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ORIGINAL PAPER

Isolation, screening, characterization, and optimization of bacteria isolated from calcareous soils for siderophore production

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Abstract

The most effective agricultural practice to prevent iron deficiency in calcareous soils is fertilizing with synthetic chelates. These compounds are non-biodegradable, and persistent in the environment; hence, there is a risk of leaching metals into the soil horizon. To tackle iron deficiency-induced chlorosis (IDC) in crops grown on calcareous soils, environmentally friendly solutions are needed rather than chemical application as it affects the soil health further. Hence, the present work focused on isolating and screening calcareous soil-specific bacteria capable of producing iron-chelating siderophores. Siderophore-producing bacteria (SPB) was isolated from the groundnut (*Arachis hypogea* L.) rhizosphere region, collected from Coimbatore district, Tamil Nadu, of which 17 bacterial isolates were positive for siderophore production assayed by chrome azurol sulphonate. The performance of SPB isolates was compared for siderophore kinetics, level of siderophore production, type of siderophore produced and iron-chelating capacity under 15 mM $KHCO_3$. Four best performing isolates were screened, with average siderophores yield ranging ~60–80% under pH 8, with sucrose as carbon source and NH_2SO_4 as nitrogen source at 37 °C. The four efficient SPB were molecularly identified as *B. licheniformis*, *B. subtilis*, *B. licheniformis,* and *O. grignonense* based on 16S rDNA sequencing. The simultaneous inhibition method showed *T.viride* has the highest antagonistic effect against *S.rolfsii,* and *M.phaseolina* with a reduction of mycelial growth by 69.3 and 65.1%, respectively, *compared to control.* Our results indicate that the optimized conditions enhanced siderophores chelation by suppressing the stem and root rot fungi, which could help in a cost-effective and environmentally friendly manner.

Keywords *Arachis hypogea* · Calcareous soils · Iron · Phytopathogenic and siderophores

Introduction

Even though Fe is one of the most predominant elements in the Earth's crust (Taylor and Konhauser 2011), its availability to plants and microorganism is insufficient. A transition metal can be found in two oxidation states, Fe (III) and Fe (II). It is a crucial limiting factor for plants and microorganisms as it occurs as Fe^{3+} (ferric form) which is not soluble at physiological pH (Bou-Abdallah 2010) and is insoluble in the form of iron (Saha et al. 2016). Iron is an essential element for plant growth and development (Barker and Pilbeam 2015). Though Fe is not a chlorophyll component, it is vital for photosynthesis and the functioning of the photosynthetic apparatus (Rangani et al. 2018). It also controls the biosynthesis of antibiotics, porphyrins, pigments, toxins, siderophores, cytochromes, and aromatic compounds (Messenger and Barclay 1983). The iron deficiency of plants results in chlorosis, characterized by the yellow young leaves due to lack of iron for efficient chlorophyll production (Barhoumi et al. 2021). These effects of chlorosis in plants result in lower biomass and yield, decreased flowers and fruits, and in critical cases total crop fatigue (Kabir et al. 2016).

Because of iron chemical composition, low solubility, low bioavailability, and dissolubility kinetics, the absorption of iron on calcareous soils becomes a problem. IDC is widespread in plants grown in alkaline and calcareous soils due to low Fe (Fe^{2+}) levels. Calcareous soils are estimated to

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cover about 30% of the world's cultivated soils (Barker and Pilbeam 2015) which underscores the importance of iron deficiency as a major global agricultural challenge. This significantly affects crop yields and hinders low bioavailability in soil (Martins et al. 2017). Synthetic chelating agents viz., *o,o*-ethylenediamine—di (*o*-hydroxyphenylacetate)-Fe(III) and *o,o*-EDDHA/Fe(III) chelate (Bin et al. 2016) have been commonly used to overcome chlorosis in calcareous soils, but their non-biodegradable nature poses a hazard to soil health. Since these are powerful chelating agents, the pursuit of environmental protection and the proper chelating of iron fertilizers has therefore become a major challenge.

Microbes have developed many strategies to scrounge iron from their atmosphere in response to iron limitation. One mechanism of microorganisms and plants is to produce low molecular (500–1000 Daltons) iron chelators termed siderophores (Greek *sideros* meaning iron and phores meaning bearer) to enhance the acquisition of iron from the soil environment, particularly under the Fe-limited circumstances, which selectively complex iron (III) with very high affinity (Chowdappa et al. 2020). They exhibit strong interaction constants for iron complexation. Siderophores are usually classified into three major classes, such as hydroxamates, catecholates, and carboxylates based on their structural units (Hider and Kong 2010). Some bacteria possess more than one form of siderophores and multiple absorption pathways for iron acquisition. The main feature of the siderophores is the chelating of Fe (III) and accumulation of complex heavy metals such as Fe^{3+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cd^{2+} and the production and retention of toxic metals such as Cu^{2+}, Zn^{2+}, and Pb^{2+} affecting metal mobility (Braud et al. 2009).

Siderophore first bonds to the ferric form of iron and then forms a siderophore–iron complex, which enters the cells through unique cell membrane siderophore receptors. Many siderophores produced by microorganisms are attributable to the low atmospheric abundance of Fe and are more selective to Fe (III) than divalent metals (Chowdappa et al. 2020). Approximately 500 biomolecules have been listed under siderophores where many genes and regulators are involved in their synthesis, distribution, and re-import into cells (Chu et al. 2010). Previous studies reported that the siderophore beneath iron-deprived conditions forms complex with iron (Narendra Babu et al. 2015).

Several siderophore-producing rhizobacteria (SPR) have been assessed and reported as biocontrol agents against plant pathogens (Sulochana et al. 2013; Sasirekha and Srividya 2016), including the species of *Pseudomonas* (Ghazy and El-Nahrawy 2021), *Bacillus* (Miao Sheng et al. 2020; Ghazy and El-Nahrawy 2021), and *Enterobacter* (Sinha and Parli 2020). These strains develop siderophores under iron-restricting conditions that chelate the available iron and deprive the respective phytopathogens of iron nutrients (Santoyo et al. 2016), thus limiting the dissemination of phytopathogens and root colonization. SPR is also known to impart induced systemic resistance (ISR) to plants (Pieterse et al. 2000;) and has been documented in biocontrol of *M. Phaseolina, Rhizoctonia solani, Phytophthora nicotianae* var. *Parasitic, Pythium spp*., and *Fusarium spp*. (Haas and Défago 2005; El-Shabrawy and Shehata 2018). Narendra Babu et al. 2015 also observed strong protection against *Alternaria solani* in tomato plants when the plants were pretreated with PGPR which is in accordance with the presence of siderophore. The solvent-extracted siderophores inhibited *Ralstonia solanacearum* in groundnut and *Xanthomonas oryzae pv. oryzae* in rice (Chowdappa et al. 2020). With this context, our research focuses on the isolation and characterization of calcareous soil siderophore-producing bacteria and the culture conditions for higher siderophore production have also been optimized.

Materials and methods

Micro-organisms and culture conditions

Soils were sampled from both rhizosphere and bulk soils of groundnut, grown under calcareous soils in June 2019 near the villages of Thondamuthur block of Coimbatore, Tamil Nadu, India (10°99′N, 76°79′E). These sites were located about at an altitude of 473 m above sea level. The annual average precipitation is 618 mm and an annual average temperature is 28.9 °C. The bedrock in the area is charnockite and gneiss. The soil samples for this study were taken from the rhizosphere soil and kept cold (4 °C) until further analysis.

Isolation and identification of siderophore-producing bacteria from soil

The aerobic and facultative anaerobic siderophore-producing microorganisms were isolated from the soil solution. The samples were serially diluted (to 10^{-5}) and inoculated in nutrient agar (NA) medium and incubated for 24 h at room temperature. The colonies were distinguished, sub-cultured, and purified (Gaonkar and Bhosle 2013). The purified colonies were further tested for the production of siderophore using CAS (Schwyn and Neilands 1987). The positive colonies were purified by three times subculture method on CAS agar plates and stored in 20% (v/v) glycerol at − 20 °C.

Identification of strains

Bacterial isolate showing efficient siderophore production was further characterized based on the morphological, biochemical, and molecular levels. Isolates were gram stained to understand the cell shape, size, arrangement, and gram nature. The purified isolates were subjected to biochemical characterization (Aneja 2007) for the detection of organisms up at the genus level. Further, the molecular characterization was carried out using fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACG GCTACCTTGTTACGACTT 3') primers. The reaction mixture in each tube consisted of 20 µl with DNA template 50 ng, 1 × Taq buffer, 0.2 mM of each dNTP mixture, 1 µM of each primer, 1.5 mM $MgCl_2$, and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed and products were separated by electrophoresis on 1% agarose gel (Sambrook et al. 1989). The 16S rRNA gene was sequenced in both directions and the obtained sequences were compared to sequences in the NCBI GenBank database using the BlastN (Altschul 1997). The recovered sequences, as well as the closely identified relatives, were aligned in Molecular Evolutionary Genetics Analysis (MEGA) Software ver. 10.0. All the sequences were submitted to the GenBank and accession numbers were acquired.

Partial purification of siderophore

The cultures were grown in Fiss-glucose minimal media (Vellore 2001) for 24 h at 28 °C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7000 rpm for 30 min. The supernatant was then acidified to pH 2.0 with 6 M HCl in order to make the siderophore less soluble in water. This acidified supernatant passed through a 30 × 5 cm column packed with Amberlite XAD-2 (Sigma, USA), which binds cyclic compounds were dissolved in distilled water and kept overnight for soaking. The loaded column was pre-washed twice with water, methanol, and water. The aqueous supernatant was allowed to slowly pass through the column at the rate of 5 mL min^{-1}. Loading of supernatant was continued until the saturation of the column, which was indicated by the browning of the column. The column was then washed with five to ten bed volumes of distilled water to remove all unbound components of the medium. The column was then equilibrated by changing the solvent to 50% methanol to facilitate the extraction of siderophores. Different fractions were separately collected; filtrate, water wash, and eluted fractions were checked for CAS test. Fractions positive for siderophore were combined in a 100 ml boiling flask and the pH was adjusted to 3.0 with H_2SO_4 and 50% ammonium sulfate solution was added to deproteinize and evaporated to dryness using a rotary evaporator. This aqueous phase was concentrated in a lyophilizer (Lark) and set aside in cold to crystallize. The filtrate was neutralized, reduced to dryness, extracted in dry hot methanol and were then separated on Whatman filter paper no. 44. The obtained purified siderophore crystals obtained were then subjected to Fourier transform infrared spectroscopy (FTIR)-ATR analysis (JASCO FT/IR-6800) for determination of the functional groups. The spectrum was recorded in the range from 400 to 4000 cm^{-1}. The infrared spectrum wavelengths were determined based on their functional groups (Tank et al. 2012).

Quantitative and qualitative estimation of siderophores

The purified bacterial isolates were grown in an iron-deficient succinate medium and incubated for 48 h with constant shaking at 120 rpm. All the isolates were screened for siderophore production viz spectrophotometric means at A630 nm (Grimm and Allen 1954) which was further confirmed by the chrome azurol sulphonate (CAS) agar test. The production of siderophore by the isolate was quantitatively determined using CAS as described by Schwyn and Neilands (1987). From the prepared CAS solution, 0.5 ml was taken to which 0.5 ml of culture supernatant was added and incubated for 5 min. Then the mixture was measured at 630 nm and calculated for the siderophore production (Fazary et al. 2016). The percent of siderophore was intended in terms of % of siderophore units by the following formula:

$$\%\ \text{of siderophore units} = [\text{Ar} - \text{As})/\text{Ar}] * 100$$

where Ar = absorbance of reference (CAS reagent); As = absorbance of the sample at 630 nm. Further, the qualitative confirmation was performed by CAS agar test, where the bacterial isolates streaked on the CAS plates show orange zone.

Screening of bacteria for calcareousness tolerance

The succinate medium (SM) was used to screen calcareous tolerant bacteria. The succinate medium concentration per liter: KH_2PO_4, 3.0 g; K_2HPO_4, 6.0 g; $MgSO_4.7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1.0 g; succinic acid, 4.0 g; peptone, 1.0 g; final pH 6.5. This medium was enriched with $KHCO_3$ (calcareous source) to mimic calcareous soil. The $KHCO_3$ were added at different concentrations viz., 10, 15, 20, and 25 µM at room temperature. The production of siderophore by the isolates under calcareous stress was quantitatively determined using CAS (Schwyn and Neilands 1987).

Succinate medium (SM)	KH_2PO_4 3.0 g per litre; K_2HPO_4 6.0 g per litre; $MgSO_4.7H_2O$ 0.2 g per litre; $(NH_4)_2SO_4$ 1.0 g per litre; succinic acid 4.0 g per litre; peptone 1.0 g per litre; distilled water 1000 ml; final pH 6.5
Treatment 1	SM + 10 mM $KHCO_3$
Treatment 2	SM + 15 mM $KHCO_3$
Treatment 3	SM + 20 mM $KHCO_3$
Treatment 4	SM + 25 mM $KHCO_3$

Chemical characterization of siderophores

The type of siderophore was determined by specific tests using culture supernatants prepared as described above. Hydroxamate type of siderophore was detected by tetrazolium test (Snow 1970), catecholate type of siderophores was detected by Arnow's test (Arnow 1937), and carboxylate type was detected using Vogel (1992).

Optimization for maximum siderophore production

The bacterial isolates were allowed to grow in succinate broth under different fermentation conditions, such as pH (5, 6, 7, 8, and 10), temperature (27, 37, and 45°C), nitrogen sources (urea, ammonium sulfate (NH_2SO_4), and sodium nitrate ($NaNO_3$)), carbon sources (sucrose, glucose, fructose, and lactose), and iron concentration (1, 2, and 3 ppm) for 48 h to investigate the maximum level of siderophore production. For siderophores analysis, the supernatant was centrifuged at 5000 rpm for 10 min and cell-free supernatant was analyzed using CAS assay test. The production of siderophore was measured at 630 nm and calculated (Fazary et al. 2016).

Antagonism tests in vitro

Growth of bacterial strain and fungal mycelium in dual culture

The antimicrobial activity of every bacterial strain was surveyed by the dual culture method (Campanile et al. 2007) to screen antagonism. Petri dishes (9 cm) containing 20 ml of sterile PDA were inoculated with a loop of potential antagonists of bacterial strains and the 0.5 cm sterile plug of a 3 day-old pure culture pathogens *S. rolfsii* and *M. phaseolina,* which were collected from the department of plant pathology, Tamil Nadu Agricultural University, Coimbatore. The distance between the pathogen and the antagonist was 4 cm. Each combination of pathogen/antagonist was repeated three times and as negative controls. All Petri dishes were incubated at 25°C in the dark and randomly distributed. The experiment was replicated three times. Radial growth was recorded by measuring colony diameter at 1-day intervals for the time required to reach the margin of the dish in controls. The Antagonism Index (AI) was assessed according to the following formula: AI = (RM − rm)/RM * 100, where rm = ray of the colony towards the antagonist and RM = average of the three rays of the colony in the other directions. The zone of inhibition around the spots was recorded as a positive result.

Interactions between bacterial strains and antagonistic fungi in dual culture

The antagonistic ability of the selected bacterial cultures against *S. rolfsii* and *M. phaseolina* was tested using the dual-culture method described by Badalyan et al. (2002, 2004). A loop of bacterial cultures and a plug (0.5 cm diameter) of antagonistic fungus were cut from the margin of a 3-day-old culture and placed, respectively, on opposite sides (4 cm from the margin) of Petri dishes containing PDA. Each combination of pathogen/antagonist was repeated three times and plates were randomly incubated at 25°C for 3 days. As negative controls, three Petri dishes were inoculated with selected bacterial cultures and a water agar plug. Interactions were examined daily. Antagonism towards bacterial cultures was scored using the Badalyan et al. (2002) rating scale into three types (A, B, and C) and four subtypes (CA1, CA2, CB1, and CB2), where: *A* = deadlock with mycelial contact, *B* = deadlock at a distance, *C* = replacement, overgrowth without initial deadlock; CA1 and CA2 = partial and complete replacement after initial deadlock with mycelial contact, CB1 and CB2 = partial and complete replacement after an initial deadlock at a distance. The following scores were assigned to each type or subtype of reaction: *A* = 1.0; *B* = 2.0; *C* = 3.0; CA1 = 3.5; CB1 = 4.0; CA2 = 4.5; and CB2 = 5.0. The AI was calculated for each species using the formula: AI = SN x *I*, where *N* = number (frequency) of each type or subtype of reaction and *I* = the corresponding score.

Statistical analysis

All the data were analyzed using SPSS software 23.0 version (SPSS IBM). The significant differences in the means were analyzed based on Tukey's multiple comparison test ($p < 0.05$).

Results

Isolation and screening of siderophores-producing bacteria

A total of 33 bacterial isolates were obtained, purified, and cultured. The siderophore-producing bacterial isolates were

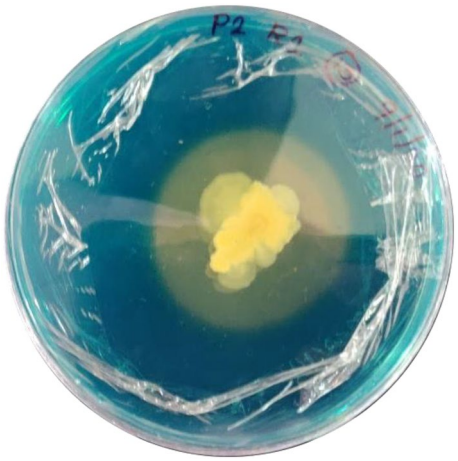

Fig. 1 The appearance of orange color and zone formation indicating siderophore production in CAS agar plate assay

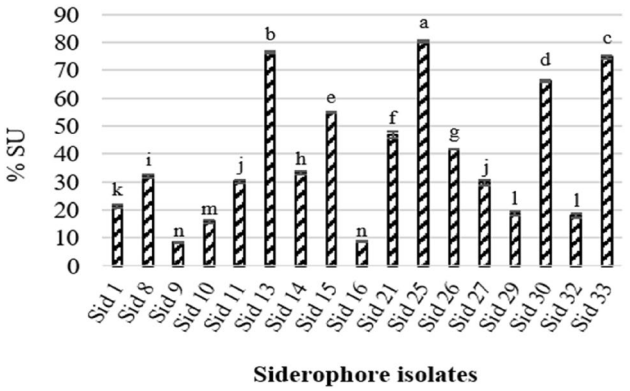

Fig. 2 Percentage siderophore production by the bacteria isolated from calcareous soils

screened using a CAS assay (both qualitatively and quantitatively). The cultures were grown in a succinate medium and incubated for 48 h before being tested for CAS in a spectrophotometer. Seventeen of the thirty-three isolates tested positive for turbidity in the SA medium and turbidity in the CAS test. The presence of siderophores was confirmed on CAS agar plates by the presence of a distinctively orange-colored zone (Fig. 1) indicating siderophore production.

Quantitative screening of siderophore-producing bacteria

For quantitative estimation, CAS assay was employed and their cell concentration was measured at A630 nm. Out of 33 isolates, 17 were found to be positive for siderophore and selected for quantitative estimation of siderophore production. Thirteen isolates produced siderophore units in the range of 8.4 – 54.9%, while four isolates SID 13, SID 25, SID 30, and SID 33 produced significantly higher quantities of siderophores (< 65% siderophore units (SU) (Fig. 2).

Screening of bacteria for calcareousness tolerance

Potassium bicarbonate ($KHCO_3$) is used in the culture medium to simulate calcareousness artificially and their influence was studied in terms of bacterial population and siderophore production. All the isolates followed similar trend, siderophore production increased with increasing the concentration of sodium bicarbonate. Among the four isolates, *Bacillus licheniformis* showed marked increase in bacterial colony forming units (cfu) and siderophore production with rising concentration of bicarbonate. At higher concentrations, *i.e.*, at 25 mM $KHCO_3$, the siderophore production percentage ranges from 65.9 to 74.1% irrespective of the bacterial strains (Table.1).

Characterization of efficient siderophore-producing isolate

Further, the isolates SID 13, SID 25, SID 30, and SID 33 were taken for morphological, molecular, and biochemical characterization. The results of biochemical characterization are given in Table.2. The molecular identification was confirmed by 16S rDNA sequencing of bacterial isolates where SID 13 was showing similarity to *B. licheniformis* (98%)*;* SID 25 was showing similarity to *B. subtilis* (100%); SID 30 was showing similarity to *B. licheniformis* (99.9%), and SID 33 was more close to *O. grignonense* with the percent identity of 93%. The evolutionary relationship of the identified 16S rDNA sequencing and the acquired accession numbers from NCBI are shown in Fig. 3.

Table 1 Bacterial population and siderophore production (%) on different calcareousness levels

Isolates code	Bacterial population (log_{10}cfu)				Siderophore production (%)			
	10 mM $KHCO_3$	15 mM $KHCO_3$	20 mM $KHCO_3$	25 mM $KHCO_3$	10 mM	15 mM	20 mM	25 mM
B. licheniformis (MW279241)	5.80	6.11	6.52	7.21	62.3	69.5	70.1	70.9
B. subtilis (MW279240)	6.98	6.52	6.53	6.62	79.8	76.4	74.7	74.1
B. licheniformis (MW279255)	6.47	6.06	6.20	6.91	59.1	61.7	63.9	65.9
O. grignonense (MW279256)	6.68	6.63	6.72	6.61	70.4	69.9	72.2	72.9

Table 2 Characterization of siderophore-producing bacterial isolates

	Bacillus licheniformis	*Bacillus subtilis*	*Bacillus licheniformis*	*Ochrobactrum grignonense*
Morphological parameters				
Gram reactivity	+	+	+	+
Shape	Rod	Rod	Rod	Rod
Colony color	white	white	white	White
Elevation	Raised	Raised	Raised	Raised
Opacity	Translucent	Translucent	Translucent	Translucent
Biochemical characteristics test				
Citrate utilization test	–	+	+	+
Catalase	+	+	+	+
MR (methylene red)	+	–	+	–
VP (Voges–Proskauer)	+	+	+	–
Indole acetic acid	–	+	+	–

Positive and negative show the results of biochemical results

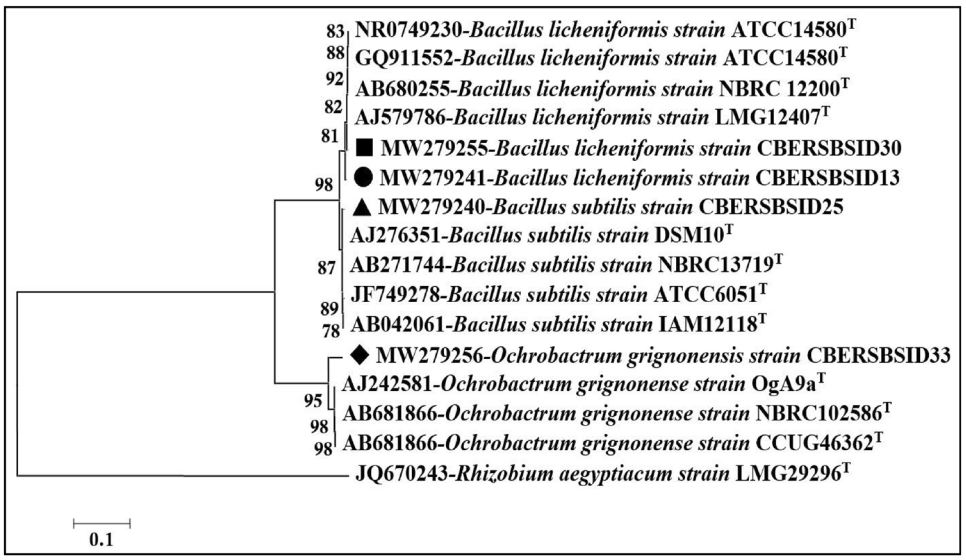

Fig. 3 Phylogenetic tree of 16S rDNA sequences obtained using neighbor joining method. Bootstrap probabilities are shown in percentages of 1000 replicates. Scale bar, 0.1 divergent residues per site. The tree was rooted by out grouping sequence LMG29296^T—Type strain

Siderophore chemotyping

The type of siderophore present in each medium filtrate was subjected to Arnow's, Snow's, and Vogel's tests. Both *B. licheniformis* strains and *O. grignonense* were positive for all three tests, indicating the presence of catechol, carboxylate, and hydroxamate siderophores in the filtrate of the culture medium. *B. subtilis* filtrate was positive only for hydroxamate. (Table 3; Fig. 4).

Table 3 Chemotyping of the siderophore type produced by the bacteria

Bacteria	Arnow[a]	Snow[b]	Vogel[c]
Bacillus licheniformis	+	+ +	+
Bacillus subtilis	–	+ +	–
Bacillus licheniformis	+ +	+ +	+ +
Ochrobactrum grignonense	+ +	+ +	+

+ + Strong positive result; + positive result; – negative result

[a]Catechol

[b]hydroxamate

[c]carboxylate type siderophores were identified using Arnow's, Snow's, and Vogel's tests, respectively

FTIR analysis

The infrared spectrum analyses of the partially purified SPB cultures extract pellet showed a broad peak at 3221, 3242, 3264, and 3265 cm^{-1} indicating the presence of aromatic OH

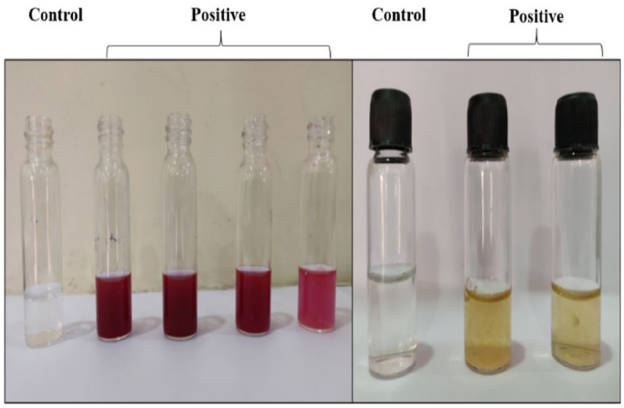

Fig. 4 Results of hydroxamate-type and catechol-type siderophore activity, using cell-free culture supernatants

moiety of siderophores. The peak at 3170 cm^{-1} is attributed to NH stretching. The appearance of a peak at 2950 cm^{-1} showed the presence of saturated alkanes. The spectrum also provides evidence for the presence of amide linkage in the structure. The intense peak at 1640 cm^{-1} typically indicated an amide C=O stretching suggesting a secondary amide functionality. A conjugation and intramolecular H bonding may have caused the lowering of the C=O stretch peak. The peak observed at 1451 and 1452 cm^{-1} revealing the presence of one –C–H bending with functional group $–CH_2$ and one –N–O structure shows similarity towards hydroxamate functional groups. Furthermore, a peak at around 1200 cm^{-1} indicated a –C–O–C– bond of the ether linkage. Thus, the spectrum indicated the presence of catechol siderophores (Fig. 5).

Different culture conditions for optimum production of siderophores

To understand the significant effect of various culture conditions that relates the bacterial growth and siderophore production, the cultures were grown under different growth conditions such as pH, temperature, carbon source, nitrogen source, and iron concentration. Hence, we tried to optimize conditions for the maximum production of siderophore by the SPB strains.

Influence of pH

The optimum siderophore production in all the four SPB strains was achieved at pH 8. The maximum amount of siderophore produced (84.8% SU) was found with SID 30 strain at pH 8. Irrespective of the strains, the lowest siderophore yield was found at pH 10 in SID 13 with the value of 52.8% SU. This might be because the alkaline pH of the medium decreases the solubility of iron, thus making it unavailable to the growing bacteria creating an iron-depleted environment suitable for siderophore production (Fig. 6).

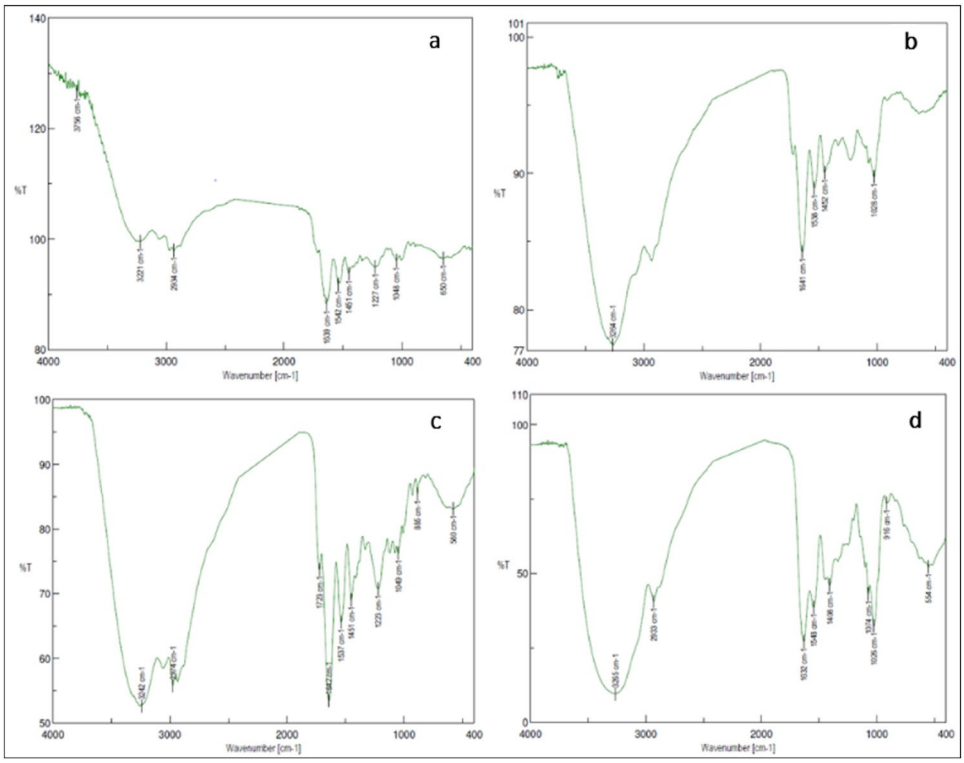

Fig. 5 FTIR spectrum of siderophore positive bacterial extract (**A**: *Bacillus licheniformis* (SID 13); **B**: *Bacillus subtilis* (SID 25); **C**: *Bacillus licheniformis* (SID 30) and **D**: *Ochrobactrum grignonense* (SID 33))

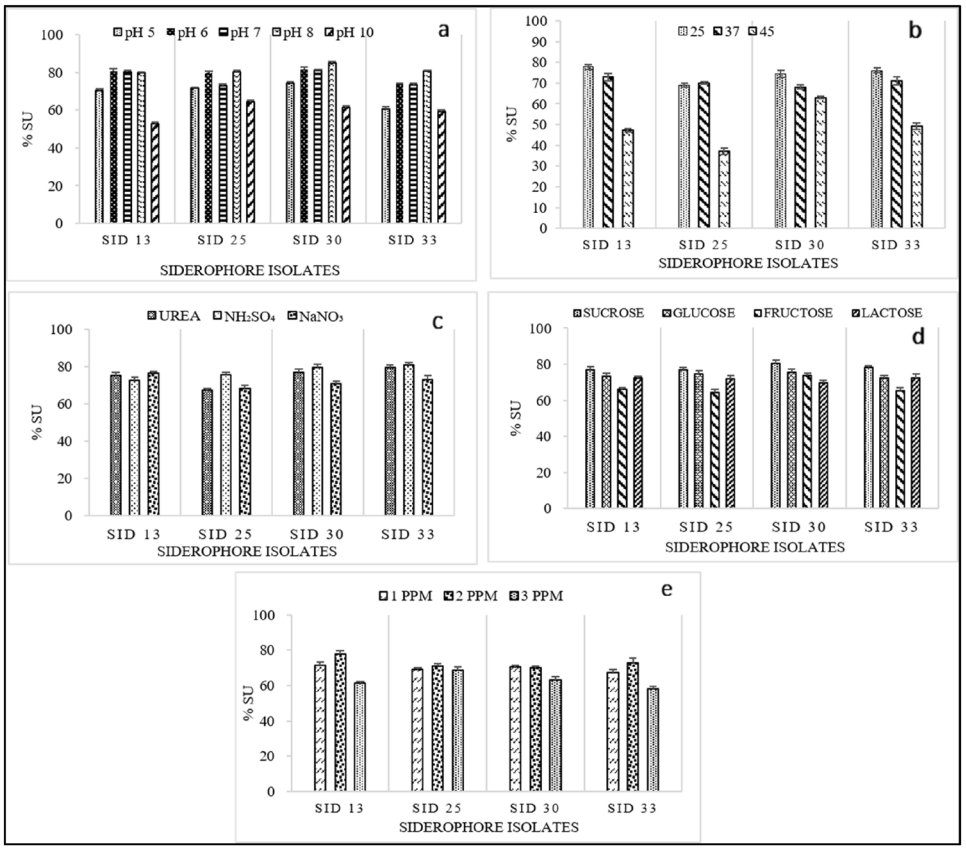

Fig. 6 Effect of different **a** pH, **b** temperature, **c** nitrogen source, **d** carbon source, and **e** iron concentration on the production of bacterial siderophores

Influence of temperature

Further, the culture conditions were changed with constant pH and different temperatures. Our study also showed higher production of siderophore accounting for ~ 70% at 25 °C in all the strains. Upon increasing the temperature levels, the siderophore production showed a sheer decline to 37.2% at 45^{0}°C in SID 25 (Fig. 6).

Influence of nitrogen sources

All the four selected strains from this study were able to synthesize siderophore > 67% irrespective of applied nitrogen source. Different nitrogen sources influenced siderophore production significantly. The highest % SU was detected with ammonium sulfate (80.6%) as the nitrogen source in SID 30, whereas the lowest siderophore production was registered with SID 25 (67.2%) with urea as a nitrogen source as shown in Fig. 6.

Influence of carbon sources

The SPB strains were assessed for their optimal requirement of carbon source for maximal production of the iron-chelating siderophores. Sucrose was found to be a favorable carbon source for all the four tested SPB (Fig. 6). The production of siderophore was comparatively higher ~ 80.4% in *B. licheniformis* (showing an evolutionary relationship with the *Bacillus sp.*).

Influence of iron concentrations

The addition of $FeSO_4$ in the medium significantly affected the siderophore production. The addition of 2 PPM of $FeSO_4$ in the medium resulted in higher siderophore production up to 77% SU in SID 13. This might have induced an enhanced rate of siderophore production to bind with the available iron and provide it to the cell. Upon increasing iron concentration, there was a steep decline in the % SU to 61.3. This could be because once iron concentration in the medium reached above the threshold value required for siderophore production, it negatively regulates iron acquisition genes (Fig. 6).

Antagonistic potential of SPB in dual culture

Varying degrees of mycelial growth inhibition of *S. rolfsii* and *M. phaseolina* were observed with antagonistic bacterial and fungal isolates. *Trichoderma viride* had the maximum inhibitory effect on mycelial growth of both *M. phaseolina* and *S. rolfsii* with a reduction of 69.3 and 65.1, respectively, compared to control. The isolates *B.*

licheniformis, *B. subtilis*, *B. licheniformis*, and *O. grignonense* expressed significantly lower mycelial growth inhibition with reductions in the range of 37.3–57.5% for *M. phaseolina* and 48.3–52.6% for *S. rolfsii* ($P < 0.05$, compared to controls and other antagonists) (Table 4).

The antagonistic index in the dual-culture method, based on the AI values for both the *M. phaseolina* and *S.rolfsii* in the presence of antagonistic isolates was divided into three groups: active (*T. viride*), moderate (*B. licheniformis and B. subtilis*) and weakly active (*O. grignonense*) only for *S. rolfsii*. The most important parameter determining antagonistic activity was the inhibiting speed of colony growth (Table 5 and Fig. 7).

Table 5 Antagonism Index (AI) and type/subtype of interaction between antagonistic isolates and SR, MF scored on Badalyan's scale

Antagonistic isolate	AI[a]		Type/Subtype[a]	
	MP	SR	MP	SR
Bacillus licheniformis	10	10	B	B
Bacillus subtilis	10	10	B	B
Bacillus licheniformis	10	10	B	B
Ochrobactrum grignonense	5	20	A	CA1
T. viride	5	5	A	A

[a]Mean of three replicates

A deadlock with mycelial contact; *B* deadlock at a distance; *CA1* partial replacement after initial deadlock with mycelial contact

Discussion

Siderophores are low molecular weight compounds (between 500 and 1500 Dalton), of high affinity and selectiveness to bind and complex Fe (III). As a part of a strategy to extract iron from the atmosphere because of the poor bioavailability of iron, these siderophores are produced both from plants and microbes (Hider and Kong 2010). In the last few years, several researchers have drawn attention to this particular function. They have wider applications in plant growth, biocontrol activity, and several other ecological factors. In the present study, the siderophore-producing bacteria were isolated from the soil samples and the optimal fermentation condition was configured to understand the culture medium capable of a high level of siderophore production. Similar results were reported by Ferreira et al. (2019) under alkaline conditions where they evaluated five bacterial strains for siderophore kinetics, percent siderophore production, type of siderophore produced, and iron-chelating capacity at pH 9.0. The CAS or HDTMA forms a strong complex with a ferric ion to produce a blue color to the medium; when iron chelators like siderophores are added to the medium, the iron is separated from the dye complex and the color gradually changes from blue to orange (Louden et al. 2011).

Out of 17, 4 isolates (SID 13, SID 25, SID 30, and SID 33) produced higher siderophore units (SU) above 65% in CAS-liquid assay. When these siderophore-producing isolates were subjected to calcareous stress, they showed positive results even under 25 mM K HCO_3 concentration, without any decline in % siderophore units. Earlier results showed that *E. coli, P. aeruginosa, S. aureus, S. agalactiae, E. faecalis* and *H. influenza* growth significantly inhibited

Table 4 Effect of antagonistic isolates on the growth of MP and SR

Antagonistic isolate	Mycelial growth of pathogens (% reduction over control)[f]	
	MP	SR
Bacillus licheniformis	57.5 (±3.04)[b]	52.6 (±2.78)[c]
Bacillus subtilis	48.2 (±2.55)[c]	51.0 (±2.70)[c]
Bacillus licheniformis	54.1 (±2.86)[b]	76.3 (±4.04)[a]
Ochrobactrum grignonense	37.3 (±1.97)[d]	48.3 (±2.56)[c]
T. viride	69.3 (±3.67)[a]	65.1 (±3.44)[b]
Control[g]	0.0[e]	0.0[d]

Different letters indicate the existence of significant differences according to Tukey test ($\alpha = 0.05$)

[f]Mean of three replicates

[g]Growth of *Macrophomina phaseolina* (MP) *and Sclerotium rolfsii* (SR) in the absence of antagonistic fungal isolates

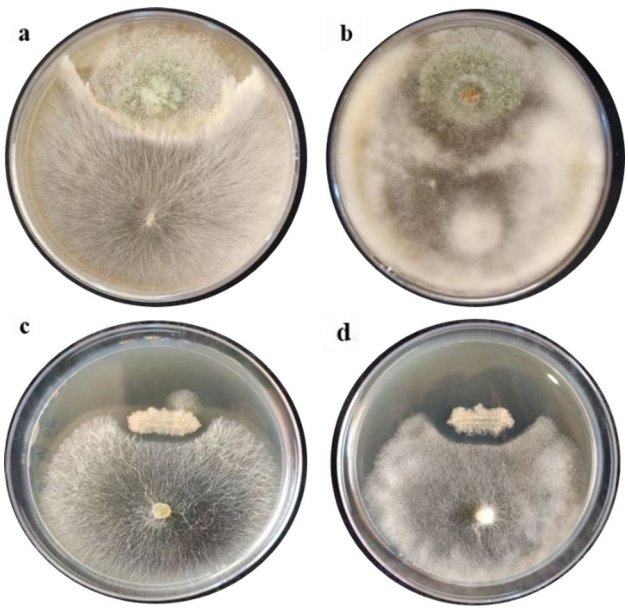

Fig. 7 **a** and **b**, Complete replacement with *T. viride* mycelium on *M. phaseolina* and *S. rolfsii* after 7 and 6 days of inoculation in dual culture, respectively; **c**, partial replacement after initial deadlock at a distance between *B. licheniformis* isolate against *M. phaseolina*; **d** shows deadlock at a distance between *B. licheniformis* isolate against *S. rolfsii* in dual culture method

when subjected to $NaHCO_3$ (100 mmol l^{-1}), suggesting that HCO_3^- can suppress bacterial growth in general (Dobay et al. 2018). As several researchers have also confirmed that high bicarbonate concentration directly caused iron deficiency chlorosis (Coulombe et al. 1984), our strain proved to withstand at all the four levels of bicarbonate concentration showing its ability of tolerance against calcareousness along with higher siderophore production.

B. licheniformis (MW279241) and *B. licheniformis* (MW279255) showed a good adaptation to the calcareousness, and this was reflected in the progressive increase in bacterial population intern in siderophore production even with increasing levels of calcareousness. This might be due to the fact that insolubility of iron increases with increasing pH (kumar et al. 2017) and iron becomes more insoluble and it might have stimulated the production of maximum siderophore. On the other hand, *B. subtilis* (MW279240) and *O. grignonense* (MW279256) presented a sustained growth with almost constant count of bacterial population and siderophore production irrespective of calcareous levels, which could be due to environmental stress. Microbial siderophores are usually grouped as catecholates, hydroxamates, carboxylates, and mixed type since these compounds shows the highest affinity towards iron. Evolutionary relationship of our bacterial strains showed maximum relative percentage towards *Bacillus sp.* Earlier, researchers also found that most of the siderophore-producing bacteria are subsiding under the *Bacillus sp* (Shaikh et al. 2016). Most of our isolates were tested positive for more than one type of siderophore. Earlier reports showed that several soil bacteria could produce more than 65% of SU and multiple types of siderophore (Hider and Kong 2010; Ferreira et al. 2019).

The partially purified siderophores using Amberlite XAD-2 shows similar trends of Sayyed and Chincholkar (2006) who purified siderophores of *Alcaligenes faecalis* on Amberlite XAD-400 resins. Further, the FTIR spectrum of LSBS2 showed the adsorption bands at 3445, 2951, 1652, 1455, and 1143 cm^{-1}, respectively, which indicates the presence of (–OH), aromatic (–CH), (–C=O), (–CH2) and (C–O–C) linkage. These functional groups are present in the catecholate type of siderophore so that the purification of siderophore production in FTIR analysis confirmed the presence of a catecholate type of siderophore that is specific to 2,3 dihydroxybenzoic acid (Nithyapriya et al. 2021).

To optimize the bacterial strains capable of siderophores potential ability, the performance of the bacteria was optimized with the following characteristics: culture media composition (carbon and nitrogen sources), iron complexation capacity, pH range, and under different temperatures conditions. Though iron is the major factor involved in siderophore production, other cultural conditions also play a significant role in siderophore production. Tailor and Joshi (2012) reported that bacteria can grow optimally in the physiological environment. Storey et al. (2005) indicated that under normal conditions with Fiss-glucose medium, the hydroxamate siderophore was lower and this can further be optimized to higher siderophore production with certain modifications in the growth medium.

Because its solubility and availability are affected by the pH of the medium, pH plays an important role in microbial proliferation. We detected that the increased siderophore concentration was recorded when the pH was lower than 8. Given that Fe^{2+} is soluble and Fe^{3+} is insoluble at physiological pH (7.35–7.40) (Bou-Abdallah 2010), alkaline pH decreases iron solubility, rendering it unavailable to bacteria, resulting in an iron-depleted environment ideal for siderophore synthesis. Gérard (2016) discovered that the insolubility of iron increases at high pH values, which supports our observations. At pH 8, iron becomes more insoluble in soil solution, which may have accelerated siderophore production. The present finding agrees with Calvente et al. (2001) who reported similar results as pH near 8 stimulates higher siderophore production. In some cases, it was reported that the highest iron-chelating capacity, at pH 9.0, was obtained by *B. megaterium* followed by *B. subtilis* and *A. vinelandii* (Ferreira et al. 2019).

The percentage of siderophore production varied with incubation temperature, being optimum at 27°C, lesser at 35°C, and least at 45°C. The incubation temperature considerably influenced siderophore production; these findings are in accordance with Dave and Dube (2000) who have reported that maximum siderophore production occurred at 30 °C. Percent siderophore units were recorded maximum at 30 °C, *i.e.,* 83.9% by *A. oryzae* (Singh and Mishra 2015). The *P. fluorescens* strain was also found to produce maximum siderophore at 29^{0}C (Tailor and Joshi 2012). Kumar et al. (2017) reported that 35 °C (room temperature) showed optimum bacterial siderophore production in VITVK5 and VITVK6 strains.

The optimization was carried out with different sources of nitrogen such as ammonium sulfate, sodium nitrate, and urea. All the above mentioned isolates have produced siderophore more than 65% with all the nitrogen sources and the maximum was registered with ammonium sulfate, while *Ochrobactrum* sp. influenced to produce higher siderophore with N source as shown in our results (Louden et al. 2011; Tailor and Joshi 2012). The results were similar with Tailor and Joshi (2012) who also confirmed maximum siderophore yield up to 96% in *P. fluorescens* and *P. putida* in the presence of ammonium sulfate and urea.

Based on previous reports, we have chosen to evaluate four major carbon sources namely glucose, fructose, lactose, and sucrose. Among these, sucrose had a stimulating effect on the production of siderophore followed by glucose. The present findings prove that the presence of sugars such as sucrose in the growth media increased the

growth of *Nocardia levis* MK-VL_113 for the elaboration of bioactive metabolites (Kavitha et al. 2009). Sucrose concentration at 2% roused the growth and siderophore production in *Rhizobium* strains (Sridevi and Mallaiah 2007). A previous study of siderophore production by *Rhodotorula sp.* reported by Calvente et al. (2001) also shows similar results of enhanced siderophore production upon sucrose supplementation. Supplementing the growth media with carbon sources increases the growth and metabolism of bacteria and the siderophore production capability. The siderophore production by an organism depends on the availability of organic and inorganic nutrients in the medium (Abd-Alla 1998).

Among various abiotic factors, the pH of the medium plays a major role in Fe availability. Notably, increasing concentration beyond a threshold level suppresses the siderophore production. Our findings also showed that the percentage siderophore enhanced with increasing pH and Fe concentration up to 8 and 2 PPM, respectively, and beyond that siderophore production started to decline, which might be due to native availability of iron. Tailor and Joshi (2012) showed similar results, where the concentration of siderophore produced from *P. fluorescens* decreased after 1 µM. Excess iron concentration harmed siderophore production along with the morphology alterations and growth (Singh and Mishra 2015).

Identification and selection of effective antagonistic organisms is the first and foremost step in biological control (Kamalakannan et al. 2004). Our findings witness the significant antagonistic activities of *B. licheniformis*, *B. subtilis*, *B. licheniformis*, and *O. grignonense* against *M. phaseolina* and *S. rolfsii* in *in vitro* dual cultures. Siderophores are also thought to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth (Champomier-Vergès et al. 1996). Siderophore positive *P. fluorescens* is known to control bacterial pathogens including bacterial soft rot of potato, the bacterial canker, and bacterial wilt of tomato (David et al. 2018). These results are also in agreement with Solanki et al. (2014) who also reported efficient siderophore production using *Enterococcus sp.* and its activity against plant pathogen *R. solani*. These beneficial effects of siderophore include both plant growth enhancement and biological control of phytopathogenic fungi (Ghazy and El-Nahrawy 2021). Secondary metabolite like siderophore production is a key for plant growth-promoting bacteria by fulfilling the plants' iron requirements and protecting against plant pathogens. Nursery and field trials in cardamom with siderophore-producing bacteria, by *P. putida* TAUC10, had higher ligand formation constants than those of the soil pathogens (Panchami et al. 2020).

In the present study, 17 bacterial isolates were found to be positive for qualitative CAS agar assay. Upon qualitative and quantitative analysis, four isolates (*B. licheniformis*, *B. subtilis*, *B. licheniformis*, and *O. grignonense*) were found to produce above 65% siderophore units having higher potential use for iron-induced chlorosis amendment in calcareous soils. The morphological and molecular characterizations of siderophore-producing bacteria depict close resemblance to the bacterial species of *Bacillus sp.* and *Ochrobactrum sp.* Results of the influence of different culture conditions showed that the bacterial isolate had the efficiency of producing siderophore in higher concentration at pH 8 and temperature 37 °C with sucrose as carbon source and Na_2SO_4 as nitrogen source. The increase in iron concentration increased the production of siderophore, which saturated after a threshold limit. As these strains are capable of producing higher siderophore units even up to 25 mM calcareous level, it can be used as a tool for overcoming iron-related stress under calcareous soils (> 30% of world area), where iron deficiency is a major constrain. The present investigation also revealed that these bacterial isolates exhibited other plant growth-promoting traits viz. citrate utilization, catalase activity, IAA production and subdues the root and stem rot fungi in groundnut viz., *S. rolfsii* and *M. phaseolina* has added advantage. However, further research is needed to elucidate the tolerance limits for calcareousness and optimization of siderophore under field conditions, which can lower the impact of ion-induced chlorosis.

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Declarations

Conflict of interest Authors have declared that no competing interests exist.

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