isolates.

3.6. Greenhouse study

Based on the results of the laboratory experiments, only ZnSB2 qualified for the subsequent greenhouse study on turmeric. ZnSB2 was applied alone or in combination with different levels of Zn (0, 75% and 100% Zn applied as $ZnSO_4$) to soils under turmeric. Soil available Zn levels were determined at 120 DAP and immediately after harvest (Fig. 8). At 120 DAP, it was greatest in the treatments with 100% Zn (12.74 ± 2.63 mg kg^{-1}) and 75% Zn + ZnSB2 (12.69 ± 2.96 mg kg^{-1}). The available Zn levels in the other treatments were lower (11.10–11.21 mg kg^{-1}), though significantly ($P < 0.05$%) higher than control (1.03 ± 0.45 mg kg^{-1}). Similar trend did not exist at harvest, wherein the available Zn levels in treatments involving chemical Zn application (100% Zn and 75% Zn) showed a decrease (8.34 ± 2.45 and 8.91 ± 2.13 mg kg^{-1}, respectively). Contrarily, the treatments involving ZnSB2 (ZnSB2 only and 75% Zn + ZnSB2) registered relatively higher available Zn levels (11.67 ± 2.56 and 13.76 ± 2.21 mg kg^{-1}, respectively). Indeed, the treatment with 75% Zn + ZnSB2 maintained 65.0% and 54.4% higher available Zn levels than 100% Zn and 75% Zn, respectively in the harvest soil samples.

The rhizome yield (Fig. 9) was significantly higher ($P < 0.05$) in the treatment with 100% Zn (177.2 ± 36.7 g pot^{-1}), which was, however, not significantly different from ZnSB2 + 75% of Zn (154.2 ± 36.0 g pot^{-1}). The rhizome yield in the latter was greater by 81.0% compared to control (no Zn), by 46.2% compared to 75% Zn and 56.6% compared to only ZnSB2. The Zn uptake (Zn content x dry matter production; Fig. 10) was significantly higher ($P < 0.05$) in the treatment with ZnSB2 + 75% Zn (40.5 ± 3.5 mg kg^{-1}), which was at par with 100% Zn (35.9 ± 3.2 mg kg^{-1}). Compared to control, the Zn uptake level was greater by a staggering 98.5%. Further, sole application of 75% Zn and ZnSB2 registered 42.0% and 27.7% lower Zn uptake than combined application of 75% Zn + ZnSB2.

3.7. Other phytobeneficial traits of ZnSB2

We also wanted to investigate whether ZnSB2 possessed other diverse set of traits related to plant growth promotion and disease suppression. The results suggested that ZnSB2, while being positive to protease, α-amylase, oxidase, NH_3, H_2S, and P solubilization, did not possess any biocontrol traits related to HCN, EPS and siderophore production. This was validated through dual culture experiments involving ZnSB2 and plant pathogens like *Fusarium oxysporum* causing wilt, *Rhizoctonia solani* causing collar rot, root rot or damping off, *Phytophthora capsici* causing quick wilt in black pepper, *Pythium myriotylum* causing soft rot and *Sclerotium rolfsii* causing stem rot or tuber rot or rhizome rot. The results revealed that ZnSB2 had no inhibitory effect, whatsoever, on these plant pathogens (Fig. 12). Interestingly, ZnSB2 was found to be compatible with an array of previously reported rhizobacteria with proven growth promotion & bicontrol characteristics (Fig. 13) like *B. amyloliquefaciens*, *Micrococcus luteus*, *Enterobacter aerogenes*, *Micrococcus* sp. and IISR6 - *Pseudomonas fluorescens*.

4. Discussion

In general, the soil was very acidic with very little variations in organic C, available P, exchangeable-K, available-Fe, -Mn, -Cu and -Zn

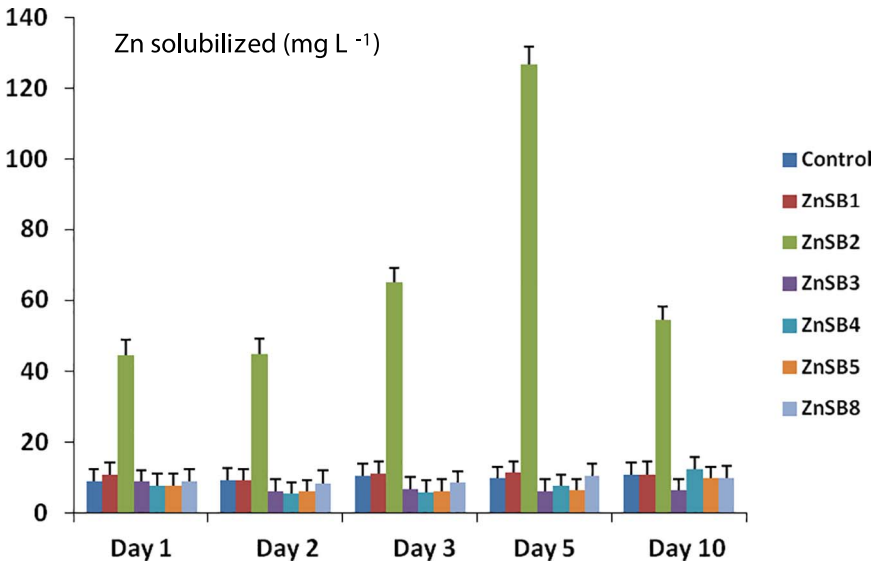

Fig. 3. Zn solubilized (mg L^{-1}) by promising Zn solubilizing bacteria (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified) in liquid medium at different days of incubation [Vertical bars indicate standard deviation; LSD ($P < 0.05$) for Day 1–2.59; Day 2–1.90; Day 3–3.54; Day 5–3.66; Day 10–4.55].

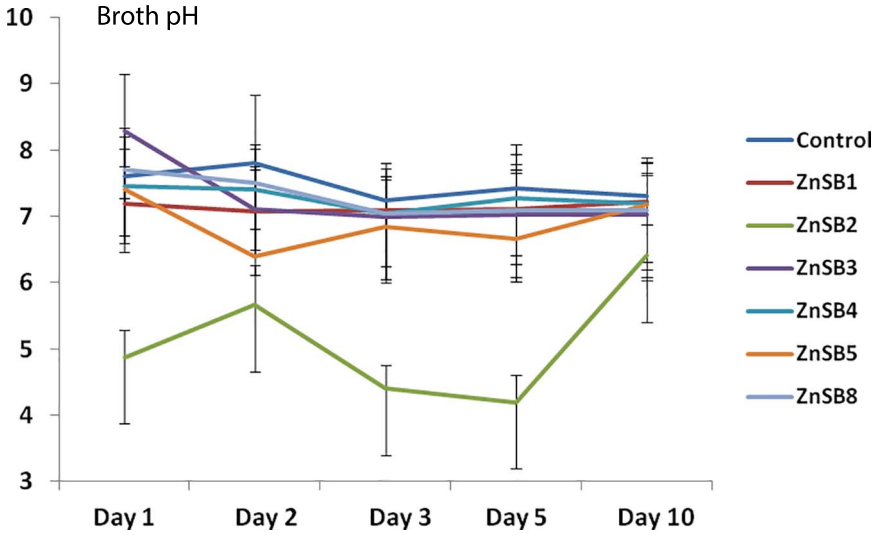

Fig. 4. Variations in pH of the liquid medium inoculated with promising Zn solubilizing bacteria (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified) at different days of incubation.

levels among the black pepper and cardamom rhizospheres. Nevertheless, with respect to the nutrient of interest viz., Zn, about 43.0% of the soil samples registered > 2.0 mg kg^{-1} available Zn (range 2.04–4.72 mg kg^{-1}; Supplementary Table 1) indicating that the Zn level in these soils are well above the critical Zn concentration of 0.38–2.0 mg Zn kg^{-1} reported in a wide range of soils from different climatic zones of India (Alloway, 2009).

Among the 70 bacterial strains obtained from these soil samples, the genera *Bacillus* followed by *Pseudomonas* was found to be the most dominant. Earlier reports also indicated that in the soil (rhizosphere and non- rhizosphere) and other environments, *Bacillus* is the most dominant group of bacteria though genera like *Pseudomonas*, *Azospirillum*, *Enterobacter*, *Arthrobacter*, *Flavobacterium*, *Azotobacter*, *Micrococcus*, *Clostridium*, *Achromobacter* etc. are also common (Dinesh et al., 2015; Felici et al., 2008; Forchetti et al., 2007; Swain and Ray, 2009).

Seven bacterial strains were found to outperform others with regard to Zn solubilization in vitro, but showed variable degrees of solubilization as indicated by the variation in the diameter of halo formation. This corroborated with earlier reports that bacterial strains differ in their ability to solubilize $ZnCO_3$, $Zn_3(PO_4)_2$ and ZnO (Kumar et al., 2016a; Sunithakumari et al., 2016; Sharma et al., 2012). The halo formation signified the solubilization zone and can be attributed to lowering of pH and consequent solubilization of the insoluble Zn compound in the immediate vicinity of the bacteria. Among the Zn sources we found the dissolution zones to be markedly larger with ZnO followed by $ZnCO_3$ and $Zn_3(PO_4)_2$. However, reports on the size of the dissolution zone are contradictory, varying with the C source (sucrose/glucose) provided during the assay (Saravanan et al., 2007b) and greater solubilization zone has been observed with ZnO (Fasim et al., 2002; Kumar et al., 2016a), or $Zn_3(PO_4)_2$ (Sharma et al., 2012) or $ZnCO_3$ (Vidyashree et al., 2016).

Since the plate assay does not reflect the true capability of the bacterial strains to solubilize/mineralize insoluble compounds (Bashan et al., 2013), the shortlisted ZnSB were further assayed in liquid medium (broth assay) containing a mixture of Zn compounds viz., $ZnCO_3$, $Zn_3(PO_4)_2$ and ZnO. The broth assay also confirmed the differential abilities of the ZnSB to solubilize Zn, with only ZnSB2 consistently registering markedly higher labile Zn at all stages of sampling. The enhanced labile Zn level was also accompanied by a significant reduction in pH of the medium suggesting that ZnSB2 produced organic acids that reduced the pH, which in turn helped in the dissolution of the added insoluble Zn compounds. The maximum labile Zn registered by ZnSB2 (126.8 mg L^{-1}) suggested that 42.3% of the added Zn was solubilized by the bacteria on the 5th day.

To determine if the Zn solubilization pattern observed in plate and

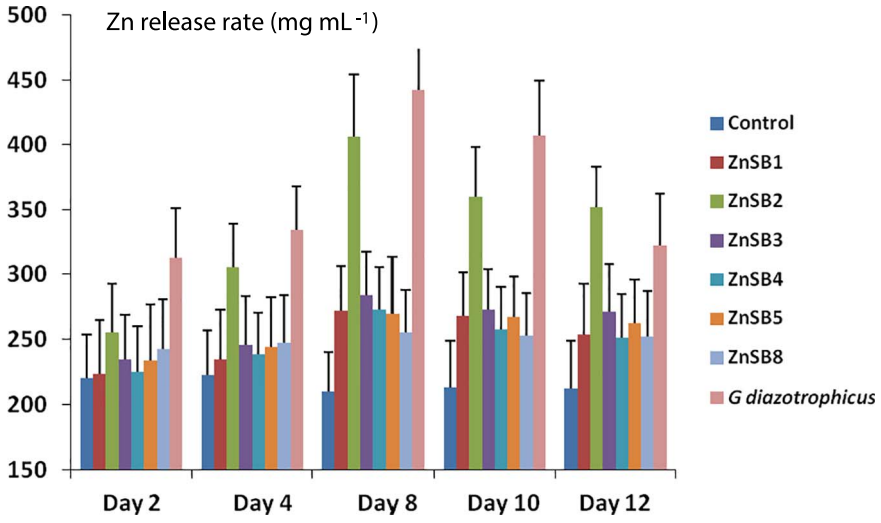

Fig. 5. Zn release rate (mg kg^{-1}) in soils inoculated with Zn solubilizing bacteria (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified) and reference Zn solubilizer (*G. diazotrophicus*) at different days of incubation [LSD ($P < 0.05$) at Day 2–64.12; Day 4–45.75; Day 8–50.77; Day 10–83.0; Day 12–62.5].

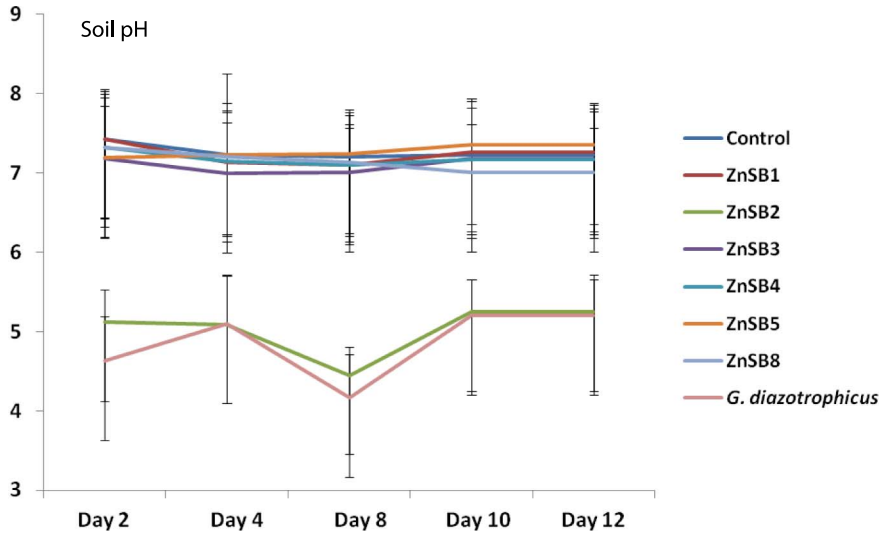

Fig. 6. pH variations in soils inoculated with Zn solubilizing bacteria (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified) and reference Zn solubilizer (*G. diazotrophicus*) at different days of incubation [LSD ($P < 0.05$) at Day 2–1.33; Day 4–1.24; Day 8–1.16; Day 10–1.27; Day 12–1.32].

broth assays could be emulated in soils per se, an incubation study was conducted using the shortlisted strains. The aim was to study the rate of release of Zn in soils inoculated with the shortlisted ZnSB over a period of time. A reference Zn solubilizer viz., *Gluconacetobacter diazotrophicus* was also included in this experiment for comparison. It is an endophytic bacteria belonging to acetic acid group with proven Zn solubilizing credential (Sarathambal et al., 2010; Saravanan et al., 2007b). The Zn release pattern in soils treated with the shortlisted ZnSB was almost identical to that observed in the plate and liquid assays. Among the isolates, *G. diazotrophicus* registered the greatest Zn release rates up to the 10th day of incubation, while ZnSB2 registered the greatest Zn release rate on the 12th day. The rate of release of net available Zn in treatment with ZnSB2 was greater by 16.1–72.9% compared to control, while the corresponding values were as low as 1.4–34.7% among the other ZnSB. To all intents and purposes, this suggested the overwhelming influence of ZnSB2 on Zn mineralization and release in soil, which is perhaps due to the increased organic acid production as evidenced by the simultaneous reduction in soil pH at different days of incubation. Solubilization of fixed or insoluble metal complexes by organic acids is an important property in soil nutrient cycling (Goteti et al., 2013; Rashid et al., 2016; Saravanan et al., 2007b) and the active involvement of bacteria in such solubilization processes is considered to be an effective mechanism to enhance the labile form of nutrient elements in soil (Archana et al., 2012).

The dissolution of insoluble Zn in broth assay and in the soil can be attributed to the production and efflux of gluconic acid and its derivatives like 2- and 2, 5-keto-derivatives by ZnSB2 (Saravanan et al., 2011). Gluconic acid synthesis in gram-negative bacteria is achieved by oxidation of glucose and the reaction is catalysed by membrane-bound glucose dehydrogenase with pyrroloquinoline quinine as the cofactor. The subsequent oxidation of gluconic acid to 2-ketogluconate is catalysed by gluconate dehydrogenase (de Werra et al., 2009). Gluconic acid and its derivatives have been implicated in enhancing the dissolution of insoluble Zn compounds through reductions in the pH of the medium (Di Simine et al., 1998; Gontia-Mishra et al., 2017; Saravanan et al., 2008; Sharma et al., 2012).

With regard to ZnSB2, the dissolution rate was inversely proportional to the pH of the medium ($r = -0.72$ at $P < 0.01$) and, therefore, acidification by gluconic acid production appears to be the major mechanism by which ZnSB2 enhanced the labile Zn level. This was well supported by the gluconic acid production levels which was greatest with ZnSB2. Higher gluconic acid production by ZnSB2 tended to shift

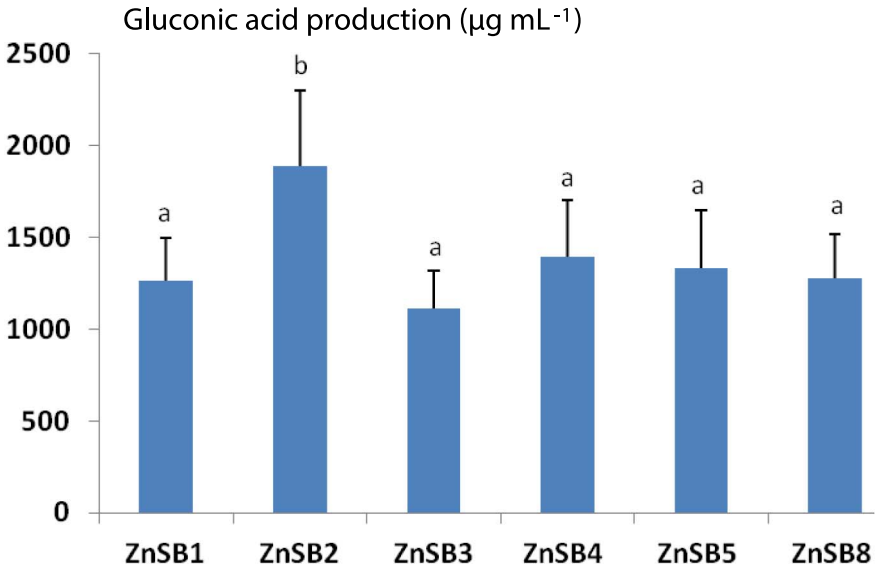

Fig. 7. Gluconic acid production (µg mL^{-1}) by promising Zn solubilizing bacteria (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*, ZnSB8 - Unidentified) [Vertical bars indicate standard deviation; different letters indicate significant difference at $P < 0.05$].

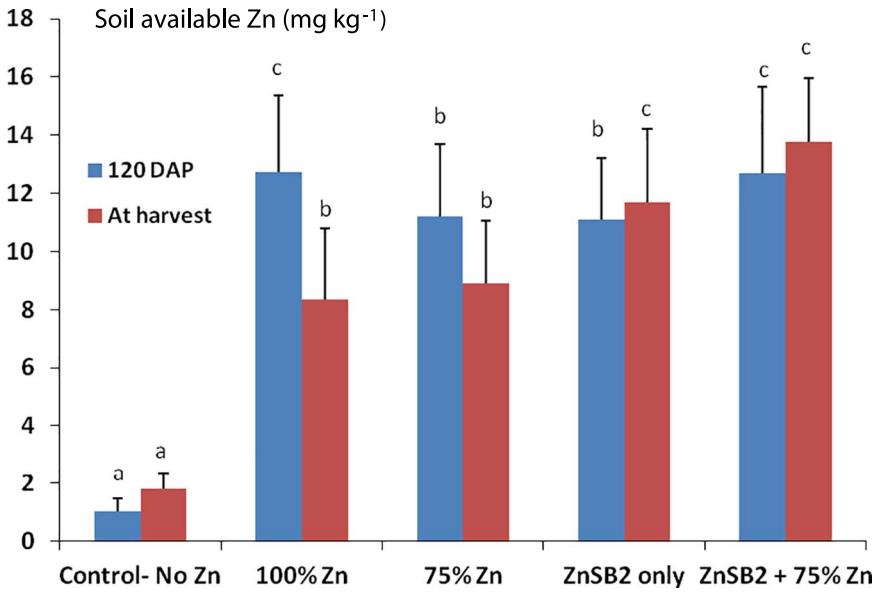

Fig. 8. Effect of ZnSB2 (*B. megaterium*) applied alone and in combination with varying rates of chemical Zn on soil available Zn levels (mg kg^{-1}) at 120 days after planting (DAP) and at harvest of turmeric [100% Zn - 6.0 kg ha^{-1}; Vertical bars indicate standard deviation; different letters at each sampling stage indicate significant difference at $P < 0.05$].

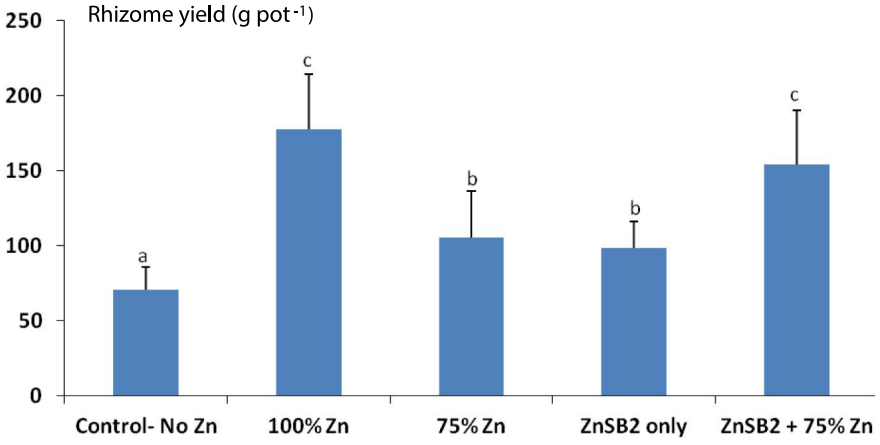

Fig. 9. Effect of ZnSB2 (*B. megaterium*) applied alone and in combination with varying rates of chemical Zn on turmeric rhizome yield (g pot^{-1}) [100% Zn - 6.0 kg ha^{-1}; Vertical bars indicate standard deviation; different letters indicate significant difference at $P < 0.05$].

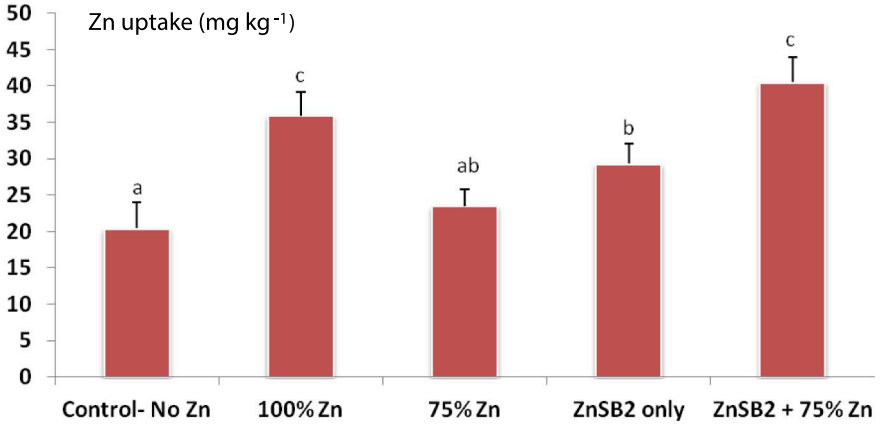

Fig. 10. Effect of ZnSB2 (*B. megaterium*) applied alone and in combination with varying rates of chemical Zn on Zn uptake in rhizome (mg kg^{-1}) [100% Zn - 6.0 kg ha^{-1}; Vertical bars indicate standard deviation; different letters indicate significant difference at $P < 0.05$].

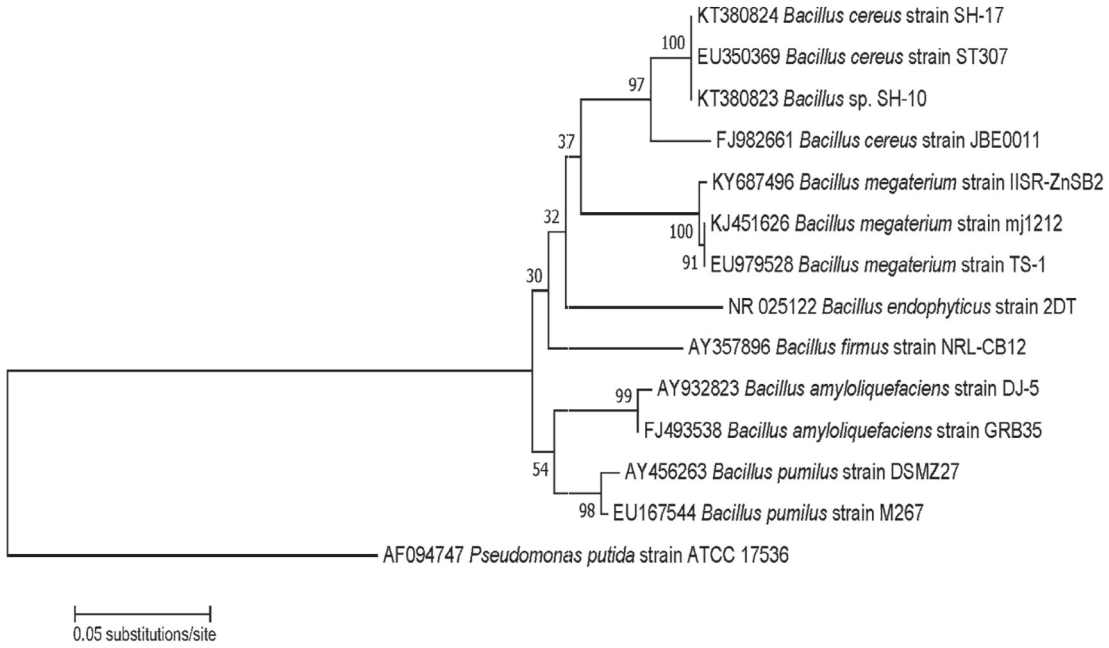

Fig. 11. Neighbour-joining tree based on analysis of partial 16S rRNA nucleotide sequences of *B. megaterium* (IISR-ZNSB2; KY687496). The sequence of ZnSB2 along with gene sequences of Zn solubilizing bacteria reported in the literature were retrieved from the GenBank and aligned using MUSCLE incorporated in MEGA 7.0. Bootstrapping was performed with 1000 replicates and the evolutionary distance was computed using Kimura 2 + G parameter model.

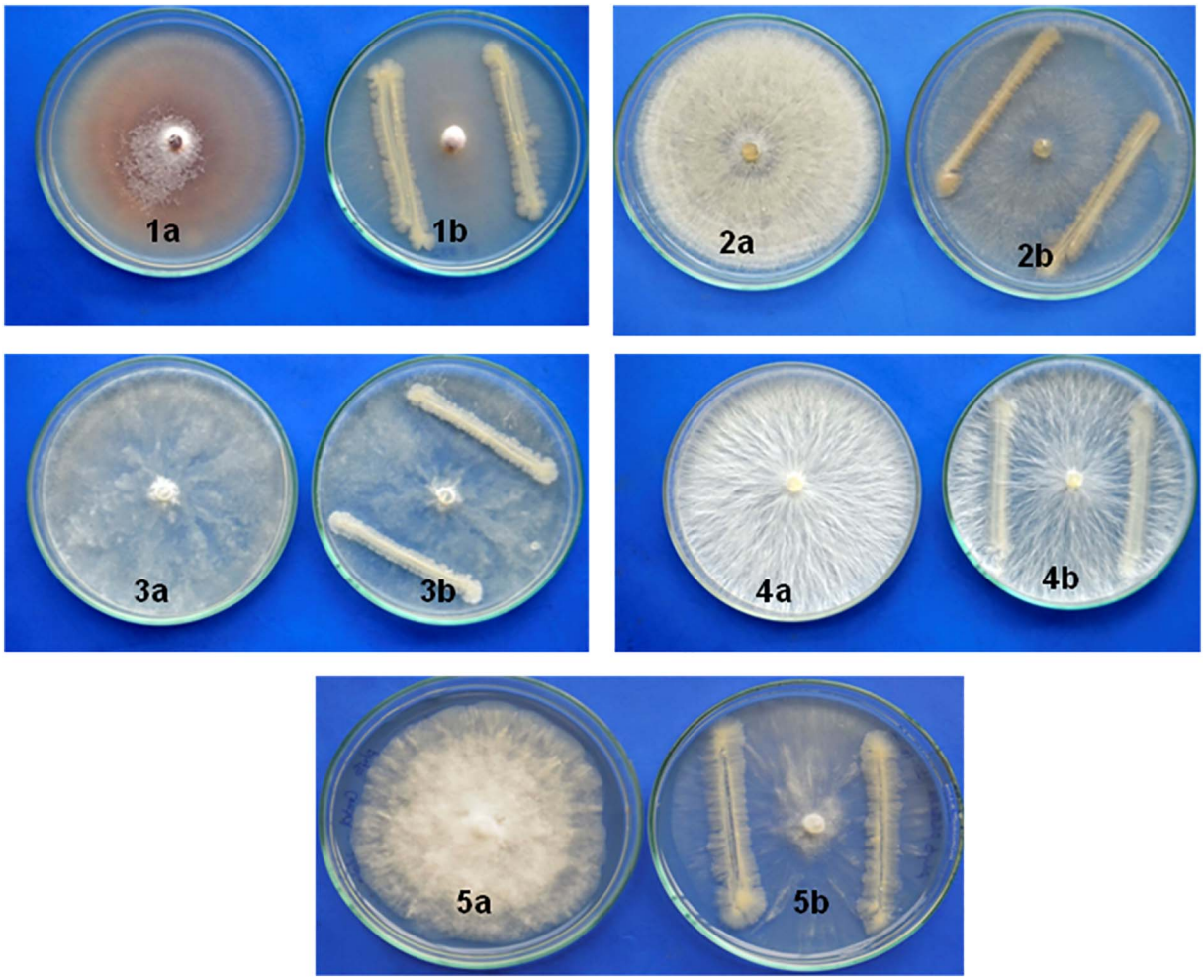

Fig. 12. In vitro interaction (dual culture) between ZnSB2 and some common plant pathogens 1a - *Fusarium oxysporum*, 1b - *F. oxysporum* vs ZnSB2; 2a - *Rhizoctonia solani*, 2b - *R. solani* vs ZnSB2; 3a - *Pythium myriotylum* 3b - *P. myriotylum* vs ZnSB2; 4a - *Sclerotium rolfsii*; 4b - *S. rolfsii* vs ZnSB2.

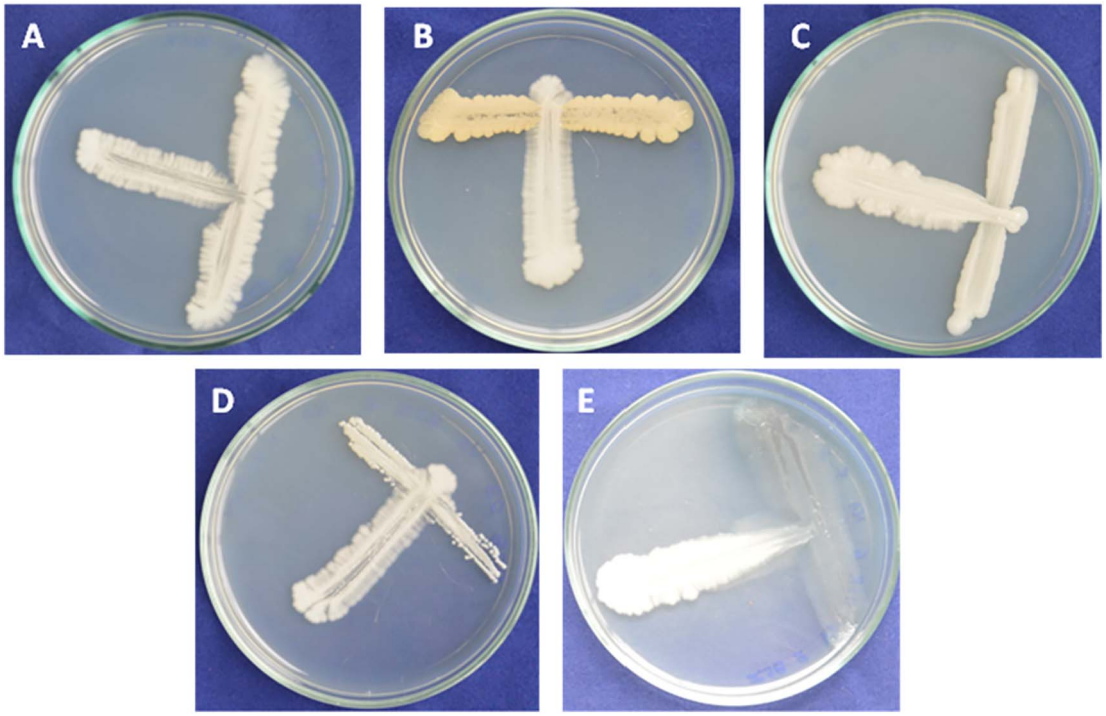

Fig. 13. In vitro interaction of ZnSB2 with some promising PGPR viz., (A) GRB35 - *B. amyloliquefaciens*, (B) BRB-3 (*Micrococcus luteus*), (C) BRB-13 (*Enterobacter aerogenes*), (D) BRB-23 (*Micrococcus* sp.) and (E) IISR6 - *Pseudomonas fluorescens*.

the pH towards acidic levels, which consequently resulted in greater Zn solubilization rates.

The subsequent greenhouse study involved only ZnSB2 due to its markedly higher Zn solubilization potential in the broth and soil. ZnSB2 was applied alone or in combination with varying levels of Zn (0, 75% & 100% Zn applied as $ZnSO_4$). The results showed that the rhizome yield was significantly ($P < 0.05$) higher in the treatment involving 100% Zn, which was statistically ($P < 0.05$) at par with the treatment involving combined application of ZnSB2 and 75% Zn. This indicated the possibility of reducing chemical Zn application rate by 25% while at the same time maintaining sufficient yield when chemical Zn form is applied in tandem with ZnSB2. Higher rhizome yield levels in 100% Zn treatments and ZnSB2 + 75% Zn can be ascribed to enhanced available Zn in the soil at both 120 DAP and at harvest. In fact, the treatments with ZnSB2 were able to maintain available Zn level for sufficiently longer period of time (up to harvest) than the treatments with chemical form of Zn. This suggested sustained bioavailability of Zn due to solubilization by ZnSB2.

Besides yield, Zn uptake by the crop was also highest when ZnSB2 was combined with 75% of the recommended Zn rate, which further underlined the positive influence of ZnSB2 on labile Zn pool. This is expected since enhancing soil available Zn levels can result in marked increases in their concentration in crop produce (Graham et al., 2007; Bouis and Welch, 2010). Similar positive effects of ZnSB like *Burkholderia* sp. SG1, *Acinetobacter* sp. SG2 & *Acinetobacter* sp. SG3 (Vaid et al., 2014), *Acinetobacter* sp. AGM3 & 9 (Gandhi and Muralidharan, 2016), *Pseudomonas aeruginosa*, *Ralstonia picketti*, *Burkholderia cepacia* & *Klebsiella pneumoniae* (Gontia-Mishra et al., 2017) on rice, *Exiguobacterium aurantiacum* strain MS-ZT10 on wheat (Shaikh and Saraf, 2017) and *Bacillus* sp. (ZM20), *Bacillus aryabhattai* (ZM31 and S10) & *Bacillus subtilis* (ZM63) on maize (Ramesh et al., 2014; Mumtaz et al., 2017) have been reported.

The promising ZnSB strain viz., ZnSB2 was identified by 16S rRNA as *Bacillus megaterium*. The sequence data of ZnSB2 was deposited in GenBank and accession number KY687496 was assigned. The phylogenetic tree constructed using 16S rRNA sequences shows the relationship between ZnSB2 and other reported nutrient solubilizing bacteria (Fig. 11). Our strain was very closely related to *Bacillus megaterium* strain KJ451626 (Kang et al., 2014) and *Bacillus megaterium* strain EU979528 (Kumar and Rai, 2017) known for their P solubilizing potential. *B. megaterium* occurs in terrestrial and aquatic environments in close proximity to animals, humans and plants. It is a gram positive, rod shaped bacterium capable of producing endospores, which are thick walled and capable of persisting and surviving in sub-optimal environments and can withstand nutrient depletion. It is commonly employed in wastewater treatment, biodegradation and bioremediation, septic and drain treatment, cleaning and deodorizing, as well as enzyme production. More importantly, *B. megaterium* has been used in crop production due to its bicontrol & growth promoting capabilities (Kang et al., 2014; Nguyen et al., 2011; Trivedi and Pandey, 2008; Wyciszkiewicz et al., 2017) and is also reported to be a remarkable P solubilizer in soils (Ortiz-Castro et al., 2008; Wyciszkiewicz et al., 2017). Though several *Bacillus* sp. have been reported as efficient Zn solubilizers (Shakeel et al., 2015; Sharma et al., 2012), there are very few reports on the ability of *B. megaterium* to solubilize Zn in soils.

Studies on the biocontrol traits of ZnSB2 revealed that it did not inhibit plant pathogens like *Fusarium oxysporum* causing wilt, *Rhizoctonia solani* causing collar rot, root rot or damping off, *Phytophthora capsici* causing quick wilt in black pepper, *Pythium myriotylum* causing soft rot and *Sclerotium rolfsii* causing stem rot or tuber rot or rhizome rot. It is, therefore, apparent that in addition to lacking the ability to produce antibiotics and siderophores in sufficient quantities to suppress these pathogens, ZnSB2 lacked the trappings of a typical plant growth promoting rhizobacteria (PGPR) possessing important disease suppressing mechanisms like antibiosis, competitiveness and parasitism. Nonetheless, to negate this drawback, it would be possible to deploy ZnSB2 in a consortium mode since we found this strain to be compatible with several previously reported PGPR with proven growth promotion & bicontrol characteristics like GRB35 - *B. amyloliquefaciens* (Dinesh et al., 2015), BRB-3 (*Micrococcus luteus*), BRB-13 (*Enterobacter aerogenes*) & BRB-23 (*Micrococcus* sp.) (Dinesh et al., 2014), IISR6 - *Pseudomonas fluorescens* (Diby et al., 2005).

5. Conclusions

An array of bacteria were isolated from undisturbed soil, characterised and studied for their Zn solubilization potential. Six bacterial isolates were found to be promising (ZnSB1 - *Burkholderia lata*; ZnSB2 - *Bacillus megaterium*; ZnSB3 - *Lysinibacillus* sp.; ZnSB4 - *Bacillus* sp.; ZnSB5 - *Burkholderia latens*; and ZnSB8 - unidentified), of which, ZnSB2 (NCBI-KY687496) was found to be the most potent strain owing to its enhanced Zn solubilization capacity exhibited in both qualitative and quantitative assays. This was also reflected in the soil incubation study wherein ZnSB2 exhibited remarkably high Zn release rates at all days of incubation. This was attributed to decreased pH owing to its high gluconic acid production capacity. Further evaluation of ZnSB2 in the green house study on turmeric revealed greater soil available Zn level in the treatment with ZnSB2 + 75.0% Zn (applied as $ZnSO_4$) compared to 100% Zn. Such positive effects of ZnSB2 were also registered on yield and Zn content of rhizomes, which suggested the possibility of reducing chemical Zn application to soil. However, the strain lacked in several disease suppression traits thereby limiting its biocontrol ability, but was compatible with several known PGPR indicating its suitability for application in a consortium mode. Further studies on the effect of these potential Zn solubilizers on various fractions of Zn in soils will provide gainful insights into the changes in the chemical pools of Zn and their subsequent contribution to phytoavailability of Zn. It would also be interesting to pursue studies involving ZnSB2 applied in conjunction with varying levels of Zn applied as insoluble ZnO and $ZnCO_3$ instead of soluble $ZnSO_4$. It also remains to be seen if the benefits of ZnSB2 observed in the green house study persists under field conditions and whether ZnSB2 applied alone or in a consortium mode would aid in enhancing Zn content in the rhizomes while simultaneously enhancing growth and yield. Nonetheless, it seems inevitable that fewer chemical inputs will be used in future with greater reliance on such beneficial microorganisms for soil nutrient solubilization/mobilization and plant growth promotion.

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